METABOLISM OF CELLOBIOSE, GENTIOBIOSE, AND CELLOBIITOL IN AEROBACTER AEROGENES

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THESIS

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ABSTRACT

METABOLISM OF CELLOBIOSE, GENTIOBIOSE, AND CELLOBIITOL IN AEROBACTER AEROGENES

By

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Aerobacter aerogenes PRL-R3 can utilize the disaccharides cellobiose, gentiobiose, and cellobiitol as sole sources of carbon and energy. The pathways by which these β-glucosides are degraded in this organism were elucidated. A β-glucoside kinase catalyzes the phosphorylation of cellobiose, gentiobiose, and cellobiitol, utilizing adenosine 5'-triphosphate as the phosphoryl donor, to yield the disaccharide-phosphates. The disaccharide-phosphates are then hydrolyzed by a phospho-β-glucosidase to yield D-glucose-6-phosphate and D-glucose (D-glucitol in the case of cellobiitol-phosphate).

The β-glucoside kinase is induced by cellobiose, gentiobiose, and cellobiitol to approximately the same specific activities, and evidence is presented that all three disaccharides induce the same enzyme. Other β-glucosides, such as salicin and arbutin, do not induce the kinase. The kinase was purified 110-fold from cellobiose

grown cells, and was characterized with respect to phosphoryl acceptor specificity, phosphoryl donor specificity, molecular weight, and pH optima in several different buffer systems. The purified enzyme catalyzed the phosphorylation of eleven different β-glucosides, including aromatic and aliphatic β-glucosides as well as disaccharides, a trisaccharide, and a tetrasaccharide; compounds which did not possess the β-glucosidic linkage did not serve as substrates. The ratios of specific activities for several substrates were not altered by purification of the enzyme. When substrates were mixed, the individual activities were not additive. These data are interpreted to mean that a single enzyme catalyzes the phosphorylation of all eleven of the β-glucosides.

The product of the kinase-catalyzed phosphorylation of cellobiose was determined by chemical, physical, and enzymatic techniques to be cellobiose monophosphate, with the phosphate group located at carbon six of the non-reducing ring.

The phospho-β-glucosidase from cellobiose-grown cells was purified 14-fold and partially characterized. Seven β-glucoside-phosphates (cellobiose-phosphate, gentiobiose-phosphate, cellobiitol-phosphate, salicin-phosphate, arbutin-phosphate, methyl-β-glucoside-phosphate, and phenyl-β-glucoside-phosphate) served as substrates. Mixed substrates gave non-additive rates, which is

consonant with one enzyme catalyzing the cleavage of the several substrates.

Mutants of A. aerogenes were isolated which failed to grow on cellobiose, gentiobiose, or cellobiitol, but grew normally on other sugars including other β-glucosides. These mutants lacked β-glucoside kinase activity. Spontaneous revertants regained the ability to grow on all three disaccharides and concomitantly regained the inducible β-glucoside kinase activity. These experiments demonstrated that, although the β-glucoside kinase showed activity on several β-glucosides, it functioned only in the biodegradation of cellobiose, gentiobiose, and cellobiitol.

This is the first pathway described for disaccharide metabolism in which the sugar is phosphorylated with adenosine 5'-triphosphate prior to cleavage of the glycosidic linkage.

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ABBREVIATIONS USED

ATP	adenosine triphosphate
Bicine	N,N-bis(2-hydroxyethyl)glycine
DEAE	diethylaminoethyl-
gl	glucoside
HPr	heat-stable protein
MES	2[N-Morpholino]ethane sulfonic acid
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ONP	o-nitrophenyl-
P_i	inorganic orthophosphate
P-	phospho-
-P	-phosphate
PEP	phosphoenolpyruvate
PIPES	piperizine-N-N'-bis[2-ethane sulfonic acid]
PNP	p-nitrophenyl-
sal	salicin
TEG	ethyl-1-thio-β-glucoside
TES	N-(hydroxymethyl)methyl-2-aminoethane sulfonic acid
TMG	methyl-1-thio-β-glucoside

TPG	phenyl-1-thio-β-glucoside
Tricine	N-(hydroxymethyl)methyl glycine
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol

INTRODUCTION

Aerobacter aerogenes PRL-R3 can utilize the β-glucosides cellobiose, gentiobiose, or cellobiitol as well as D-glucose and many other sugars as sole sources of carbon and energy. Two different pathways for the metabolism of cellobiose, involving either hydrolysis or phosphorolysis of the disaccharide, have been reported to occur in a variety of organisms (see Literature Review), but the pathway of cellobiose degradation has not been elucidated for any member of the *Enterobacteriaceae*. The pathways of gentiobiose and cellobiitol metabolism have not been previously determined for any organism.

This thesis describes the pathways of cellobiose, gentiobiose, and cellobiitol degradation in *A. aerogenes* PRL-R3, and characterizes the participating enzymes. The pathways involve ATP-dependent phosphorylation of the disaccharides, followed by hydrolysis of the glycosidic linkage of the resulting disaccharide phosphates. Thus, the pathways are distinct from any previously described pathways for the degradation of disaccharides or β-glucosides in any organism.

LITERATURE REVIEW

This literature review concerns the degradation of natural disaccharides and aromatic and aliphatic β-glucosides by enzymes from microbial, insect, protozoan, and mammalian sources. The following α- and β-glucosides form the basis for this discussion: cellobiose, gentiobiose, sophorose, laminaribiose, maltose, trehalose, nigerose, isomaltose, salicin, and arbutin. A consideration of specific α- and β-glucosidases, phosphorylases, and a general α- and β-glucosidase, to show the specificity within a class of compounds (i.e., α- and β-linked disaccharides), are covered in the first section. The second section is devoted to an analysis of the work done by other workers on cellobiose degradation. The third section reviews the work done in the last few years on the mode of transport and mechanism of phosphorylation of various β-glucosides.

1. α- and β-Glucoside Metabolism

Pertinent data on the α- and β-glucosides plus several other oligosaccharides discussed in this thesis are shown in Table I. The majority of work done with these disaccharides has been concerned with their occurrence in polysaccharides--whether or not a given polysaccharide

TABLE I.--Comparison of α- and β-glucosides.

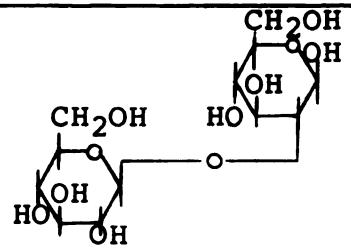
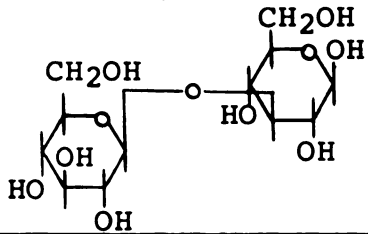
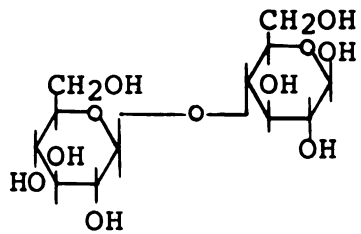
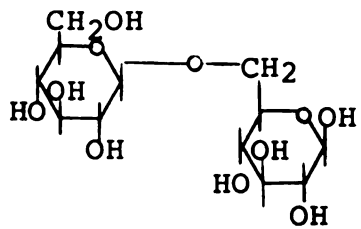
Glucoside	Linkage	Source[a]	Nomenclature	Structure
Sophorose	β 1→2	*Sophora japonica* (1,2) *Agrobacterium radiobacter* Polysaccharide (3)	2-O-β-D-Gluco-pyranosyl-D-glucose	
Laminaribiose	β 1→3	paramylon (4) laminarin (5,6,7)	3-O-β-D-Gluco-pyranosyl-D-glucose	
Cellobiose	β 1→4	cellulose (8,9) *Pinus silvistris* (10)	4-O-β-D-Gluco-pyranosyl-D-glucose	
Gentiobiose	β 1→6	gentianose (11) yeast glucan (12)	6-O-β-D-Gluco-pyranosyl-D-glucose	

TABLE I.--Continued

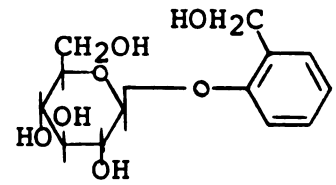
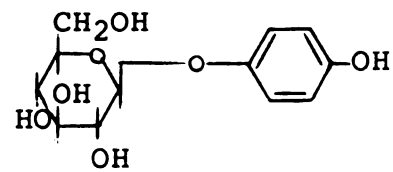
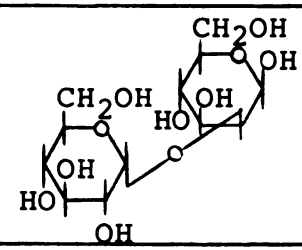
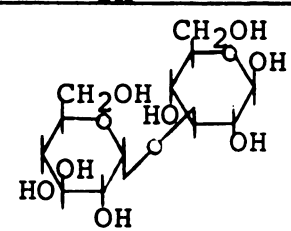
Glucoside	Linkage	Source[a]	Nomenclature	Structure
Salicin	β	Hot water extracts of the bark from poplar and willow trees (13)		
Arbutin	β	Ericaceae (14)		
Kojibiose	α 1→2	Typha latifola (15) Trisaccharide produced by L. mesenteroides (16)	2-O-α-D-Gluco-pyranosyl-D-glucose	
Nigerose	α 1→3	Typha latifola (16) Nigeran (17)	3-O-α-D-Gluco-pyranosyl-D-glucose	

TABLE I.--Continued

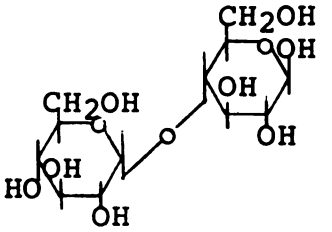
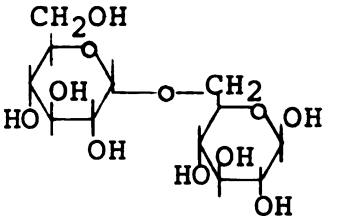
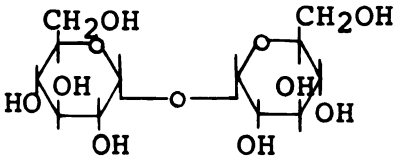
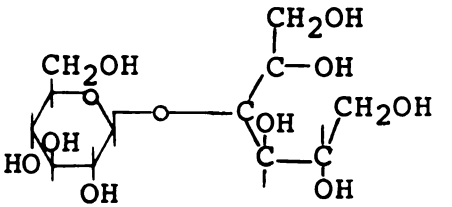
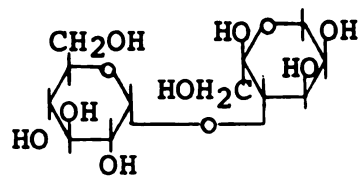
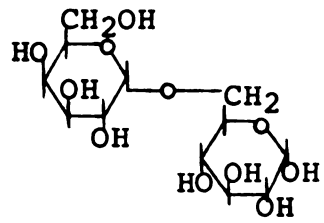
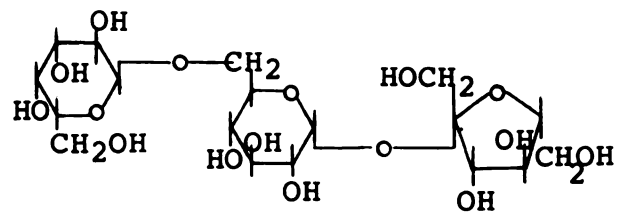
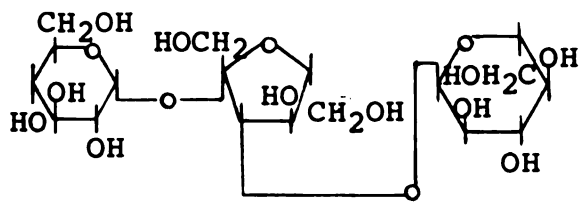
Glucoside	Linkage	Source[a]	Nomenclature	Structure
Maltose	α 1→4	amylopectin (18) starch (19)	4-O-α-D-Gluco-pyranosyl-D-glucose	
Isomaltose	α 1→6	Dilsea edulis polysaccharide (20) Typha latifola (15)	6-O-α-D-Gluco-pyranosyl-D-glucose	
Trehalose	α 1→1	Trehala manna (21) Hyalophora cecropia (22)	1-O-α-D-Gluco-pyranosyl-D-glucose	
Cellobiitol	β 1→4	$NaBH_4$ reduction of Cellobiose (23)		

TABLE I.--Continued

Glucoside	Linkage	Nomenclature	Structure
Turanose	α 1→3	3-O-α-D-Glycopyranosyl-D-fructose	
Melibiose	α 1→6	6-O-α-D-Galactopyranosyl-D-glucose	
Raffinose		O-α-D-Galactopyranosyl-(1→6)-O-α-D-glycopyranosyl-(1→2)-β-D-fructofuranoside	
Melezitose		O-α-D-Glucopyranosyl-(1→3)-O-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside	

[a]The references given in this table are not meant to be exhaustive, but are only exemplary of the sources available. For an exhaustive source of references, see reference 24.

contains the particular linkage, hence the compound, and if so, how much there is. This type of study will not be described here.

Cellobiose, gentiobiose, laminaribiose, and sophorose constitute the disaccharide population of the large group of compounds termed β-glucosides. Of these only cellobiose and laminaribiose have been studied with regard to their metabolism. Recently, some work was reported on paramylon (a polysaccharide containing only β 1→3 linkages of glucose) breakdown and laminaribiose metabolism in Euglena gracilis. Fellig in 1960 reported the isolation of a laminarinase from E. gracilis which serves to cleave this glucose polymer to smaller units (25). Since his assay consisted of following only the increase in reducing sugar, no conclusion could be drawn as to the distribution of products among the one-, two-, three-, four- or higher-unit oligomers possible from this type of cleavage. In light of the papers subsequently to be described, the data on oligomer distribution are important.

Goldemberg and coworkers described the isolation of a laminaribiose phosphorylase from E. gracilis (26, 27). The reaction catalyzed by this enzyme is shown below.

$$\text{Laminaribiose} + P_i \rightleftharpoons \alpha\text{-D-Glucose-1-P} + \text{D-Glucose}$$

The specificity is especially interesting in that the enzyme also catalyzes the phosphorolysis of the higher

homologues of laminaribiose, namely laminaritriose, laminaritetraose, and laminaripentaose. The K_m values for laminaribiose and laminaritriose are 5 and 6 mM, respectively. Since large amounts of paramylon are stored in E. gracilis and there are no amylases present, the laminarinase and laminaribiose phosphorylase together function to mobilize the carbohydrate reserve, and the products constituting the β 1→3 linked homologous series (through the five-unit oligomer) can be altered to a freely metabolizable form. The laminaribiose phosphorylase is specific for β 1→3 bonds, as maltose, cellobiose, and gentiobiose do not serve as substrates. The enzyme is specific for α-D-glucose-1-phosphate as the glycosyl donor, and the best acceptors (normalized to D-glucose, 100) are phenyl-β-glucoside 104, arbutin 92, and salicin 79.

At approximately the same time Manners and Taylor reported the presence of laminaribiose phosphorylase in Astasia ocellata (28, 29). The enzymes from A. ocellata and E. gracilis seem very similar, although Manners and Taylor make no mention of whether the enzyme from the former source used the higher homologues of the β 1→3 series as substrates. The glucosyl acceptor specificity (normalized to glucose, 100) was somewhat different as shown by the following order: methyl-β-glucoside 164, glucosyl-mannitol 92, laminaribiose 87, cellobiose 63, and gentiobiose 59.

Two phosphorylases active on α-linked disaccharides also have been reported. Euglena gracilis served as the source for isolation of a phosphorylase active on trehalose by Belocopitow and Maréchal (30). The reaction catalyzed is shown below:

$$\text{Trehalose} + P_i \rightleftharpoons \beta\text{-D-Glucose-1-P} + \text{D-Glucose}$$

No specificity studies were reported in this article, and therefore no observations can be made as to whether it requires an α 1→1 bond or not.

The first in-depth study of a disaccharide phosphorylase was reported by Fitting and Doudoroff using Neisseria meningitidis (31). The enzyme catalyzes the phosphorolysis of maltose by the reaction shown below:

$$\text{Maltose} + P_i \rightleftharpoons \beta\text{-D-Glucose-1-P} + \text{D-Glucose}$$

None of the following compounds could substitute for maltose: methyl-α-glucoside, trehalose, isomaltose, cellobiose, or gentiobiose.

Two different mechanisms are possible for this type of reaction, as shown by the reactions below:

$$(1) \quad A\text{-}B + C \overset{E}{\rightleftharpoons} B + A\text{-}C$$

$$(2)\quad A\text{-}B + E \rightarrow E\text{-}A + B$$

$$E\text{-}A + C \rightarrow A\text{-}C + E$$

where E represents the enzyme, A-B and C are substrates for the enzyme, A-C and B are products of the reaction, and E-A represents an enzyme-substrate complex.

In the first mechanism one would expect to see no isotopic exchange between reactants and products if one of the reactants were omitted from the mixture. The reaction would involve a direct displacement of C for A, and if the compound A-B were optically active, a change in optical rotation would occur. One could predict an intermediate A-B-E-C complex interconvertible with an A-C-E-B complex. Unless the enzyme does not have rigid structural specificity, little transferase activity should be found during this type of mechanism.

However, if the second mechanism were operative, one would expect to see isotopic exchange for each reaction separately and for the whole process as shown below:

$$A\text{-}B + A^* \overset{E}{\rightleftharpoons} A + A^*\text{-}B$$

$$A\text{-}C + C^* \overset{E}{\rightleftharpoons} A\text{-}C^* + C$$

$$A^*\text{-}C + A\text{-}B \overset{E}{\rightleftharpoons} A^*\text{-}B + A\text{-}C$$

where E represents the enzyme, A-B and C are substrates for the enzyme, A-C is a product of the reaction, A is a moiety which can form a complex with the enzyme, and * indicates radioactivity.

Since this mechanism involves a double displacement, the configuration of the products should be the same as that of the reactants; therefore, if asymmetric centers are involved, no inversion will occur and the optical rotation will remain of the same sign and the configuration remain the same. Since there is an enzyme-bound intermediate, transferase activity would be predicted for this type of mechanism.

In light of this discussion the important data needed to determine the mechanism of a phosphorylase reaction include the cleavage point of the reaction (C-O$\overset{\downarrow}{-}$P or C$\overset{\downarrow}{-}$O-P), isotopic exchange data, and optical inversion data. The mechanism of the maltose phosphorylase was investigated and the following exchange data were obtained: first, no exchange occurred between $^{32}P_i$ and β-D-glucose-1-phosphate without the presence of D-glucose; second, no exchange was observed between labeled D-glucose and unlabeled maltose in the absence of added phosphate. Third, arsenate could substitute for phosphate to cleave maltose to two moles of D-glucose. Both donor and acceptor were required for the catalysis. These data can be explained by the formation of a maltose enzyme-phosphate intermediate, interconvertible

with a β-D-glucose-1-phosphate-enzyme-glucose complex. When the ability of the enzyme to produce D-glucose from β-D-glucose-1-phosphate in the presence of arsenate was tested, it was found that β-D-glucose-1-phosphate was not directly arsenolyzed. This is further evidence that there is no enzyme-glucose intermediate formed and that the mechanism is a single, concerted displacement reaction. The interesting feature of the overall reaction is that the sequence involves a Walden inversion at C_1. This mechanism can be explained if a transfer of phosphate occurs between substrates and not between enzyme and each substrate separately. Transglycosylase activity is absent. The carbon-oxygen bond must be broken in the phosphate ester to account for the inversion.

The most common method for glucoside breakdown involves the α- and β-glucosidases. Only enzymes which function specifically in the metabolism of the α- and β-glucosides or have a limited specificity will be covered here. Enzymes such as almond emulsion have been reviewed extensively before (32). However, three enzymes, one an α- and the other two β-glucosidases, will be covered to indicate the broad specificity possible in these enzymes in terms of the diversity of bonds that they may cleave. With one enzyme it is possible to cleave a whole class of compounds.

Larner reported the occurrence of an oligo-1,6-glucosidase in hog intestine which acts on panose

(isomaltosyl-D-glucose), isomaltose, and isomaltotriose (33, 34). The K_m values for isomaltose and isomaltotriose were both 7×10^{-4}M. Gentiobiose did not serve as a substrate, thereby making the enzyme specific for α 1→6 bonds.

Again, Larner published a paper reporting the hydrolysis of nigerose in hog intestine (35). The K_m for nigerose was found to be 3×10^{-4}M. The enzyme seems to be an α-1,3-glucosidase. However, neither laminaribiose nor any other α- or β-glucoside was tested for substrate activity.

Maltase activity was detected and purified from equine serum by Lieberman and Eto (36). After a 1400-fold purification the enzyme cleaved the following compounds in addition to maltose: phenyl-α-glucoside, methyl-β-maltoside, turanose, and isomaltose. No activity was detected with cellobiose, methyl-α-glucoside, lactose, melibiose, and trehalose. The K_m for maltose was found to be 3.8×10^{-4}M.

Larner and Gillespie found maltase activity in hog intestinal mucosa (36a). The enzyme was purified just 6-fold, but during the process, contaminating oligo-1,6-glucosidase activity was removed. The K_m for maltose was determined to be 1×10^{-3}M. No other sugars were tested for substrate activity with this enzyme, but inhibition by D-glucose occurred with a K_i of 4×10^{-5}M.

Specific enzymes for the hydrolysis of trehalose have been reported from several sources. These include

Galleria mellonella (37); hog intestine (38); Schistocerca gregaria (39); Neurospora crassa (40); baker's yeast (41); hybrid yeast (42); Phormia regina, Meig (43); and Streptomyces hygroscopicus (44). The pH optima for these enzymes ranged from 5.0 to 7.0. The K_m values ranged from 10^{-2} to 10^{-4}M, those enzymes from Neurospora, baker's yeast, and Phormia regina having the lowest values. The specificity studies showed a remarkable similarity among enzymes. The enzyme from Schistocerca showed some activity on maltose with the crude extract and that from baker's yeast some activity with raffinose. However, no activity was obtained with the following compounds: cellobiose, lactose, methyl-α-glucoside, methyl-β-glucoside, phenyl-α-glucoside, phenyl-β-glucoside, salicin, PNP-α-glucoside, and turanose. The most extensive disaccharide substrate specificity study was done by Elbein (44) in which kojibiose, sophorose, nigerose, laminaribiose, isomaltose, and gentiobiose were tried as substrates. None was acted upon by trehalase. None of the trehalases studied showed transglucosylase activity.

Halvorson and Ellias reported the 100-fold purification of an α-glucosidase from Saccharomyces italicus (45). None of the following compounds was hydrolyzed by the enzyme: raffinose, isomaltose, cellobiose, methyl-α-glucoside, thiophenyl-β-glucoside, ONP-galactoside, or PNP-β-glucoside. The enzyme showed the following substrate specificity in terms of relative V_{max} values: phenyl-α-

glucoside 1.00, turanose 0.87, sucrose 0.79, methyl-β-maltoside 0.76, maltose 0.57, and PNP-α-glucoside 0.60. The K_m for PNP-α-glucoside was determined to be 2.5×10^{-4}M. No K_m values were determined for the other sugars which served as substrates for the enzyme.

A recent paper by Han and Srinivasan reported that 130-fold purification of a β-glucosidase from Alcaligenes faecalis which exhibits a wide range of specificity (46). The enzyme specificity (normalized to cellobiose, 100) is shown for the following compounds: cellobiose 100, laminaribiose 65, cellotriose 62, cellotetraose 50, sophorose 42, lactose 8, sucrose 2, salicin 2, gentiobiose 0, maltose 0, melibiose 0, methyl-β-glucoside 0. The enzyme is semi-constitutive. Cellobiose, lactose, and methyl-β-glucoside induced the enzyme to approximately the same specific activity, whereas growth on D-glucose and melibiose resulted in a specific activity one-tenth as high. The K_m for PNP-β-glucoside was determined to be 1.25×10^{-4}M. The rate of hydrolysis depends on the aglycon group, the fairly strict requirement for glucose in the non-reducing ring, and the type of glycosidic linkage.

The β-glucosidase of Saccharomyces cerevisiae was characterized by Duerksen and Halvorson (47). The enzyme was purified 121-fold and the K_m for PNP-β-glucoside found to be 8.1×10^{-5}M. This β-glucosidase failed to hydrolyze any α-glucosides such as phenyl- or PNP-α-glucoside or

β-galactosides such as ONP-β-galactoside. Also, it exhibited a very marked preference for an aromatic-aglycon group as shown by the V_{max} values given for the following sugars: cellobiose 12, methyl-β-glucoside 33, phenyl-β-glucoside 163, benzyl-β-glucoside 116, salicin 82, arbutin 187, and PNP-β-glucoside 126.

2. Biochemical Transformations of Cellobiose

Cellobiose is one of the main products of the enzymatic breakdown of cellulose. For many years the only pathway known for its degradation was through the direct hydrolysis by a general β-glucosidase. Simon and Schubert, working with the wood-destroying mold, Poria vaillantii, attempting to ascertain the mechanism of cellulose degradation, reported the presence of a cellobiase which works in conjunction with a cellulase (48). The cellulase breaks cellulose down to cellobiose and a mixture of the homologous series of oligosaccharides of the β 1→4 series. Cellobiase acts on all of these oligosaccharides and cleaves all to D-glucose. However, no substrate studies were undertaken to determine the K_m values for these various saccharides. The activity was determined with three sugars, and the order of decreasing activity was PNP-β-glucoside, cellobiose, and salicin.

Another mechanism for cellobiose degradation was found and studied extensively by Alexander in Clostridium thermocellum (49), a strict anaerobe; by Ayers in Ruminococcus flavefaciens (50), an anerobic rumen bacterium; and

by Hulcher and King in Cellvibrio gilvus (51, 52) a soil bacterium. All three of these organisms degrade cellulose by means of a cellulase, the sole product of which is cellobiose (determined by paper chormatography of reaction products). In all three systems the researchers found a pathway which consisted of a phosphorolytic cleavage of cellobiose by the enzyme, cellobiose phosphorylase. The reaction catalyzed is shown below:

$$\text{Cellobiose} + P_i \rightleftharpoons \alpha\text{-D-Glucose-1-P} + \text{D-Glucose}.$$

Ayers worked with crude enzyme since two important contaminating enzymes, phosphoglucomutase and hexokinase, were absent. Since the reaction is reversible, it was measured in both directions. In the forward direction the only products detected were D-glucose and α-D-glucose-1-phosphate, while in the reverse direction, only cellobiose was found. Specificity studies showed that neither maltose nor salicin was cleaved phosphorolytically, and in the reverse direction that D-glucose was the only glucosyl acceptor for the synthesis of cellobiose.

Cellobiose phosphorylase from Clostridium thermocellum has been partially purified and characterized (49). The equilibrium constant in the direction of cellobiose synthesis is 4.3, and the pH optimum was determined to be 7.0, but exhibited a wide peak from 4.6 to 8.1. Specificity

studies showed the enzyme to be unable to catalyze phosphorolysis of gentiobiose, salicin, lactose, or maltose. However, in the reverse direction, several sugars other than D-glucose could serve as the glucosyl acceptor. These include D-xylose, L-xylose, D-glucosamine, and 2-deoxyglucose. The mechanism was investigated (49), and the following data were obtained: first, the enzyme is specific for α-D-glucose-1-phosphate as the glucosyl donor; second, an exchange experiment involving arsenate and α-D-glucose-1-phosphate in the absence of D-glucose failed to produce any exchange; third, no exchange occurred between cellobiose and the acceptor D-xylose in the absence of phosphate; and fourth, arsenate can substitute for phosphate in the overall reaction and cause the arsenolysis of cellobiose to yield two moles of D-glucose. From these data it was deduced that the mechanism of cellobiose phosphorylase is the same as that of maltose phosphorylase. Namely, a Walden inversion occurs at C_1 in a single displacement reaction between the substrates and not between the enzyme and each substrate separately.

An important study by Swisher and King involved a determination of whether or not the two glucose moieties of cellobiose were metabolically equivalent (53). C. thermocellum and R. flavefaciens do not grow on D-glucose; however, C. gilvus does grow on D-glucose after prolonged lag.

Hulcher and King reported the following data to demonstrate that D-glucose and cellobiose follow independent metabolic pathways (51, 52). A comparison of growth rates on the two sugars showed a 30 to 40 percent greater rate of growth on cellobiose than on D-glucose. Cells grown on D-glucose, cellobiose, and an equimolar mixture (on a hexose basis) of D-glucose and cellobiose showed a linear oxidation of both sugars indicating that the transport process was not responsible for the differences in growth rates for the two sugars. The oxidation of cellobiose was approximately 10 percent higher than that of D-glucose, but a mixture of the two showed a distinctly higher rate of oxidation than that of either alone, showing the effect not to be additive and suggesting further that independent metabolic pathways might be involved prior to the terminal oxidase. Fermentation acid production was measured and the results showed that there was 85 percent more acid produced from glucose than cellobiose per hexose equivalent utilized. Inorganic phosphate stimulated the oxidation of cellobiose but not of D-glucose, which is logical since the mechanism of cellobiose degradation involves phosphorolysis. Gluconate was one of the acids detected in both D-glucose- and cellobiose-grown cells and these workers postulated that D-glucose is metabolized through the gluconate shunt. ATP should stimulate D-glucose utilization in a cell-free system if the D-glucose were being metabolized via the phosphogluconate

shunt, and ATP addition resulted in a 3-fold increase in utilization. If the D-glucose from cellobiose were being metabolized by the same route, the same effect should be seen. However, ATP inhibited D-glucose utilization from cellobiose. If D-glucose from cellobiose were being metabolized via D-fructose-6-phosphate and D-fructose diphosphate, ATP addition would inhibit D-fructose-6-phosphate kinase and result in an overall decrease in cellobiose utilization.

The data from these two papers (51, 52) represent only an attempt by the authors to demonstrate that the pathways of metabolism for D-glucose, as a single carbon growth source, and D-glucose, generated by the phosphorolysis of cellobiose, are different. The data are not conclusive and contain many unexplained effects which have a substantial bearing on the evidence presented for their hypothesis. In another paper (53) they present the most convincing data contained in the three papers that two independent pathways are followed by D-glucose from the two different sources.

These workers synthesized radioactive cellobiose labeled with ^{14}C in the reducing moiety, measured manometrically the respiratory CO_2, and looked for the percent of ^{14}C in the CO_2. If the two halves of the molecule were equivalent, the CO_2 should be derived equally from both moieties and the specific radioactivity should remain constant. However, the specific radioactivity was much higher than predicted and approached 80 percent of total CO_2 released.

If, then, the reducing glycosyl moiety of cellobiose, yielding D-glucose after phosphorolysis, and exogenous D-glucose are metabolically equivalent, the respiratory CO_2 evolved from a mixture of ^{14}C-cellobiose and D-glucose (equivalent on a hexose basis) should approach the specific activity of the mixture. Again, the observed specific activity of $^{14}CO_2$ evolved was higher than predicted, 41 percent. Since the reducing moiety of cellobiose contained only 25 percent of the total carbon present and it contributed 41 percent of the CO_2 evolved, these data indicated that at least in C. gilvus, the two halves of the cellobiose molecule are metabolized differently.

Hayano and Fukui recently reported a novel conversion of cellobiose to 3-ketocellobiose by the microorganism Agrobacterium tumefaciens (54). The 3-ketocellobiose was prepared by incubating cells of A. tumefaciens aerobically with cellobiose for three hours and isolating the product from the culture medium. The enzyme involved, D-glucoside-3-dehydrogenase, can convert several D-glucosides to their corresponding 3-ketoglucosides; namely, sucrose, trehalose, maltose, and lactose. The keto group is located in the non-reducing ring.

An epimerase acting on cellobiose was reported recently by Leatherwood and coworkers (55, 56). The enzyme was partially purified from Ruminococcus albus. The product of the epimerase reaction is 4-O-β-D-glucosylmannose. The

structure of this compound was proven by reducing the glycosylmannose with sodium borohydride followed by hydrolysis in 1N HCl for one hour at 100°C. Paper chromatographs of the hydrolyzed compound when sprayed with aniline hydrogen phthalate showed only D-glucose; therefore, the mannose was formed from the reducing moiety. This is the only biochemical metabolic reaction on the reducing moiety of a disaccharide that has been reported. Another interesting fact is that no keto intermediate was found during this epimerization, and the authors have proposed a carbanion-type intermediate.

3. Recent Work on β-Glucoside Phosphorylation and Transport

In 1964 Kundig and Roseman reported a system for the phosphorylation of sugars which has completely changed the emphasis of studies in the areas of transport and phosphorylation of sugars (57). This PEP-dependent phosphotransferase system consists of at least three proteins which function according to the reactions below (57-63):

$$\text{PEP} + \text{HPr} \xrightarrow[\text{Mg++}]{\text{Enz I}} \text{P-HPr} + \text{Pyruvate}$$

$$\text{P-HPr} + \text{Sugar} \xrightarrow[\text{Mg++}]{\text{Enz II}} \text{Sugar-P} + \text{HPr}.$$

The first reaction consists of the phosphorylation of a heat-stable protein, HPr, using PEP as the phosphoryl donor

and an enzyme, designated Enzyme I, as the catalyst. Enzyme I is a soluble enzyme which has no sugar specificity. Enzyme II, on the other hand, is sugar specific, inducible, and membrane bound. This system originally was reported as existing in Escherichia coli, Aerobacter aerogenes, and Aerobacter cloacae, but it was extended by several groups while working with the β-galactosidase system to include Staphylococcus aureus (64-67). Since Enzyme I and HPr are not sugar specific but function in the phosphorylation of many different sugars and hence must be present during the metabolism of many sugars, a mutation in the genes which code for either of these proteins would have a pleiotropic effect. That is, a point mutation could affect the growth of a microorganism on many different sugars, which is not the usual single effect shown after a point mutation. A mutation in the Enzyme II gene would result only in the loss of ability to grow on the sugar or sugars for which that particular Enzyme II is specific. Many papers have now been reported documenting this pleiotropic effect in A. aerogenes and S. aureus (60, 66, 68-70, 59, 71-75).

Schaefler and coworkers were the first to publish results of experiments designed specifically to ascertain the mechanism for the utilization of β-glucosides in Enterobacteriaceae (76, 77). Working with Escherichia coli, the wild type of which does not metabolize β-glucosides ($\beta\text{-gl}^-$), he found that inducible mutants ($\beta\text{-gl}^+$) can be isolated

which do so. This inducible system consists of a β-glucoside permease and an aryl-β-glucoside splitting enzyme(s). Both of these proteins are induced by aryl and alkyl-β-glucosides. In $\beta\text{-gl}^-$ and noninduced $\beta\text{-gl}^+$ cells, ^{14}C-TEG is taken up by the constitutive D-glucose permease which has low affinity for aryl β-glucosides. In induced $\beta\text{-gl}^+$ strains a new permease is induced with high affinity for β-glucosides. The aryl β-glucoside splitting enzyme(s) had the following specificity (expressed in specific activities): ONP-β-glucoside 180, PNP-β-glucoside 102, phenyl-β-glucoside 98, salicin 82, arbutin 60. Inducers of the enzyme include salicin, PNP-β-glucoside, ONP-β-glucoside, phenyl-β-glucoside, esculin, methyl-β-glucoside, TPG-β-glucoside, TEG, and TMG. Cellobiose, gentiobiose, amygdalin, and methyl-α-glucoside were not inducers. TEG is reported in this publication to be phosphorylated only when it enters the cell through the D-glucose permease and not through the β-glucoside permease. When the β-glucosides are phosphorylated, they do not serve as inducers for the cleavage enzyme(s) and inhibit competitively the induction by the unphosphorylated derivative. Therefore, all of the β-glucosides which serve as inducers for the cleavage enzyme(s) must enter in an unmodified form through the β-glucoside permease. However, some of these data were refuted by Fox and Wilson and will be dealt with later.

Schaefler's second paper (77) treats the genetics of the system. Two mutants were obtained by treatment of

β-gl$^+$ E. coli with nitrosoguanidine; one was constitutive for aryl-β-glucoside cleavage enzyme(s) and for the aryl-β-glucoside permease, and the second had lost the ability to ferment salicin but retained the capacity to ferment arbutin and other aryl-β-glucosides. The β-gl$^+$ sal$^-$ organism does not cleave salicin, but does cleave other aryl-β-glucosides, does possess the β-glucoside permease, and salicin acts as a gratuitous inducer for both enzymes. Induced or constitutive β-gl$^+$ sal$^-$ cells transport ^{14}C-TEG and salicin through the β-glucoside permease, but the lack of cleavage of salicin indicates that the splitting enzyme is not induced or present. Therefore, it seems that there are two aryl-β-glucoside cleavage enzymes. The system for β-glucoside metabolism, then, seemed to consist of four genes, one for a β-glucoside permease, two for β-glucoside cleavage enzymes, and one for a regulatory gene.

The situation was clarified somewhat by a later publication by the same group using Aerobacter aerogenes (78). Here Schaefler and his coworker report evidence for the existence of two permeases. β-glucoside permease I is induced by aryl and alkyl β-glucosides with high affinity for aryl-β-glucosides and low affinity for cellobiose. This permease is similar to the β-glucoside permease already described. A second permease, β-glucoside permease II, is induced by cellobiose and lactose and has high affinity for both aryl-β-glucosides and cellobiose. Enzymatic data on

the cleavage enzymes showed a 2- to 3-fold increase in induced versus noninduced cells. Crude extract was chromatographed on DEAE-cellulose in an attempt to purify the cleavage enzymes. However, three peaks were obtained, one containing a gl-phosphotransferase and the other two phospho-β-glucosidases, designated A and B. The glucoside (gl) phosphotransferase functions to phosphorylate β-glucosides. Glucose-6-phosphate, β-glycerophosphate, and fructose diphosphate are the best phosphoryl donors, while PNP-β-glucoside, ONP-β-glucoside, phenyl-β-glucoside, and salicin serve as acceptors. Both phospho-β-glucosidases cleave only the phosphorylated derivatives of the sugars. Substrate specificities distinguish the two phospho-β-glucosidases from one another. Phospho-β-glucosidase A has the following specificity (in terms of units, 1 U = 1 mμmole aglycon liberated/min.): P-ONP-β-glucoside 56, P-phenyl-β-glucoside 49, P-PNP-β-glucoside 31, and P-Salicin 4, In contrast, phospho-β-glucosidase B shows the following reactivity: P-Salicin 40, phenyl-β-glucoside 30, P-ONP-β-glucoside 34, P-PNP-β-glucoside 16. It is important to note that cellobiose is not a substrate for the gl-phosphotransferase and, therefore, not a substrate for the phospho-β-glucosidase enzymes. The main idea that Schaefler tries to emphasize is that the coupling of the PEP phosphotransferase system is required for the accumulation of β-glucosides by both permeases, an idea that is substantiated in part but not to a complete extent.

Fox and Wilson then published a paper on the role of the PEP-dependent phosphotransferase system in β-glucoside catabolism in E. coli (60). They concluded that, in contrast to Schaefler's work (76), any β-glucoside entering the cell by the β-glucoside permease was phosphorylated by the system of Kundig and Roseman, and the sugar then accumulated as its phosphorylated derivative. A species of Enzyme II was found with high specificity for β-glucosides, II-β-gl. Enzyme I mutants do not accumulate either methyl-β-glucoside or TPG. They found that enzymes I and II, β-gl, PEP, and Mg^{++} are required for the hydrolysis of PNP-β-glucoside by extracts. In other words, the phosphorylated derivative is cleaved, not the free sugar. In E. coli two phospho-β-glucosidases are present; A is found constitutively in both $\beta\text{-gl}^-$ and $\beta\text{-gl}^+$ strains, and B is under the same regulator gene as enzyme II β-gl. Both are soluble enzymes, and again the distinguishing feature is that A does not cleave P-salicin and B does. Since extracts of enzyme I and enzyme II negative mutants contain high levels of phospho-β-glucosidase, the phosphorylated derivative does not serve as the inducer for this enzyme.

Discussion

The main function of this literature search was to bring the field of α- and especially β-glucoside metabolism into perspective by discussing the state of knowledge of this area of biochemistry at the initiation of the research

presented in this thesis. Prior to 1964 there were only two known pathways for glucoside metabolism; they were hydrolysis and phosphorolysis. The hydrolytic mechanism was accomplished either by a specific or non-specific glucosidase. Specific glucosidases have rigid substrate structural requirements: (i) the glycosidic linkage be α (for α-glucosidases) or β (for β-glucosidases), (ii) the glycosidic linkage occur between specific positions of the two moieties involved, and (iii) two specific moieties be present in the compound. Non-specific glucosidases have one general requirement--that the glycosidic linkage be α (for α-glucosidases) or β (for β-glucosidases). This single structural requirement for a non-specific glucosidase can be met by a myriad of different compounds, with the end result that a whole class of compounds may be metabolized by one enzyme. However, even within these flexible structural requirements a hierarchy of substrate activity may be formulated, such as having a non-specific glucosidase with increased affinity for aromatic or aliphatic glucosides. Specific glucosidases, although less common, are functional for the metabolism of several α- and β-glucosides from many different sources. The other mechanism, phosphorolysis, is much less widespread as a metabolic pathway at this time. Those phosphorylases which have been reported (for cellobiose, maltose, laminaribiose, and trehalose) are specific enzymes. Cellobiose phosphorylase is the only enzyme which can utilize several sugars other than D-glucose as acceptors

for α-D-glucose-1-phosphate in the direction of synthesis. In 1964 the PEP-dependent phosphotransferase system was reported (57), and with it a third method for glucoside metabolism had been found. The initiation of the metabolism of the following glucosides by phosphorylation with the components of the PEP phosphotransferase system has been reported: salicin, arbutin, TPG, methyl-α-glucoside, TEG, PNP-β-glucoside, phenyl-β-glucoside, methyl-β-glucoside, and ONP-β-glucoside.

Cellobiose is one of the most ubiquitous compounds in nature. Work on cellobiose degradation had shown two mechanisms for its biodegradation, one by hydrolysis and the other by phosphorolysis. Only two other biochemical transformations of cellobiose have been reported--epimerization and oxidation. However, with the advent of the PEP-phosphotransferase system, Schaefler, in working on β-glucoside metabolism, implied that cellobiose also was phosphorylated by this system. However, no data were ever reported to substantiate this implication.

Therefore, at the initiation of work on this thesis, some knowledge of cellobiose metabolism had accumulated, but nothing was known of gentiobiose of cellobiitol metabolism in any organism. The investigation undertaken was to elucidate the biodegradative pathways for cellobiose, gentiobiose, and cellobiitol metabolism in Aerobacter aerogenes.

EXPERIMENTAL METHODS

Bacterial Strains

A uracil-requiring auxotroph of *Aerobacter aerogenes* PRL-R3 was used as the wild-type organism. Mutants 47 and 41 were obtained from the wild type as described in the text of Part I of the Results section. Revertants 47R7 and 47R13 were obtained using strain 47 as the parental organism, and revertant 41R4 was obtained from strain 41 as outlined in Results, Part I.

Media

Mineral Medium

This medium consisted of the following components: 1.35 percent of $Na_2HPO_4 \cdot 7\ H_2O$, 0.15 percent of KH_2PO_4, 0.3 percent of $(NH_4)_2SO_4$, 0.02 percent of $MgSO_4 \cdot 7\ H_2O$, 0.005 percent of $FeSO_4 \cdot 7\ H_2O$, 0.005 percent of uracil (Sigma), and 0.5 percent of a specified sugar (autoclaved separately). The concentration of sugar was 0.5 percent unless stated otherwise.

Nutrient Broth Medium

This medium consisted of 5.0 g of Bactopeptone (Difco) and 3.0 g of beef extract (Difco) in 1 l of water. The pH of the solution was adjusted to 7.0 before autoclaving.

Casamino Acid Medium

This medium had the following composition: 1.0 percent of KH_2PO_4, 0.01 percent of $MgSO_4 \cdot 7\ H_2O$, 0.1 percent of $(NH_4)_2SO_4$, 0.005 percent of uracil, and 1.0 percent of casamino acid preparation (Difco). The concentration of sugar used was 1.0 percent.

Growth of Cultures

Growth curves were done in 18 X 150 mm culture tubes containing 7.0 ml of mineral medium (0.3% sugar). The tubes were incubated at 32°C on a reciprocal shaker. Optical density readings were made at 520 nm with a Coleman Jr. spectrophotometer.

For induction studies, cellobiose-positive strains (wild type and revertants) were grown as above in mineral medium (0.5% inducing sugar and harvested after about 8 hours of growth (O.D. of about 0.8). Cellobiose-negative mutants were grown in casamino acid medium. When the growth reached an optical density of 0.4 (about 4 hrs after inoculation) the inducing sugar was added at a

concentration of 1.0 percent and incubation continued for 3 more hours before harvesting.

Cells to be used for enzyme purification were grown in 500 ml of mineral medium in Fernbach flasks at 32°C on a rotatory shaker.

Preparation of Cell Extracts

Cells were harvested by centrifugation and washed once with distilled water, and resuspended in distilled water. The cells were broken by treatment for 15 min in a Raytheon 10-KH_z sonic oscillator equipped with an ice-water cooling jacket. The broken-cell suspension was centrifuged at 45,000 x g for twenty minutes, and the resulting supernatant was the crude extract.

Analytical Procedures

Reducing sugars were determined by the potassium ferricyanide method of Park and Johnson (79). Inorganic orthophosphate was determined by the method of Fiske and SubbaRow (80), and total phosphate by the method of Umbreit, Burris, and Stauffer (81). Total carbohydrate was determined by the anthrone method of Scott and Melvin (82); the molar extinctions of cellobiose and cellobiose-phosphate (quantitated independently by analysis with purified phospho-β-glucosidase) were equivalent. Sugars and sugar-phosphates were chromatographed on Whatman 3 MM (citric acid washed) paper, and developed in the ethyl

acetate, water, acetic acid, formic acid (18:4:3:1, v/v) solvent system. Sugars were also chromatographed on Schleicher and Schuell 589 green ribbon paper, and developed in a solvent system of n-butanol, pyridine, and water (6:4:3, v/v). The sugars and sugar-phosphates were located with a bath of silver nitrate (83). Protein was measured either by using a nomograph distributed by California Biochemicals obtained from the data of Warburg and Christian (84) or by the method of Lowry (85). Periodate oxidations were carried out by the method of Hough and Perry (86). Optical rotations were made with a Zeiss photo-electric polarimeter. Light measurements for colorimetric assays and for bacterial growth were made on a Coleman Junior Spectrophotometer (18-mm diameter round cuvettes). Enzymatic assays were made on a Gilford absorbance recording spectrophotometer (1.0-cm light path) thermostated at 25°C.

Enzyme Assays

β-Glucoside Kinase Assays

This assay was based on the continuous spectrophotometric measurement of ADP (87). The reaction mixture consisted of the following in a volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5), 1 μmole of PEP, 0.1 μmole of NADH, 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 5.0 μmoles of cellobiose, excess crystalline lactate

dehydrogenase and pyruvate kinase, and β-glucoside kinase at concentrations which gave a linear response. A control to correct for NADH oxidase and ATPase contained all of the reaction components except cellobiose. A control for possible cellobiose reductase consisted of the complete reaction mixture minus ATP. The reaction was linear with time and enzyme concentration. A unit of β-glucoside kinase is defined as the amount of enzyme which resulted in the oxidation of 1 μmole of NADH per minute in this assay.

An alternate assay for the β-glucoside kinase used to establish the independence of the reaction for PEP and dependence for ATP consisted of the following components in 0.15 ml; 10 μmoles of glycylglycine buffer (pH 7.5), 0.1 μmole of NADP, 1.0 μmole of cellobiose, 0.5 μmole of ATP, 1.0 μmole $MgCl_2$, an excess of purified phospho-β-glucosidase, an excess of glucose-6-phosphate dehydrogenase, and an amount of β-glucoside kinase to give a linear response.

Phospho-β-Glucosidase Assay

Phospho-β-glucosidase activity was followed by the spectrophotometric measurement (340 nm) of the reduction of NADP by glucose-6-phosphate dehydrogenase. The reaction mixture consisted of the following components in a volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5),

0.1 µmole of NADP, 0.1 µmole of cellobiose-phosphate, an excess of glucose-6-phosphate dehydrogenase, and an amount of phospho-β-glucosidase which gave a linear response.

Assay for PEP-Dependent Phosphorylation of Cellobiose

An assay for the phosphorylation of cellobiose by the PEP-dependent phosphotransferase system of Kundig and Roseman was carried out by the method of Hanson and Anderson (88) with a few modifications. Notably, the reaction was run at 30°C and flouride ion and mercaptoethanol were included in the reaction mixture. Enzyme II was prepared from three different sources as follows: cells grown overnight in mineral medium plus D-fructose, cellobiose, and L-arabinose in separate experiments were suspended in 0.1 M Tris buffer (pH 7.5) plus 0.001 M mercaptoethanol, and were sonicated for ten minutes. The broken cell suspension was centrifuged at 45,000 x g for twenty minutes and the precipitate was discarded. The supernatant from each was then subjected to ultracentrifugation at 100,000 x g for two hours in a Spinco Model L-2 Ultracentrifuge. The supernatant was discarded, and the precipitate was redissolved in 0.1 M Tris buffer (pH 7.5) and 0.001 M mercaptoethanol; these preparations of enzymes II were used for the experiments to determine the ability of the PEP system to phosphorylate cellobiose.

Assay for D-Glucose with Hexokinase and Glucose-6-Phosphate Dehydrogenase

A quantitative end-point assay for D-glucose consisted of the following components: 10 μmoles of glycylglycine buffer (pH 7.5), 0.1 μmole of $NADP^+$, 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, an excess of hexokinase and glucose-6-phosphate dehydrogenase, 0.01 to 0.04 μmoles of D-glucose. The reaction was monitored at 340 nm by following the reduction of NADP. An optical density change of 0.41 was equivalent to 0.01 μmole of D-glucose.

Assay for D-Glucose with Glucose Oxidase

This assay was used when it was desirable not to have ATP in the reaction mixture. The composition was as follows, in a volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5), 1.0 μmole of $MgCl_2$, 0.01 ml of Glucostat enzyme (2 ml H_2O per vial), 0.01 ml of Glucostat chromogen (5 ml of water per vial), and D-glucose. An optical density change of 0.435 at a wavelength of 430 nm was equivalent to 0.01 μmoles of glucose.

Assay for Fructose-1-Phosphate

This assay consisted of the following components in a total volume of 0.15 ml: 0.1 μmole of NADH, 10 μmoles of glycylglycine buffer (pH 7.5), an excess of D-fructose diphosphate aldolase, α-glycerophosphate dehydrogenase,

triose phosphate isomerase. and D-fructose-1-phosphate kinase (R. W. Walter). An optical density change of 0.82 at 340 nm was equivalent to 0.01 μmole of D-fructose-1-phosphate. It should be noted that, since these experiments were done, it has been established that K^+ is required for maximal activity of D-fructose-1-phosphate kinase (101).

Assay for Glucose-1-Phosphate

This assay consisted of the following components in a total volume of 0.15 ml: 10.0 μmoles of glycylglycine buffer (pH 7.5), 0.1 μmole of NADP, and excess phosphoglucomutase and glucose-6-phosphate dehydrogenase. An optical density change of 0.41 at 340 nm was equivalent to 0.01 μmole of glucose-1-phosphate.

Assay for Peroxidase

Peroxidase was determined by measuring the rate of increase in absorbance at 460 nm in a reaction mixture consisting of 0.15 ml of 0.003 percent H_2O_2 in 0.01 M sodium phosphate buffer (pH 6.0), 2 μl of 1.0 percent o-dianisidine in methanol, and 1 μl of peroxidase solution of an appropriate dilution.

Assays for Substrate Specificity of β-Glucoside Kinase

The standard pyruvate kinase-lactate dehydrogenase-linked assay was used except that 5.0 μmoles of the

various sugars tested for substrate activity were substituted for cellobiose.

Assays for Substrate Specificity of Phospho-β-Glucosidase

The standard glucose-6-phosphate dehydrogenase linked assay was used except that 0.1 μmole of the various β-glucoside-phosphates (gentiobiose-, salicin-, arbutin-, phenyl-β-glucoside-, methyl-β-glucoside-, and cellobiitol-phosphates) or 1.0 μmole of the free sugars were substituted for cellobiose-phosphate.

Reagents

Cellobiitol was prepared by a slight modification of the procedure of Smith and coworkers (23). Details for the preparation of this compound are given below. D-fructose-1-phosphate, PEP, PNP-β-glucoside, phenyl-β-glucoside, methyl-β-glucoside, acetyl-phosphate, phenyl-phosphate, methyl-α-glucoside, glucose-6-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase, and β-glycerol-phosphate dehydrogenase were purchased from Calbiochem, Los Angeles, California; protamine sulfate, yeast hexokinase (C-309), salicin, arbutin, gentiobiose, cellobiose, raffinose, D-galactose, D-xylose, sorbitol, L-sorbose, carbamyl-phosphate, 3-phosphoglyceric acid, α-glycerophosphate, phosphocreatine, D-fructose-1,6-diphosphate, choline-phosphate, and ATP from Sigma Chemical

Company, St. Louis, Missouri; NAD, NADH, NADP, NADPH, ITP, UTP, GTP, CTP, and ADP from P-L Biochemicals, Milwaukee, Wisconsin; horseradish peroxidase (HPO 6253) and intestinal alkaline phosphatase (PC 8CA) from Worthington Biochemical Corporation, Freehold, New Jersey; Dowex 1 X 8 (Cl^-) and Dowex 50 (H^+) from the Dow Chemical Company, Midland, Michigan; L-rhmnose, D-ribose, D-mannose, and D-mannitol from General Biochemicals, Chagrin Falls, Ohio; L-arabinose and inulin from Pfanstiehl Laboratories, Waukegan, Illinois; D-fucose, melibiose, D-lyxose, melezitose, trehalose, and lactose from Nutritional Biochemical Corporation, Cleveland, Ohio; phosphoramidate was synthesized by R. L. Anderson by the method of Stokes (100); amygdalin from K and K Laboratories, Inc., Plainview, New York; phosphoglucomutase from Boehringer, Mannheim, Germany; sophorose was the generous gift of Dr. Hewitt G. Fletcher, Jr., of the National Institutes of Health, Bethesda, Maryland; cellotriose and cellotetraose were the generous gifts of Dr. E. T. Reese of the U.S. Army Natick Laboratories, Natick, Massachusetts.

All other chemicals were obtained from standard chemical sources.

Purification of Chemicals

Cellotriose and cellotetraose were chromatographed on Schleicher and Schuell 589 green ribbon paper in a

solvent system consisting of n-butanol, pyridine, and water (6:4:3, v/v) to determine the purity of the preparations. Several contaminating compounds were detected using the alkaline silver nitrate detection system; therefore, preparative paper chromatography was run on these compounds in the same solvent system. The spots were cut out and the compounds eluted with water, concentrated to dryness under vacuum, weighed, and then standard solutions prepared.

Sophorose, when developed in the above solvent system, showed no contaminating compounds; therefore it was used without further purification.

Preparation of Cellobiitol

Cellobiitol was prepared by a slight modification of the procedure of Smith and coworkers (23). Five grams of cellobiose were dissolved in 100 ml of water and to this solution were added 50 ml of a 1 percent solution of $NaBH_4$. The reaction was kept at room temperature and monitored by the loss in reducing sugar using the potassium ferricyanide test (79). The reaction was run for four hours. At this time the solution was acidified with Dowex-50(H^+) to destroy the excess $NaBH_4$ and to remove the sodium ions, and then evaporated to a syrup in vacuo. The syrup was freed of borate by the methyl borate distillation method of Zill and co-workers (89). The mixture was evaporated to dryness yielding an amorphous solid after five distillations with

absolute methanol. Attempts to crystallize the white, amorphous solid were unsuccessful, as have attempts by others (90, 91). The yield of cellobiitol was 4.9 g or a yield of 96 percent. The melting-point range for cellobiitol was 141-144°C, which agrees very well with the value of 143°C obtained by Jones and Perry (90). The compound showed the following rotation: $[\alpha]_{578}^{24}$-8.2 (c 1.0 water). The literature values reported by Jones and Perry (90), Beelik and Hamilton (91), and Wolfrom and Fields (92), were respectively: $[\alpha]_{D}^{24}$ = -7.8° (c 5.0), -7.9° (c 0.94) and -7.8° (c 3.6). Cellobiitol was chromatographed on S. and S. 589 green ribbon paper in a solvent system of n-butanol, pyridine, water (6:4:3, v/v), and a single spot (developed by the alkaline silver nitrate method) was obtained with an $R_{glucose}$ of 0.66. No reducing sugar was present when tested using the potassium ferricyanide test. These data indicated that the cellobiitol was sufficiently pure for use in the studies subsequently to be described in this thesis.

Sucrose Density Gradient Centrifugation

A 3.0-ml linear gradient of 5-20 percent sucrose was employed for sucrose density gradient centrifugation. The gradient was layered with a mixture of 100 µg of β-glucoside kinase and phospho-β-glucosidase (in separate experiments) and 10 µg of horseradish peroxidase. The

centrifugation was run for 12 hours in a Spinco model L-2 centrifuge. At the end of the centrifugation, the bottom of the tube was punctured and 4 drop fractions were collected.

RESULTS--PART I

Elucidation of the Pathways of Cellobiose, Gentiobiose, and Cellobiitol Metabolism in Aerobacter Aerogenes

Since the literature contains reports on the initiation of cellobiose metabolism in various organisms either by direct hydrolysis or by phosphorolysis, the initial investigations were concentrated on determining whether either of these mechanisms was operative in Aerobacter aerogenes. Also, since the PEP-dependent phosphotransferase system of Kundig and Roseman has been shown to function in the metabolism and transport of certain α- and β-glucosides and β-galactosides in some bacteria, the possibility that it participated in cellobiose metabolism in A. aerogenes was also investigated. The results reported below indicate that none of these three mechanisms function in cellobiose metabolism in this organism; rather, a new mechanism involving ATP-dependent phosphorylation of the β-glucoside was discovered.

Direct Hydrolytic Mechanism

β-Glucosidase activity was assayed by incubating cellobiose with crude extract from cellobiose-grown cells and measuring the release of D-glucose with D-glucose

oxidase (Glucostat reagents from Worthington Biochemicals). The reaction mixture consisted of the following components in a total volume of 0.15 ml: 10.0 µmoles of glycylglycine buffer (pH 7.5), 1.0 µmole of $MgCl_2$, 0.01 ml of Glucostat enzyme, 0.01 ml of Glucostat chromogen, 1.0 µmole of cellobiose, and crude extract from cellobiose-grown cells. The reaction was thermostated at 25°C. No D-glucose release was detected by this method (< 0.0002 µmoles of D-glucose per minute per mg of protein), indicating that cellobiose was not being degraded by a hydrolytic mechanism.

A second method to test for cellobiose hydrolase activity involved linkage of the reaction to hexokinase and D-glucose-6-phosphate dehydrogenase. The results of this assay are detailed below under the section, ATP-Dependent Cleavage of Cellobiose.

Phosphorolytic Mechanism

Two different methods were employed to test the possibility of a phosphorolytic mechanism. The first method involved the detection of D-glucose-1-phosphate, which would constitute one product of a mechanism of this type. The mixture consisted of the following components in a total volume of 0.15 ml: 10.0 µmoles of glycylglycine buffer (pH 7.5), 0.1 µmole of NADP, 1.0 µmole of cellobiose, 2.0 µmoles of inorganic orthophosphate, excess phosphoglucomulase and glucose-6-phosphate dehydrogenase, and crude extract from cellobiose-grown cells. No D-glucose-1-

phosphate was detected (< 0.0002 μmoles of D-glucose-1-phosphate/minute/mg of protein). A positive control in which 1.0 μmole of D-glucose-1-phosphate was added resulted in rapid NADP reduction.

The second method involved the measurement of D-glucose release from the proposed phosphorolytic cleavage of cellobiose with D-glucose oxidase (Worthington Biochemicals). The reaction mixture consisted of the following components in a total volume of 0.15 ml: 10.0 μmoles of glycylglycine buffer (pH 7.5), 1.0 μmoles of $MgCl_2$, 0.01 ml of Glucostat enzyme, 0.01 ml of Glucostat chromogen, 2.0 μmoles of sodium phosphate, 1.0 μmole of cellobiose, and crude extract from cellobiose-grown cells. No D-glucose was detected by this method of analysis (< 0.0002 μmoles of D-glucose oxidized/minute/mg of protein). Thus, phosphorolysis, too, seemed unlikely to be the mechanism of cellobiose degradation in A. aerogenes.

Involvement of the PEP-Dependent Phosphotransferase System in Cellobiose Degradation

Another method which could serve to initiate the biodegradation of cellobiose is the formation of a phosphate derivative of the disaccharide by the PEP-dependent phosphotransferase system of Kundig and Roseman (57). The ability of the PEP-dependent phosphotransferase system to phosphorylate cellobiose was tested later in this investigation after two new enzymes had been identified and purified (the

identification and characterization of a β-glucoside kinase and phospho-β-glucosidase are examined in depth later in this Section and in Sections II and III of this thesis) and a method to detect β-glucoside-phosphates enzymatically had been developed. The possible involvement of PEP in cellobiose degradation was tested directly. Enzyme II, isolated from cells grown on L-arabinose, D-fructose, and cellobiose separately (prepared as described in the Methods section), and enzyme I and HPr (R. W. Walter), isolated from mannitol-grown cells, were incubated with D-fructose and with cellobiose. The reaction mixtures were analyzed for D-fructose-1-phosphate and cellobiose-phosphate formation respectively, by end-point assays described in the Methods section. D-Fructose-grown cells served as the positive control as growth on this sugar induces a high level of enzyme II for D-fructose; L-arabinose-grown cells served as the negative control due to the fact that no induction of the enzymes of the PEP-dependent phosphotransferase systems has been reported for this sugar. A comparison of the specific activities for cellobiose-phosphate formation in D-fructose-, L-arabinose-, and cellobiose-grown cells (Table II) showed a very low level of formation for all three; however, the level in cellobiose-grown cells was the lowest (0.0015) as compared to 0.0086 for the other two extracts. The high specific activity for D-fructose-1-phosphate formation shown by the extract of D-fructose-grown cells (0.075)

TABLE II.--Independence of cellobiose cleavage reaction sequence on components of the PEP-dependent phosphotransferase system. [The reaction mixtures consisted of the following components in a total volume of 0.21 ml; 80 µmoles of Tris-HCl buffer (pH 7.5), 0.02 µmoles of mercaptoethanol, 3 µmoles of NaF, 1 µmole of $MgCl_2$, 0.5 µmoles of PEP, 5 µmoles of sugar, and 50 µl each of saturating concentrations of enzyme I and HPr. One mg of protein of each enzyme II preparation was added to start the reaction. The reaction was carried out for ten minutes at 30°C, at which time the tubes were heated in a boiling water-bath for ten minutes, and centrifuged at 3,000 x g for ten minutes. Fifty µl of the reaction mixtures were used for product determination, as described in the Methods section.]

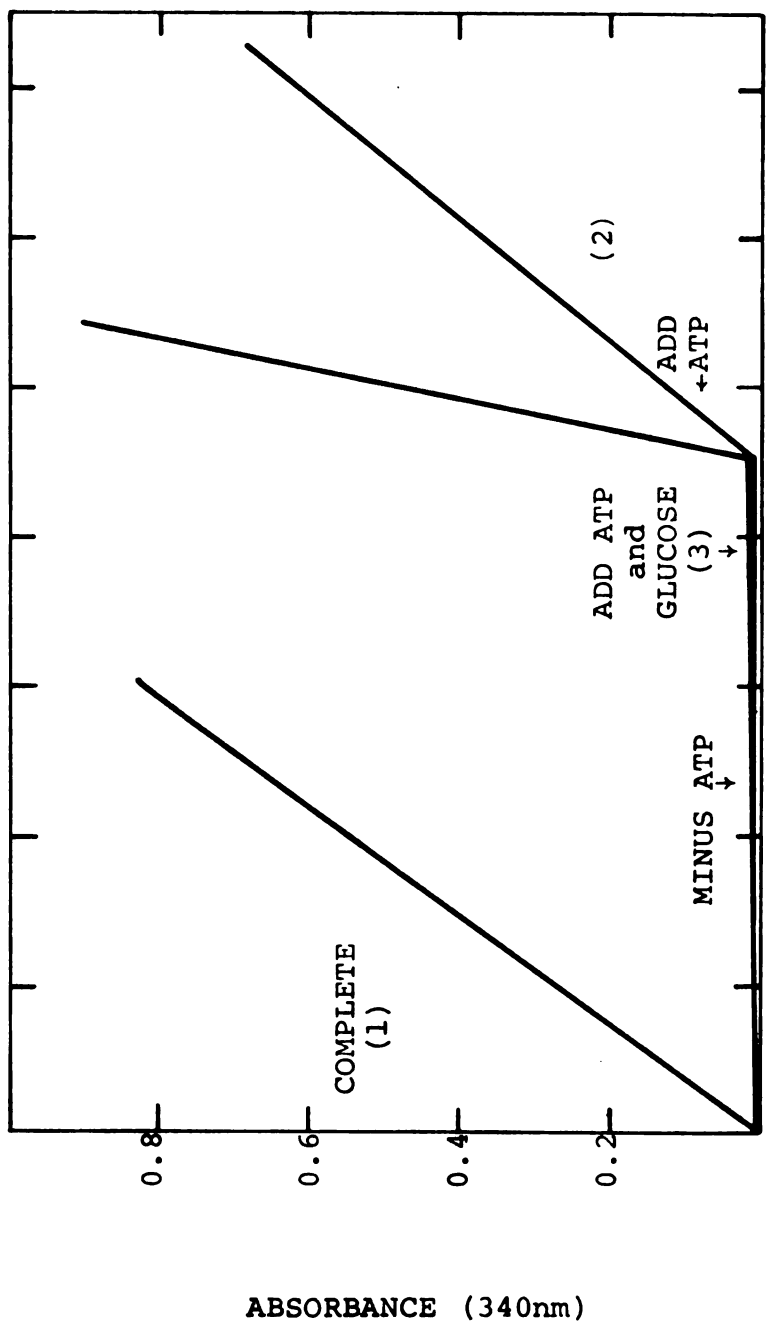
Growth Substrate of Cells Used for Obtaining Enzyme II	Products (µmoles per min per mg protein)	
	Fructose-1-Phosphate	Cellobiose-Phosphate
D-Fructose	0.075	0.0086
Cellobiose	0.038	0.0015
L-Arabinose	0.031	0.0086

indicates that under the conditions of the experiment, the components of the PEP-dependent phosphotransferase system show a high level of induction, and if they are involved in cellobiose degradation, should have been induced. These data suggest that the PEP-dependent phosphotransferase system does not serve to initiate cellobiose degradation by forming the phosphorylated derivative of cellobiose. Confirmation of this by mutant analysis will be presented later in this Section of the thesis.

ATP-Dependent Cleavage of Cellobiose

The first evidence for a pathway involving an ATP-dependent cleavage of cellobiose was obtained when an attempt was made to assay for cellobiose hydrolysis by linking the reaction to hexokinase and D-glucose-6-phosphate dehydrogenase (Figure 1). With the complete reaction mixture, shown by curve 1, NADP reduction did occur, and it was ATP-dependent, which is consistent with the hydrolytic cleavage of cellobiose, However, as shown with curve 2, when ATP was added after nine minutes to the control without ATP, NADP reduction occurred at the same rate as in curve 1. If free cellobiose were being cleaved hydrolytically, D-glucose should have accumulated during the nine-minute incubation without ATP, and the rate of curve 2 should have been that of curve 3, which had both D-glucose and ATP added at nine minutes. These data indicated that

Figure 1.--Dependence of cellobiose cleavage on ATP. The complete reaction mixture consisted of the following in a total volume of 0.15 ml: 10.0 µmoles of glycylglycine buffer (pH 7.5), 0.5 µmole of ATP, 1.0 µmole of $MgCl_2$, 0.1 µmole of NADP, 1.0 µmole of cellobiose, crude extract from cellobiose-grown cells, and an excess of both D-glucose-6-phosphate dehydrogenase and hexokinase.


ABSORBANCE (340nm)
0.8
0.6
0.4
0.2
2
4
6
8
10
12
14
TIME (MINUTES)
COMPLETE
(1)
MINUS ATP
ADD ATP
and
GLUCOSE
(3)
(2)
ADD
+ATP

the cleavage of cellobiose was somehow dependent on the presence of ATP.

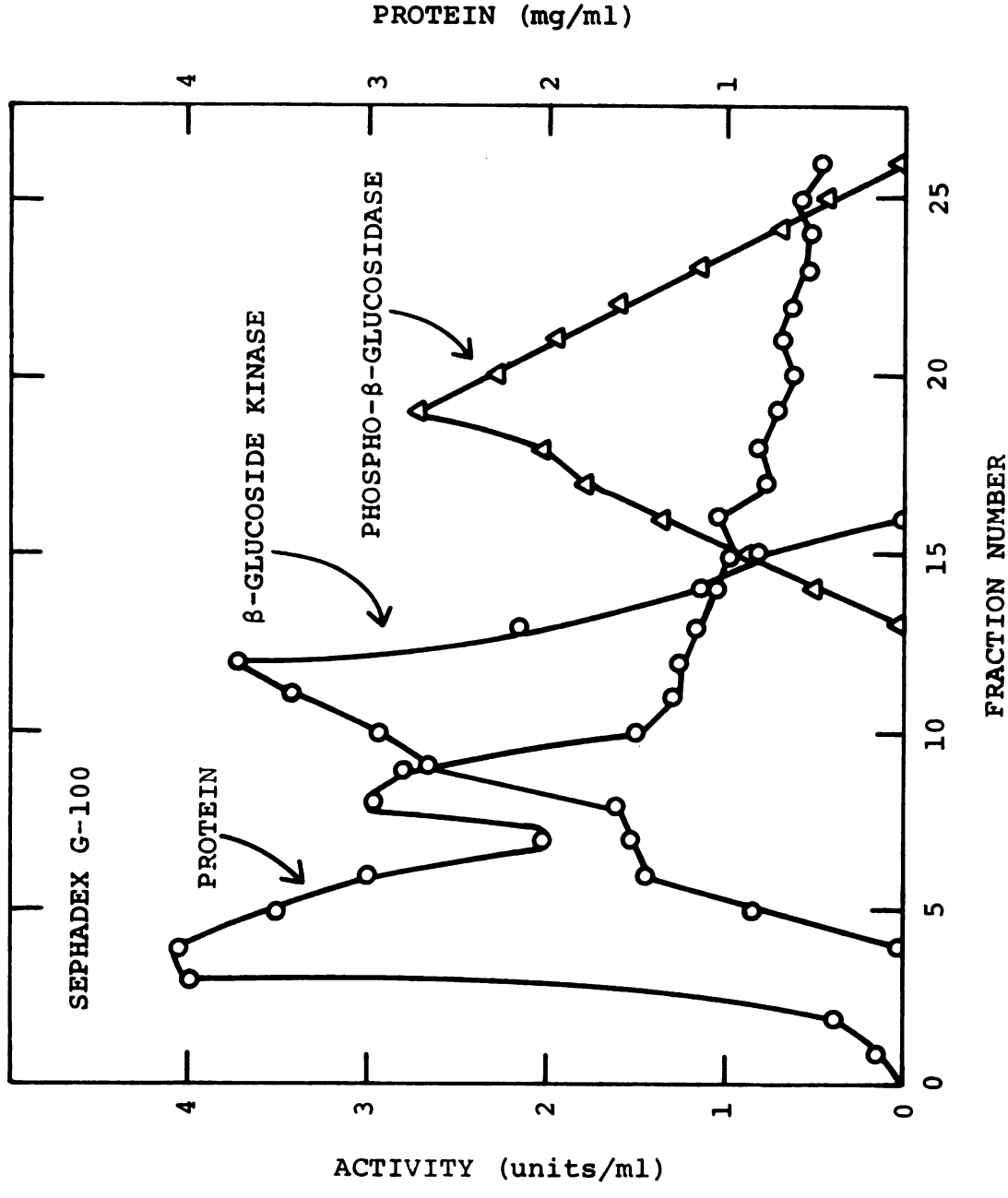
Sephadex Chromatography of the Crude Extract

The components responsible for the ATP-dependent cleavage of cellobiose were separated into two fractions by chromatography of the crude extract on Sephadex G100 (Figure 2). When the fractions collected from the column were assayed for the ATP-dependent cleavage of cellobiose by the same method as that shown in Figure 1, only a small percentage of the original activity was recovered (in fractions 14 and 15 of Figure 2). However, when fractions on either side of 14 and 15 were combined, the activity was restored, suggesting that the ATP-dependent cleavage of cellobiose involved two enzymes. As indicated on the figure, these enzymes subsequently were identified as a phospho-β-glucosidase and a previously undocumented enzyme, one which catalyzes the phosphorylation of β-disaccharides and β-glucosides with ATP, namely a β-glucoside kinase. The purification and characterization of these two enzymes will be given in Parts II and III of this thesis.

Products of the Reaction Sequence

Enzymatic Analysis.--β-Glucoside kinase and phospho-β-glucosidase, purified through Sephadex G100, were incubated with cellobiose, ATP, and $MgCl_2$ to determine the

Figure 2.--Sephadex G100 chromatography of crude extract from cellobiose-grown cells. Fractions were assayed for the ATP-dependent cleavage of cellobiose by measuring the reduction of NADP by the following reaction components: 10.0 µmoles of glycylglycine buffer (pH 7.5), 0.5 µmole of ATP, 1.0 µmole of $MgCl_2$, 0.1 µmole of NADP, 5.0 µmoles of cellobiose, an excess of D-glucose-6-phosphate dehydrogenase, and 10µl of each fraction. Ten-ml fractions were collected.


PROTEIN (mg/ml)
4
3
2
1
SEPHADEX G-100
PROTEIN
β-GLUCOSIDE KINASE
PHOSPHO-β-GLUCOSIDASE
0
5
10
15
20
25
FRACTION NUMBER
4
3
2
1
0
ACTIVITY (units/ml)

stoichiometry of the overall reaction sequence (Table III). These data show that no D-glucose-1-phosphate was formed, but equimolar amounts of D-glucose and D-glucose-6-phosphate were produced per mole of cellobiose utilized. Therefore, the sole products of the metabolism of cellobiose by this pathway appeared to be D-glucose and D-glucose-6-phosphate. This was confirmed by the chromatographic analysis given below.

Paper Chromatography.--Further identification of the products of the overall reaction sequence was obtained by chromatographing aliquants of the reaction mixture shown in Table III on Whatman 3 MM (citric acid washed) paper. Part of the reaction mixture (0.5 ml) was deionized with Dowex 50(H^+), concentrated to 0.2 ml under reduced pressure, and spotted on paper. $R_{glucose}$ values in the solvent system of ethyl acetate, H_2O, glacial acetic acid, formic acid (18:4:3:1, v/v) for D-glucose-6-phosphate, D-glucose-1-phosphate, cellobiose, cellobiose-phosphate, ATP, and inorganic orthophosphate were determined, and are shown in Table IV. In this solvent system all of these components separated after running the chromatogram for 36 hours, except for ATP and cellobiose-phosphate. However, this slight shortcoming did not interfere with the object of the experiment, since ATP does not stain with the alkaline silver nitrate developing agent used, and the other

TABLE III. Demonstration of the products of the overall reaction sequence. [The reaction mixture consisted of 4.0 μmoles of cellobiose, 4.0 μmoles of ATP, 8.0 μmoles of $MgCl_2$, and partially purified β-glucoside kinase (0.26 mg of protein, specific activity = 2.9) and phospho-β-glucosidase (0.16 mg of protein, specific activity = 0.35). The components were incubated for forty-five minutes at 30°C. The reaction mixture was then heated in a boiling water-bath for ten minutes, and the mixture was centrifuged at 45,000 x g for ten minutes. The supernatant then was assayed for D-glucose, D-glucose-6-phosphate, and D-glucose-1-phosphate by end-point assays utilizing the D-glucose-6-phosphate dehydrogenase end-point assay outlined in the Methods section. Phosphoglucomutase was not present in the enzyme preparations.]

Reactant	Products		
Cellobiose	D-Glucose-6-Phosphate	D-Glucose-1-Phosphate	D-Glucose
1.00	0.99	0.00	1.07
1.00	0.98	0.00	1.01
1.00	1.07	0.00	1.03
Average:			
1.00	1.01	0.00	1.04

TABLE IV.--$R_{glucose}$ values for descending paper chromatography of the products of the overall reaction sequence. [Descending paper chromatography on Whatman 3 MM (HCl washed) paper was run for 36 hours in a solvent system of ethyl acetate, water, glacial acetic acid, and formic acid (18:4:3:1,v/v). The reaction mixture is described in Table III and the preparation of the sample for chromatography is described in the text. The spots were developed by the alkaline silver nitrate method.]

Compound	$R_{glucose}$
Glucose	1.00
Glucose-6-P	0.57
Glucose-1-P	0.25
Cellobiose	0.44
Cellobiose-P	0.16
ATP	0.16
Inorganic Phosphate	0.00

sugars and sugar-phosphates had migrated much further down the paper.

Chromatography of the reaction mixture resulted in the identification of three spots, one each for D-glucose, D-glucose-6-phosphate, and unreacted cellobiose (see Figure 3). No D-glucose-1-phosphate was detected. Therefore, this experiment reconfirms the findings of the enzymatic analysis--that is, the sole products of the cleavage of cellobiose by the two-reaction pathway we have discovered are D-glucose and D-glucose-6-phosphate.

Enzymatic Synthesis of Cellobiose-Phosphate

The 110-fold purified β-glucoside kinase (purification procedure described in Results--Part II) was used to biosynthesize cellobiose-phosphate. The enzyme was incubated with 500 μmoles of cellobiose, 500 μmoles of ATP, 1,000 μmoles of $MgCl_2$, and β-glucoside kinase (0.9 units of enzyme). The reaction was incubated at 25°C and the extent of phosphorylation was determined by automatic titration (Sargent recording pH-stat) with 0.05 N NaOH and by enzymatic analysis using purified phospho-β-glucosidase. The progress of the reaction is shown in Figure 4. After the reaction was completed, the mixture was heated on a boiling water-bath for ten minutes, and then the mixture was centrifuged to remove the denatured protein. The solution was cooled, and then deionized with Dowex 50(H^+).

Figure 3.--Identification of reaction products by paper chromatography. Details of the reaction mixture are given in Table III, and the preparation of the sample for chromatography is described in the text. The chromatogram was developed for 36 hours with a solvent consisting of ethyl acetate, water, glacial acetic acid, formic acid (18:4:3:1, v/v).

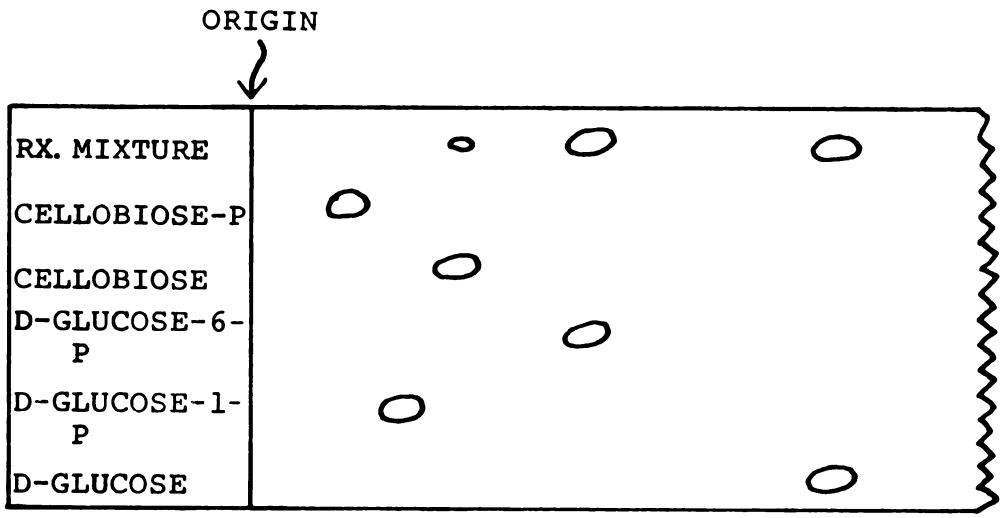
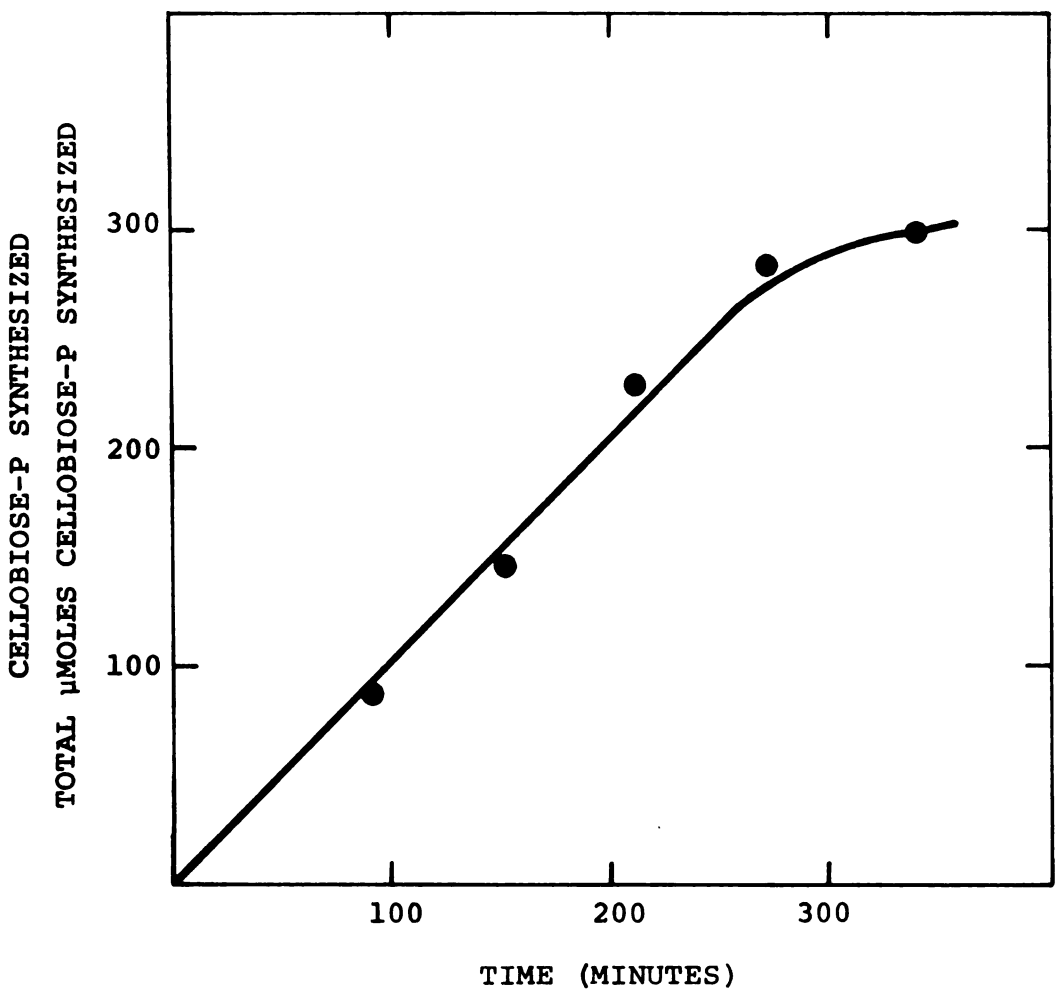

ORIGIN
RX. MIXTURE
CELLOBIOSE-P
CELLOBIOSE
D-GLUCOSE-6-P
D-GLUCOSE-1-P
D-GLUCOSE

Figure 4.--Biosynthesis of cellobiose-phosphate. Details of the reaction mixture are given in the text. The assay for the formation of cellobiose-phosphate consisted of withdrawing aliquants of the mixture, reacting them with purified phospho-β-glucosidase, and measuring D-glucose-6-phosphate release by end-point assay with the standard D-glucose-6-phosphate dehydrogenase assay described in the Methods section.

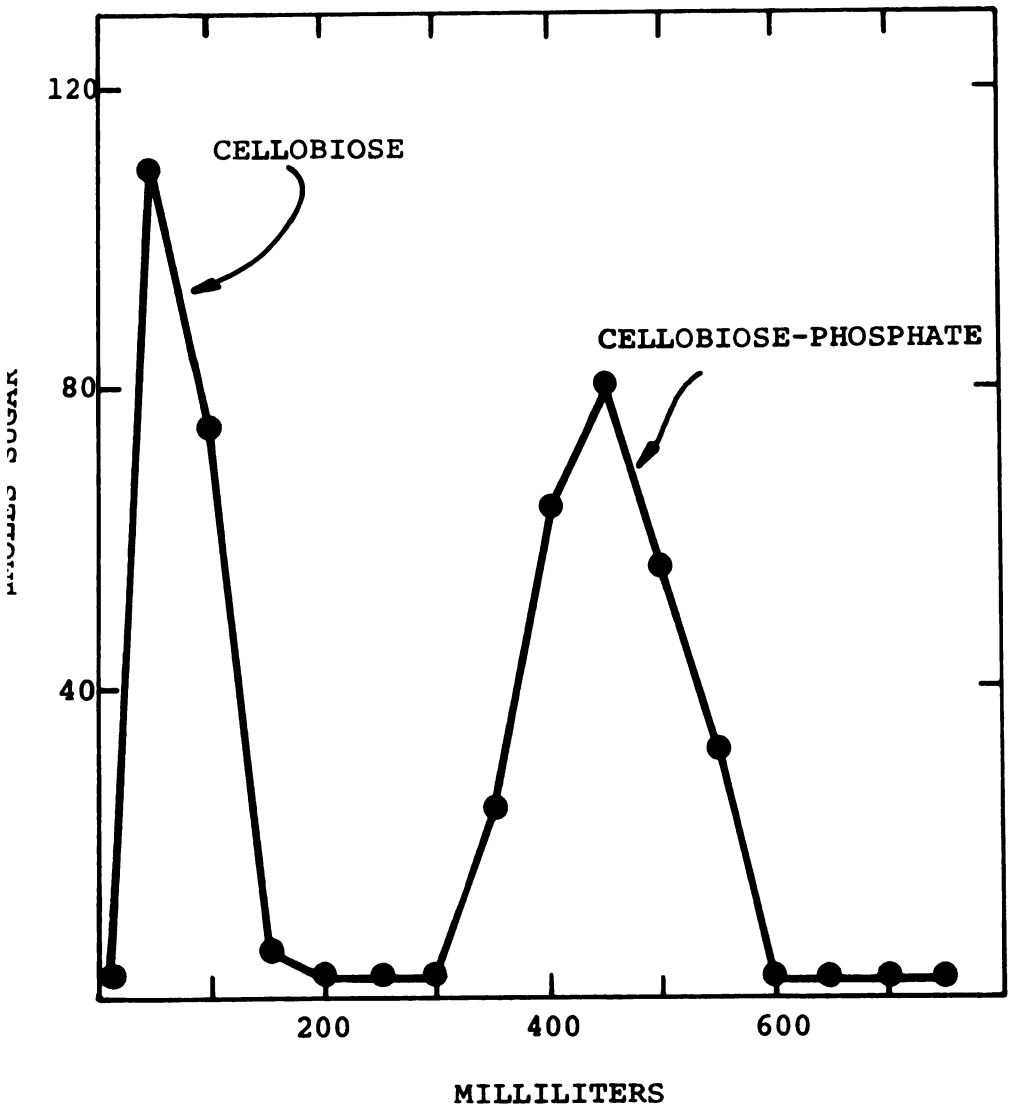

CELLOBIOSE-P SYNTHESIZED
TOTAL µMOLES CELLOBIOSE-P SYNTHESIZED
300
200
100
100
200
300
TIME (MINUTES)

Cellobiose was separated from cellobiose-phosphate by placing the reaction mixture on a Dowex-1 formate column. The column was developed by gradient elution with 1 l of a solution containing 0.4 N formic acid and 0.1 N sodium formate in the reservoir connected to a mixing chamber containing 200 ml of water. Fifty ml fractions were collected and assayed for cellobiose (anthrone test) and cellobiose-phosphate (anthrone test plus total phosphate analysis). The elution pattern for cellobiose and cellobiose-phosphate is shown in Figure 5. The fractions containing cellobiose-phosphate were combined and concentrated under vacuum to approximately 50-ml. A column of Dowex-50 X 8 (1 X 10 cm) was equilibrated with 100 ml of 10 percent cyclohexylamine, and the solution of cellobiose-phosphate (adjusted to pH 8.0 with 1.0 NaOH) was passed through the column to convert the cellobiose-phosphate to the cyclohexylammonium salt. The column was washed with 100 ml of water to elute all of phosphate ester. The eluent was dried under vacuum, and subjected to chemical and enzymatic analysis.

Chemical Characterization of Cellobiose-Phosphate

The biosynthesized cellobiose-phosphate was analyzed chemically by determining the phosphorous to cellobiose ratio and by measuring the amount of inorganic orthophosphate released after treatment of the cellobiose-phosphate with intestinal alkaline phosphatase (Worthington).

Figure 5.--Purification of cellobiose-phosphate on Dowex-1 formate. Cellobiose-phosphate was purified by fractionation on a column of Dowex-1 formate. It was eluted from the column by a formate-formic acid gradient. Details of the procedure are given in the text.


120
80
40
CELLOBIOSE
CELLOBIOSE-PHOSPHATE
200
400
600
MILLILITERS

a. Chemical Analysis.--The cellobiose to phosphorous ratio was determined by using the anthrone test to measure the cellobiose content of the compound, and then performing a total phosphate analysis by a modified procedure of Fiske and SubbaRow. Very little acid labile phosphate was observed per fraction analyzed (approximately 0.1 μmoles), but this control was subtracted from the value obtained from the total phosphate analysis in each experiment. A cellobiose to total phosphate ratio was found to be one to one, indicating that the product of the kinase reaction is cellobiose monophosphate (Table V). Another indication from this set of chemical experiments is that the phosphate is probably esterified to one of the six positions of the cellobiose molecule and not to the one position of the reducing ring, since a phosphate bonded to the one position would be acid-labile. No acid-labile phosphate was found. These results are consistent with the previous finding that the cleavage products are D-glucose-6-phosphate and D-glucose.

b. Analysis after Enzymatic Dephosphorylation.--Cellobiose-phosphate was dephosphorylated with intestinal alkaline phosphatase, and again analyzed for cellobiose and inorganic orthophosphate. After dephosphorylation the amount of inorganic orthophosphate found was again equivalent to the quantity of cellobiose, indicating a one-to-one ratio of phosphate to cellobiose and that the product of

TABLE V.--Chemical analysis of cellobiose-phosphate. [The amount of cellobiose in the sample was determined by the anthrone method (82). Total phosphate was measured by a modified procedure of Fiske and SubbaRow (80). These data are reported as μmoles/ml.]

Experiment Number	Cellobiose	Organic Phosphate
1	10.2	10.4
2	10.0	10.3
3	9.7	10.5
Average	10.0	10.3

the kinase reaction was cellobiose monophosphate (Table VI). This reaction mixture was then deionized with Dowex 50(H^+), concentrated to 0.2 ml under reduced pressure, and chromatographed on Whatman 3 MM paper in ethyl acetate, water, glacial acetic acid, formic acid solvent system (18:4:3:1, v/v) for 36 hours, and on Schleicher and Schuell 589 green ribbon paper in n-butanol, pyridine, water (6:4:3, v/v) for 12 hours. The two chromatograms were developed with the alkaline silver nitrate developing system. In both cases a single spot was detected which co-chromatographed with authentic cellobiose ($R_{glucose}$ in ethyl acetate, H_2O, acetic acid, formic acid = 0.44; $R_{glucose}$ in n-butanol, pyridine, H_2O = 0.68). Thus, it is established that the product of the cellobiose kinase reaction is cellobiose monophosphate.

Enzymatic Analysis of Cellobiose-Phosphate and Determination of the Products of the Phospho-β-Glucosidase Reaction

An experiment was designed with the dual purpose of analyzing cellobiose-phosphate enzymatically with purified phospho-β-glucosidase to determine whether the quantity of product released was equivalent to the quantity of cellobiose-phosphate reacted (as determined chemically) and concomitantly of establishing the equivalence of D-glucose and D-glucose-6-phosphate as products of the reaction. Aliquants of the cellobiose-phosphate, previously characterized chemically, were reacted with purified phospho-β-

TABLE VI.--Alkaline phosphatase cleavage of cellobiose-phosphate. [Intestinal alkaline phosphatase (Worthington), at a concentration of 1 mg/ml, was added to an aliquant of cellobiose-phosphate (total of 5.0 µmoles) in Tris buffer (pH 8.0). The reaction was run for one hour, at which time the mixture was heated in a boiling water-bath for 5 minutes, and then centrifuged at 10,400 x g for 10 minutes. Cellobiose and cellobiose-phosphate were determined by the anthrone test; inorganic orthophosphate was measured by a modified Fiske-SubbaRow procedure. The total volume was 2 ml.]

Compound Analyzed	Quantity (µmoles)
Before Alkaline Phosphatase	
Cellobiose-phosphate	5.0
Total phosphate	5.1
After Alkaline Phosphatase	
Cellobiose	4.3
Inorganic orthophosphate	4.5

glucosidase (purification described in Results--Part III), and the reaction mixture analyzed for D-glucose, D-glucose-6-phosphate, and D-glucose-1-phosphate by end-point assays using the basic D-glucose-6-phosphate dehydrogenase-linked assay. The assay for D-glucose-1-phosphate included phosphoglucomutase and that for D-glucose included hexokinase, ATP, and $MgCl_2$. The exact mixtures are described in the Methods section. The results of these experiments (Table VII) show that equimolar quantities of D-glucose and D-glucose-6-phosphate were produced from the hydrolysis of cellobiose-phosphate, and the quantity of each product released was equivalent to the quantity of cellobiose-phosphate reacted. No D-glucose-1-phosphate was detected. Of equal importance is the fact that there is precise agreement between the chemical and enzymatic characterization of cellobiose-phosphate.

Determination of the Products of the Reaction of Biosynthesized Gentiobiose-Phosphate and Cellobiitol-Phosphate Employing Purified Phospho-β-Glucosidase

Up to this point the emphasis of this investigation has been on the mechanism of cellobiose degradation. However, later on in this portion of the thesis in a section discussing the mutant analysis of the pathway, evidence is

TABLE VII.--Products of the cleavage of cellobiose-phosphate by phospho-β-glucosidase. [Aliquants of cellobiose-phosphate were analyzed for the release of D-glucose, D-glucose-1-phosphate, and D-glucose-6-phosphate after reaction with purified phospho-β-glucosidase. The end-point assays used are described in the Methods section. These data are reported as μmoles/ml.]

Experiment Number	Reactant	Products		
	Cellobiose-P	D-Glucose	D-Glucose-6-P	D-Glucose-1-P
1	10.0	9.91	10.2	0.00
2	10.0	9.94	9.82	0.00
3	10.0	10.0	9.93	0.00
Average	10.0	9.95	9.98	0.00

presented that not one, but three disaccharides--cellobiose, gentiobiose, and cellobiitol--are metabolized by similar mechanisms. Since all three disaccharides are metabolized by a common pathway, the products of metabolism of gentiobiose and cellobiitol will be presented at this time. Gentiobiose-phosphate was synthesized by incubating the following components together in a water bath at 30°C for two hours in a total volume of 2.0 ml: 66.0 μmoles of glycylglycine buffer (pH 7.5), 5.0 μmoles of ATP, 10 μmoles of $MgCl_2$, 5.0 μmoles of gentiobiose, and 0.16 unit of β-glucoside kinase (purified through the DEAE cellulose step). The solution was heated in a boiling water-bath for 5 minutes, centrifuged to remove the denatured protein, and deionized with Dowex 50(H^+). Aliquants of the biosynthesized gentiobiose-phosphate were then reacted with purified phospho-β-glucosidase, and the products--D-glucose, D-glucose-1-phosphate, and D-glucose-6-phosphate--were measured by the same assay methods employed for the product analysis of cellobiose-phosphate. The data (Table VIII) demonstrate that the sole products of this reaction are D-glucose and D-glucose-6-phosphate. No D-glucose-1-phosphate is detected. One mole of gentiobiose-phosphate yielded one mole each of D-glucose and D-glucose-6-phosphate. Therefore, the products from both the cleavage of cellobiose-phosphate and gentiobiose-phosphate, although the linkage between the

TABLE VIII.--Products of gentiobiose metabolism in A. aerogenes. [Aliquants of gentiobiose-phosphate (biosynthesis is described in the text) at a concentration of 3.25 μmoles/ml were analyzed for the release of D-glucose, D-glucose-1-phosphate, and D-glucose-6-phosphate after reaction with purified phospho-β-glucosidase. The standard D-glucose-6-phosphate dehydrogenase assay was employed for quantitative end-point analysis of the products of reaction. The assay for D-glucose-1-phosphate contained phosphoglucomutase to convert D-glucose-1-phosphate to glucose-6-phosphate. Hexokinase, ATP, and $MgCl_2$ were additional components in the assay for D-glucose. These data are normalized to the quantity of gentiobiose-phosphate reacted.]

Experiment Number	Reactant	Products		
	Gentiobiose-P	D-Glucose-6-P	D-Glucose	D-Glucose-1-P
1	1.00	0.99	0.97	0.00
2	1.00	0.98	1.02	0.00
3	1.00	0.98	0.98	0.00
4	1.00	1.03	0.98	0.00
5	1.00	0.97	1.04	0.00
verage	1.00	0.99	0.99	0.00

glucose moieties is different, are the same. These data which demonstrate that D-glucose-6-phosphate is one product of the cleavage of gentiobiose-phosphate are especially important when one is confronted with the problem of ascertaining to which moiety of cellobiose the phosphate group is esterified. Because gentiobiose has the six position of the reducing ring involved in glycosidic linkage, it has only one free six position available for esterification with phosphate, that being on the non-reducing moiety. Therefore, these data suggest that the six position of the non-reducing ring of cellobiose might also be the position of phosphate esterification.

Location of the Phosphate Group in Cellobiose-Phosphate

The following evidence convincingly demonstrated that the phosphate group of cellobiose monophosphate could not be located on the hemiacetal carbon of the reducing ring: (i) no D-glucose-1-phosphate was detected after reaction of the cellobiose monophosphate with purified phospho-β-glucosidase either enzymatically or chromatographically; (ii) no acid-labile phosphate was detected in cellobiose monophosphate by a Fiske-SubbaRow Test, and only after oxidation of the sugar moiety was inorganic orthophosphate detected (total phosphate analysis). However, since there are two free hydroxymethyl groups available (carbon six of each glucose moiety) for phosphate esterification and a phosphate at either position could account for

the observed data, an experiment was designed to determine at which position the phosphate was esterified. Strong indirect evidence, such as the facts that the β-glucoside kinase phosphorylates gentiobiose (which has only one free hydroxymethyl group, and that group is located on the non-reducing moiety) and salicin, arbutin, methyl-β-glucoside, and phenyl-β-glucoside (data on the phosphorylation of these four β-glucosides are presented in Part II of the Results section of this thesis), all of which contain only one hydroxymethyl group, that being in the glycon moiety of the compound, suggests that this phosphorylation takes place on the non-reducing moiety.

To test this hypothesis directly, cellobiitol-phosphate was prepared by sodium borohydride reduction of cellobiose-phosphate. Since cellobiitol-phosphate is a derivative of glucosylglucitol, activity with purified phospho-β-glucosidase using the D-glucose-6-phosphate dehydrogenase-linked assay could only be detected if the phosphate were esterified to the non-reducing glucosyl moiety. If phosphate esterification took place on the glucitol moiety, the product--glucitol-6-phosphate--would be inactive as a substrate for the assay system. Cellobiitol-phosphate was prepared from the following reagents in a total volume of 2.0 ml:2.24 μmoles of cellobiose-phosphate (enzymatic analysis yielded 2.24 μmoles; potassium ferricyanide reducing sugar test gave 2.20 μmoles), and

50 μmoles of $NaBH_4$. The reaction was run for four hours at room temperature, and shaken periodically. Dowex 50(H^+) was added to terminate the reaction, and then the borate was removed by two distillations in methanol under reduced pressure. The product was then dissolved in water for enzymatic and chemical analysis. The potassium ferricyanide reducing sugar test showed that no reducing sugar was present after the reduction.

The chemical analysis employed to quantitate the cellobiitol-phosphate was periodate oxidation. Upon reduction of cellobiose to cellobiitol or cellobiose-phosphate to cellobiitol-phosphate, the number of equivalents of formaldehyde released during periodate oxidation increases by one. In the case of cellobiose to cellobiitol, formaldehyde equivalents increase from two to three; for cellobiose-phosphate to cellobiitol-phosphate, the increase is from one to two. Five controls were run to assure that the analysis was valid; accurate amounts of D-glucose, cellobiose, cellobiitol, D-glucose-6-phosphate, and cellobiose-phosphate were oxidized by sodium periodate, and formaldehyde equivalents were determined by the chromotropic acid reagents (Table IX). These data show that the experimentally determined number of formaldehyde equivalents closely resemble the theoretical yield of formaldehyde, and indicate that periodate oxidation is an accurate method for carbohydrate analysis. On the basis of these data, aliquants of

TABLE IX.--Periodate oxidations of sugars and sugar-phosphates. [The periodate oxidation reaction mixture consisted of the following components in 2.1 ml: 50.0 μmoles of sodium phosphate buffer (pH 8.0), 100 μmoles of sodium metaperiodate, and 1.0 μmole of sugar. (The oxidations were analyzed for formaldehyde at 30 minutes and again after 180 minutes when the reaction should have been complete.) The liberated formaldehyde was measured by the chromotropic acid method (96), which consisted of the following procedure: the sample was diluted to 0.4 ml with water; 26 mg of sodium arsenate was added to remove excess periodate; and 5.0 ml of chromotropic acid reagent (0.2 g dissolved in 20 ml of H_2O and diluted to 100 ml with 12.5 N H_2SO_4) was added. The mixture was then heated in a boiling water-bath for 30 minutes, cooled, and the optical density was read at 570 nm.]

Sugar	μmoles of sugar in reaction mixture	μmoles HCHO / μmole of sugar	Theoretical μmoles HCHO / μmole of sugar
Glucose	1.0	1.06	1.00
Cellobiose	1.0	1.94	2.00
Cellobiitol	1.0	3.07	3.00
Glucose-6-P	1.0	0.00	0.00
Cellobiose-P	1.0	1.04	1.00
Cellobiitol-P	0.19		2.00

cellobiitol-phosphate were oxidized by sodium periodate, and using the theoretical value for equivalents of formaldehyde per equivalent of cellobiitol-phosphate of 2.0, the calculation for μmoles of cellobiitol-phosphate per reaction mixture yielded a value of 0.19. Using this value, a yield of 87 percent was calculated for the borohydride reduction of cellobiose-phosphate giving 1.9 μmoles of cellobiitol-phosphate.

It should also be noted that these data confirm the chemical analysis of cellobiose-phosphate discussed previously, in that the empirical moles of formaldehyde per mole of cellobiose-phosphate is equivalent to the theoretical value.

Based on these data, aliquants of cellobiitol-phosphate were reacted with purified phospho-β-glucosidase and assayed for D-glucose-6-phosphate release by the standard D-glucose-6-phosphate dehydrogenase end-point assay. The data in Table X show that one of the products is, indeed, D-glucose-6-phosphate, and that the quantity of D-glucose-6-phosphate was equivalent to the amount of cellobiitol-phosphate reacted. Therefore, good correlation is obtained for the chemical and enzymatic analysis of cellobiitol-phosphate. Although the reducing sugar test indicated that no reducing sugar was present after the reaction of cellobiose-phosphate, an additional assay was required to insure that the reaction formed glucitol at one end of the molecule and that cellobiose-phosphate was not

TABLE X.--Products of the cleavage of cellobiitol-phosphate by purified phospho-β-glucosidase. [Aliquants of cellobiitol-phosphate (preparation described in the text) were analyzed for D-glucose-6-phosphate after reaction with purified phospho-β-glucosidase. The standard D-glucose-6-phosphate dehydrogenase-linked assay was employed for the quantitative end-point analyses.]

Experiment Number	Reactant Cellobiitol-P	Product D-Glucose-6-P
1	1.00	1.05
2	1.00	1.02
3	1.00	1.02
Average	1.00	1.03

the compound being measured. Since an assay for glucitol was not readily available, hexokinase, which has no activity on glucitol, was used to detect D-glucose release after cellobiitol-phosphate was hydrolyzed by phospho-β-glucosidase. After the D-glucose-6-phosphate end-point was reached, excess hexokinase, ATP, and $MgCl_2$ were added to the reaction mixture. No D-glucose was detected (< 0.0002 μmoles D-glucose/min). These experiments, then, directly demonstrate that the six position of the non-reducing moiety of cellobiose is the site of phosphate esterification.

Mutant Analysis of the Pathway

If a specific biochemical pathway is of significance in the metabolism of a particular compound, isolation of mutants lacking an enzyme of that pathway should exhibit defective growth on that compound. If the enzyme functions in the metabolism of more than one compound, a pleiotropic effect should be realized, even though the mutagenesis involved only one point-mutation. Enzymatic analysis of the mutant organism must show that the defective growth was correlated with the absence of one of the enzymes specifically involved in the metabolic pathway. Since mutant analysis can provide convincing evidence for the authenticity of a pathway, mutants of the uracil requiring auxotroph of A. aerogenes PRL-R3 were prepared.

A. aerogenes was grown overnight in 7 ml of mineral medium containing 0.5 percent glucose. After eight hours

the cells were centrifuged, washed once with water, and resuspended in 14 ml of mineral medium. Ethylmethane sulfonate (0.03 ml) was added to two ml of the resuspended bacteria, which were then shaken in an incubator room at 32°C. After two hours the cells were centrifuged, washed twice with water, and resuspended in two ml of mineral medium. The mutants were then expressed by inoculating 0.05 ml of resuspended cells into 7 ml of mineral medium plus 0.5 percent glucose and were grown overnight. Mutants were selected by plating cells on MacConkey's agar containing 1.0 percent cellobiose and 0.005 percent uracil. The bacteria were allowed to grow for eighteen hours at which time the yellow colonies were picked for purification. These cells were inoculated into a tube of nutrient broth, and grown overnight. They then were streaked on plates of MacConkey's agar plus cellobiose and uracil and allowed to grow for twelve hours. At this time single clones were picked and inoculated into nutrient broth tubes and grown overnight. These mutant strains were then used for enzymatic and fermentation analyses.

Two mutants, mutant 41 and mutant 47, selected for defective growth on cellobiose, were obtained. Figures 6 through 8 show a comparison of the growth rates of the wild type, mutant 47, and mutant 41 organisms on D-glucose, D-galactose, gentiobiose, cellobiose, cellobiitol, salicin, arbutin, phenyl-β-glucoside, and methyl-β-glucoside.

Figure 6.--A comparison of growth rates of wild type and mutant 47 on gentiobiose, arbutin, cellobiitol, D-glucose, and phenyl-β-glucoside. The inocula were grown on nutrient broth.

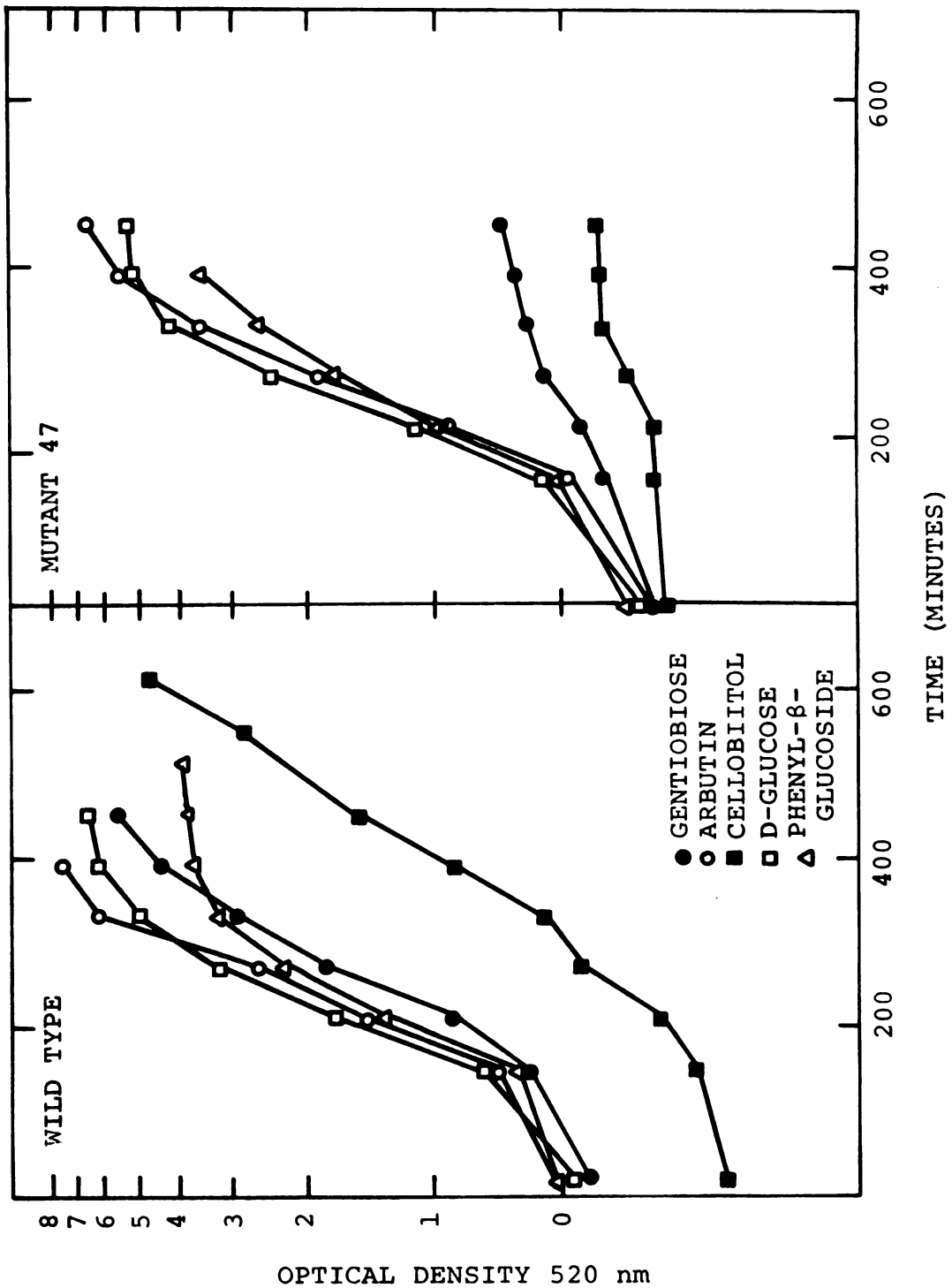

WILD TYPE
MUTANT 47
GENTIOBIOSE
ARBUTIN
CELLOBIITOL
D-GLUCOSE
PHENYL-β-GLUCOSIDE
OPTICAL DENSITY 520 nm
TIME (MINUTES)
0
1
2
3
4
5
6
7
8
200
400
600

Figure 7.--A comparison of the rates of growth of the wild type and mutant 47, on D-galactose, cellobiose, salicin, and methyl-β-glucoside. The inocula for all three were grown on nutrient broth.

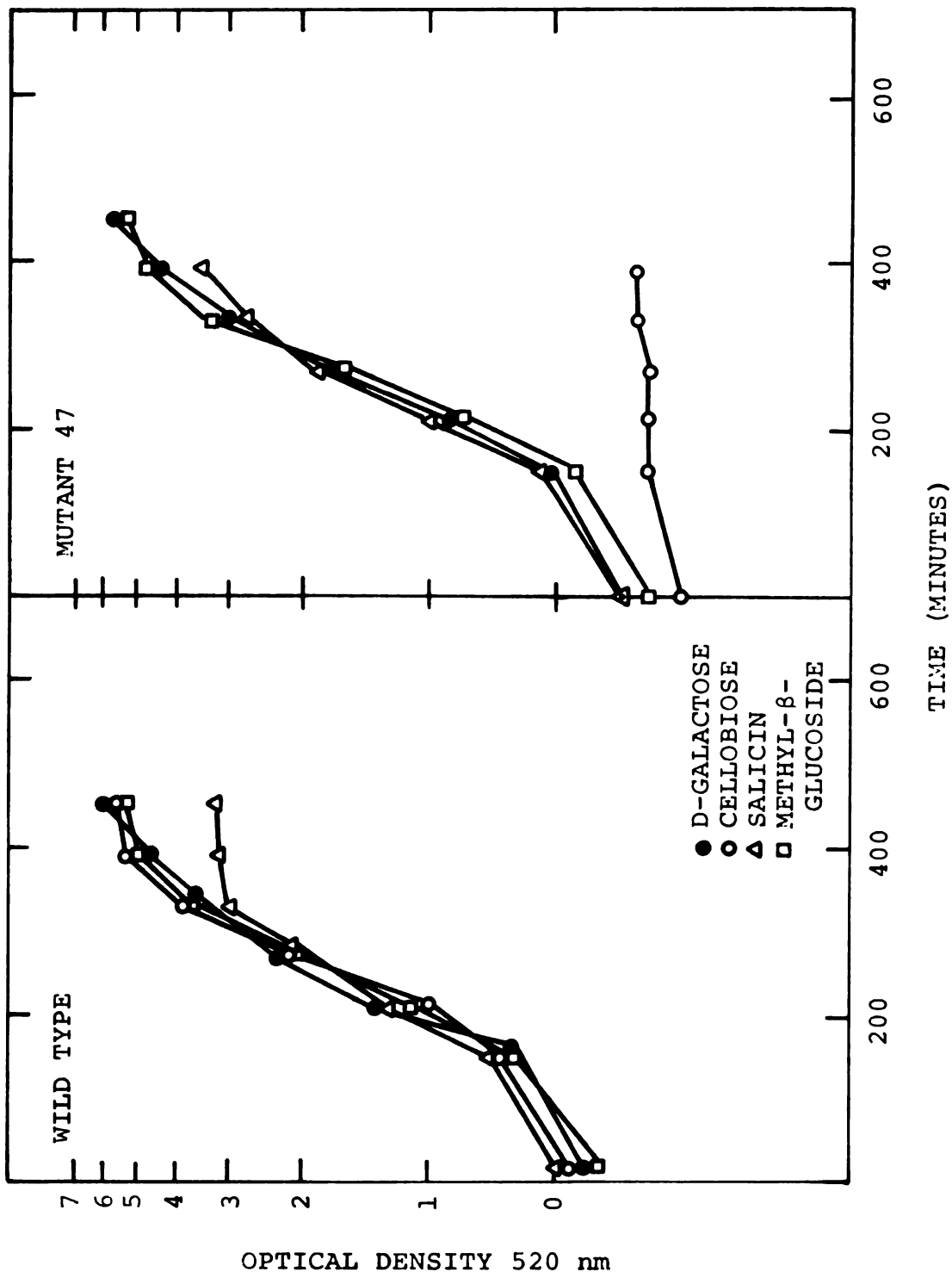
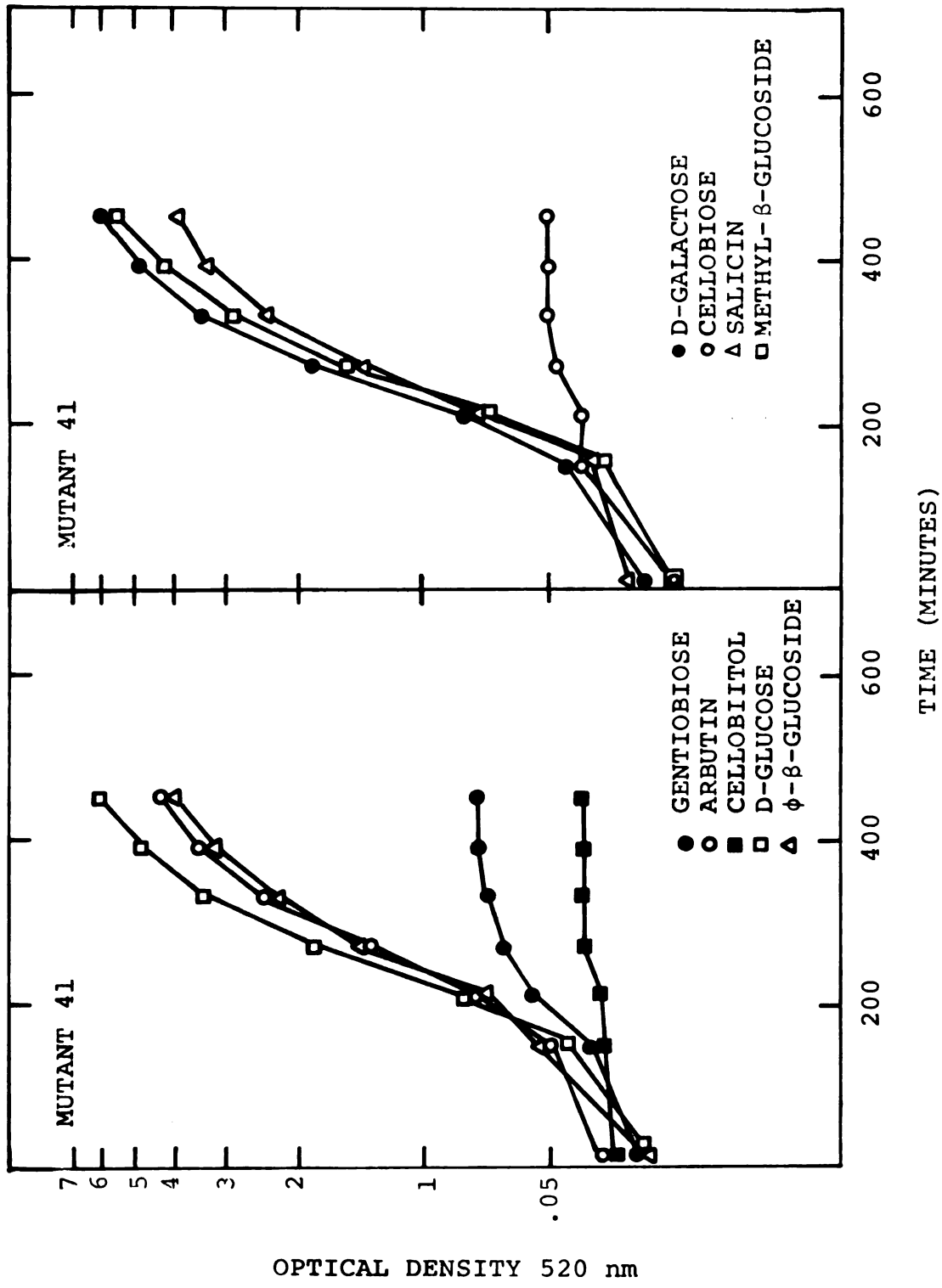

WILD TYPE
MUTANT 47
OPTICAL DENSITY 520 nm
TIME (MINUTES)
0
1
2
3
4
5
6
7
200
400
600
● D-GALACTOSE
○ CELLOBIOSE
△ SALICIN
□ METHYL-β-GLUCOSIDE

Figure 8.--Rates of growth of mutant 41 on gentiobiose, arbutin, cellobiitol, D-glucose, phenyl-β-glucoside, D-galactose, cellobiose, salicin, and methyl-β-glucoside. The inocula were grown on nutrient broth.


MUTANT 41
MUTANT 41
GENTIOBIOSE
ARBUTIN
CELLOBIITOL
D-GLUCOSE
ϕ-β-GLUCOSIDE
D-GALACTOSE
CELLOBIOSE
SALICIN
METHYL-β-GLUCOSIDE
7
6
5
4
3
2
1
.05
200
400
600
200
400
600
OPTICAL DENSITY 520 nm
TIME (MINUTES)

Generation times, calculated from these curves, are listed in Table XI. Growth of all three organisms on D-glucose, D-galactose, salicin, methyl-β-glucoside, arbutin, and phenyl-β-glucoside showed similar generation times. Mutants 41 and 47 failed to grow on cellobiose, and also failed to grow on gentiobiose and cellobiitol. The wild-type cells showed equivalent generation times of fifty-five minutes for both D-glucose and cellobiose, whereas with gentiobiose a slightly greater generation time of sixty-eight minutes is observed. Growth of the wild type on cellobiitol proceeds through an initial lag, and then attains a generation time of ninety minutes. These experiments suggest that the metabolism of β-glucosides involve more than one pathway, with cellobiose, gentiobiose, and cellobiitol being degraded by a common mechanism while salicin, arbutin, methyl-β-glucoside, and phenyl-β-glucoside having one or more alternate pathways not affected by this mutation.

An additional experiment designed to determine whether the lack of growth on cellobiose was due to cellobiose toxicity is shown in Figure 9. Wild-type cells and mutant 47 were grown in a mineral medium containing 0.3 percent of D-glucose and 0.03 percent of cellobiose as the carbon sources. These data show that cellobiose does not interfere with the growth rate of mutant 47 on D-glucose indicating that cellobiose is not toxic for this mutant.

TABLE XI.--Generation times for growth studies of wild-type U⁻ parent and mutants 47 and 41. [The generation times were computed from the growth curves in Figures 6 through 8, 9, 10, and 11.]

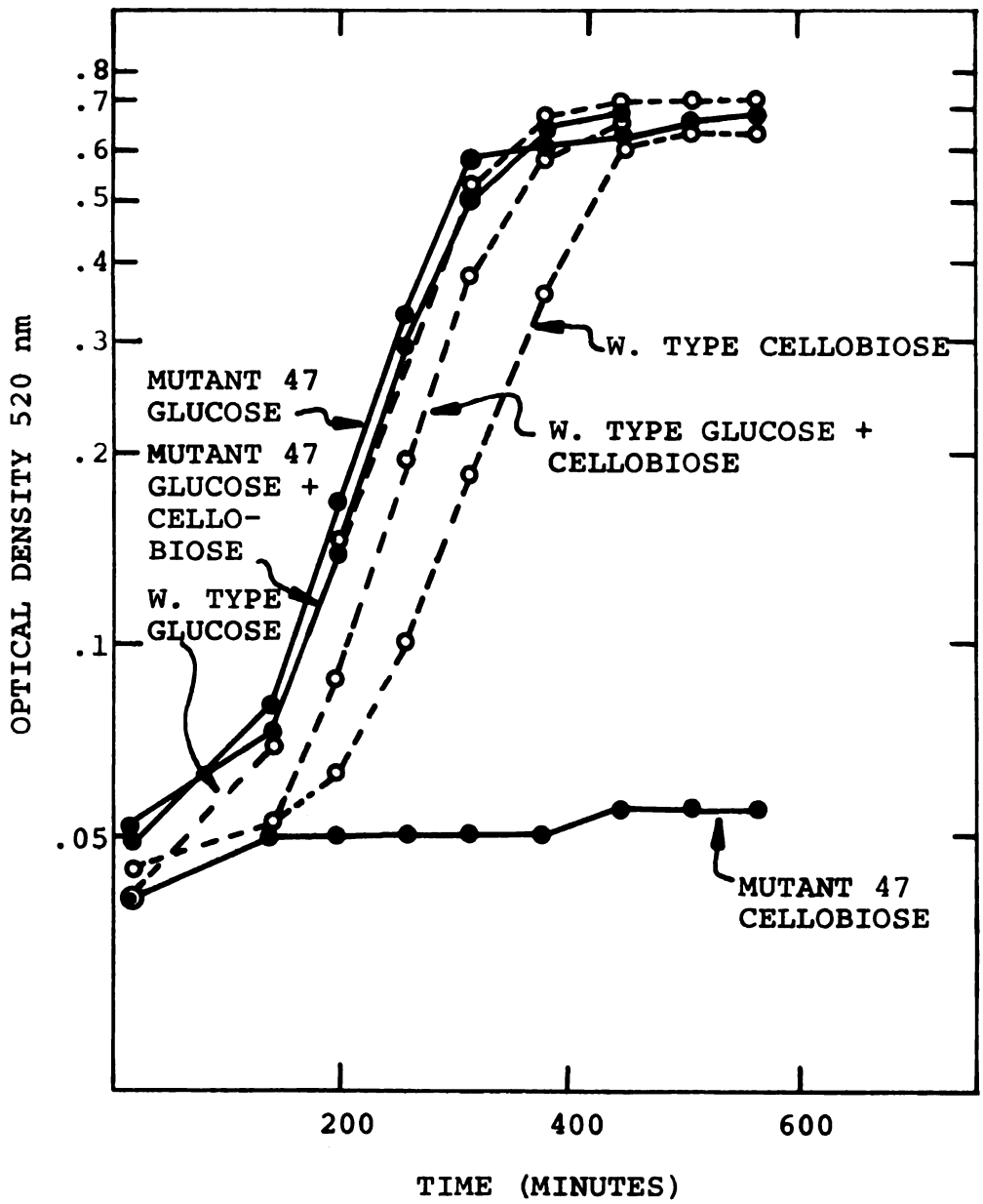
Sugar	Parent	47	41
D-Glucose	55	55	52
D-Galactose	65	65	82
Cellobiose	55	--[a]	--[a]
Gentiobiose	68	--[a]	--[a]
Cellobiitol	90	--[a]	--[a]
Arbutin	58	60	70
Salicin	75	68	70
Phenyl-β-glucoside	60	60	68
Methyl-β-glucoside	68	63	62
D-Mannose	82	78	
D-Mannitol	60	59	
Glycerol	63	72	
D-Ribose	72	74	
Maltose	60	60	
Trehalose	62	64	
Sucrose	62	65	
D-Fructose	68	70	

Note:

Generation times are in minutes.

[a]--Signifies no growth.

Figure 9.--Growth curve for mutant 47 to test for cellobiose toxicity. Mutant 47 and wild-type A. aerogenes (employed as the control) were grown on cellobiose and D-glucose at 0.3 percent concentration, and a mixture of 0.3 percent D-glucose and 0.03 percent cellobiose. The inocula were grown on nutrient broth.


.8
.7
.6
.5
.4
.3
.2
.1
.05
OPTICAL DENSITY 520 nm
MUTANT 47 GLUCOSE
MUTANT 47 GLUCOSE + CELLO-BIOSE
W. TYPE GLUCOSE
W. TYPE CELLOBIOSE
W. TYPE GLUCOSE + CELLOBIOSE
MUTANT 47 CELLOBIOSE
200
400
600
TIME (MINUTES)

To determine whether this lack of growth on cellobiose, gentiobiose, and cellobiitol was a result of an indirect effect arising from a lesion in some other pathway, the wild-type organism and mutant 47 were compared for their ability to grow on eight other mono- and disaccharides, having diverse pathways of metabolism seemingly distant from that proposed for cellobiose metabolism but which might have an indirect effect due to their contribution to the pool of metabolic intermediates. Figures 10 and 11 show a comparison of growth of the wild-type cells and mutant 47 on D-fructose, maltose, trehalose, sucrose, D-ribose, D-mannitol, D-mannose, and glycerol; generation times for growth on these sugars is shown in Table XI. Comparable rates of growth are shown by each organism, suggesting that the defect is direct and affects one of the two enzymes of the proposed pathway for cellobiose, gentiobiose, and cellobiitol.

Enzymatic Analysis of the Wild Type and Mutants 47 and 41

Experiments were designed to determine specifically what enzymatic lesion was responsible for the lack of growth of the two mutants on cellobiose, gentiobiose, and cellobiitol. The wild type and mutants 41 and 47 were grown on a casamino acid medium (medium described in Methods section) until an optical density of approximately 0.4 was reached. At this time one-half of the cells from each cell type was

Figure 10.--A comparison of the growth rates of the wild type and mutant 47 on D-fructose, maltose, trehalose, sucrose, and D-ribose. The inocula were grown on nutrient broth.

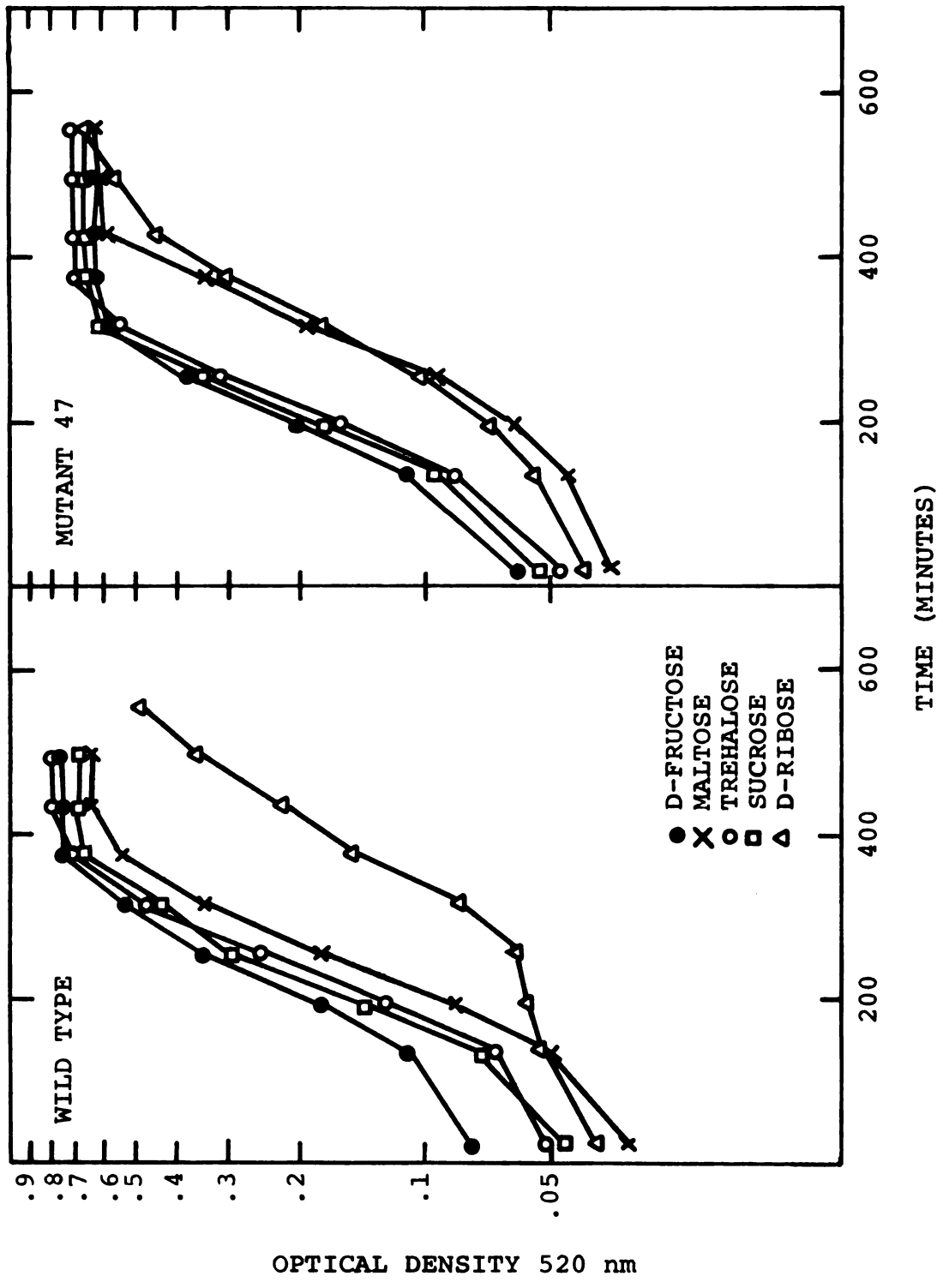
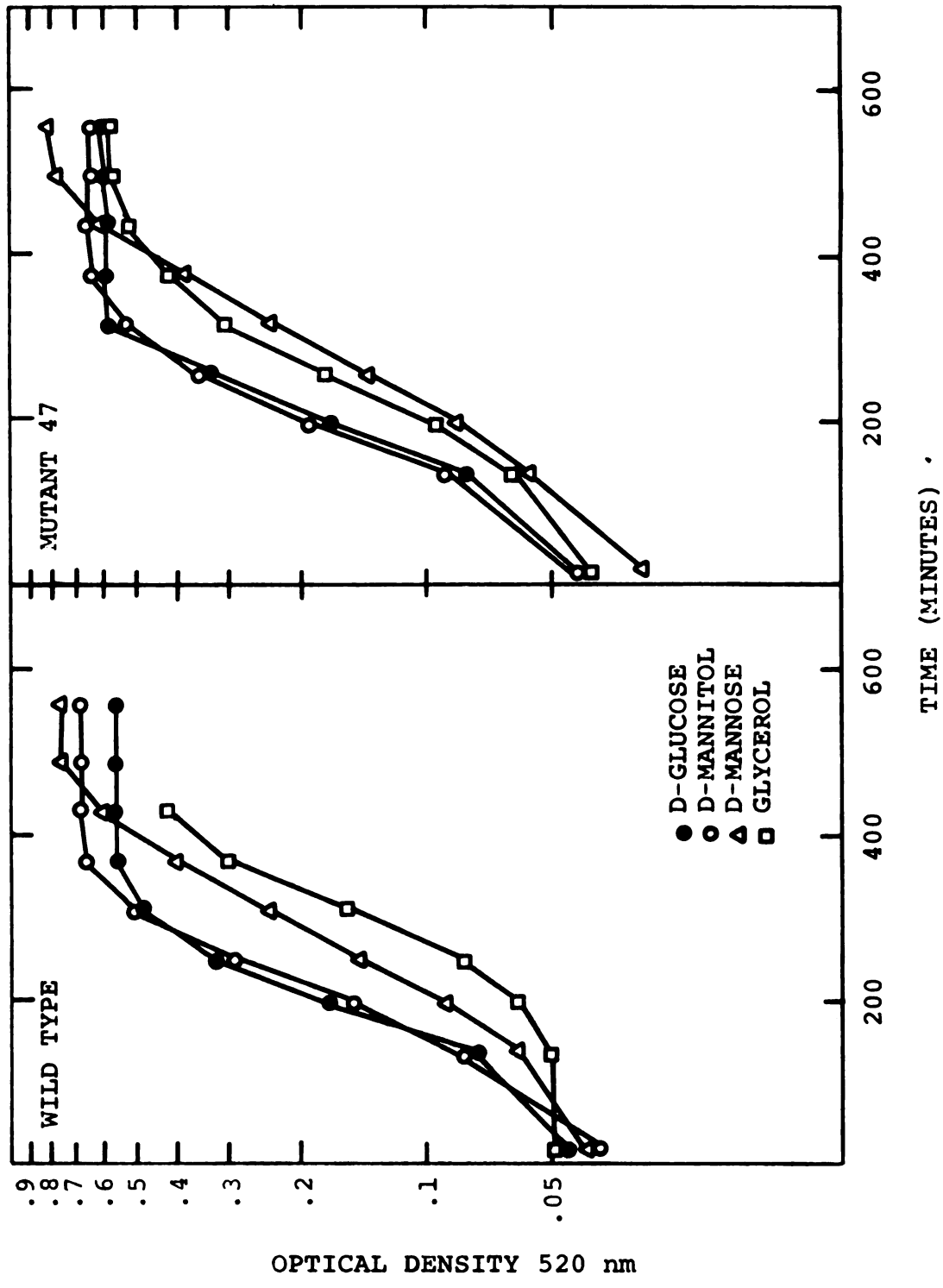

WILD TYPE
MUTANT 47
D-FRUCTOSE
MALTOSE
TREHALOSE
SUCROSE
D-RIBOSE
.9
.8
.7
.6
.5
.4
.3
.2
.1
.05
200
400
600
200
400
600
OPTICAL DENSITY 520 nm
TIME (MINUTES)

Figure 11.--A comparison of the growth rates of the wild type and mutant 47 on D-glucose, D-mannitol, D-mannose, and glycerol. The inocula were grown on nutrient broth.


WILD TYPE
MUTANT 47
D-GLUCOSE
D-MANNITOL
D-MANNOSE
GLYCEROL
.9
.8
.7
.6
.5
.4
.3
.2
.1
.05
200
400
600
200
400
600
OPTICAL DENSITY 520 nm
TIME (MINUTES)

induced with 1 percent cellobiose while the other half received no cellobiose. Both the induced and uninduced cultures were grown for an additional three hours. The cells were then centrifuged, washed, recentrifuged, and crude extracts prepared from each cell type as described in the Methods section. The crude extracts of these organisms were analyzed for both the β-glucoside kinase and the phospho-β-glucosidase. The β-glucoside kinase is an inducible enzyme in the wild-type organism (Table XII). The uninduced enzyme level is < 0.002 μmoles/minute/mg of protein; whereas in the induced organism a specific activity of 0.239 μmoles/minute/mg of protein is reached. In both mutants the induced and uninduced levels of β-glucoside kinase remain constant at the uninduced level. These results indicate that the biochemical lesion produced by the mutation is a loss of β-glucoside kinase activity. The phospho-β-glucosidase in wild-type cells is also inducible, but the enzyme is present at a constitutive level in the uninduced cells. A 4-fold increase in enzyme level occurs in the induced vs the uninduced cells, increasing in specific activity from 0.0115 to 0.0375 μmoles/minute/mg of protein. The phospho-β-glucosidase levels in the two mutants, however, remain constant at the uninduced level of approximately 0.01 μmole/minute/mg of protein. These results suggest that although cellobiose induces the β-glucoside kinase, the product of the kinase reaction,

TABLE XII.--Enzymatic analysis of wild-type cells and mutants 47 and 41. [Crude extracts from cells grown on casamino acid medium (described in Methods) were assayed for both the β-glucoside kinase and phospho-β-glucosidase by the standard assays. These data are presented in terms of specific activities (μmoles/minute/mg of protein).]

Organism		β-glucoside Kinase	Phospho-β-glucosidase
Wild-type	uninduced	<0.002	0.0115
	induced	0.239	0.0373
41	uninduced	<0.002	0.008
	induced	<0.002	0.008
47	uninduced	<0.002	0.0068
	induced	<0.002	0.0068

cellobiose-phosphate, is the inducer for the phospho-β-glucosidase. Since the β-glucoside kinase is not functional in the mutant organisms, cellobiose-phosphate is not synthesized; as a result, the phospho-β-glucosidase remains at the uninduced level.

Revertant Analysis of the Pathway

Single point-mutations causing the loss of a single enzyme activity have a high probability for reversion. Therefore, an attempt was made to prepare and isolate spontaneous revertants of mutants 41 and 47.

Preparation of Revertants of Cellobiose Negative Mutants

Both mutant 47 and 41 were inoculated into 7 ml nutrient broth tubes and grown overnight on a rotary shaker at 32°C. An aliquot of this culture then was plated on MacConkey's agar plus 0.005 percent uracil and 1.0 percent cellobiose. These plates were incubated in a room at 32°C for one to three days. At this time red, revertant colonies were picked and transferred to nutrient broth tubes for overnight incubation on a reciprocal shaker at 32°C. The strains then were purified by the same method as described for purification of mutants. Three revertants were obtained by this procedure. At this time the revertants were ready for enzymatic and fermentation analysis.

Figures 12 through 15 show a comparison of the growth rates of the wild type and revertants 47R7, 47R13,

Figure 12.--A comparison of growth rates of wild type and revertant 47R7, on D-glucose, D-galactose, phenyl-β-glucoside, and methyl-β-glucoside. All inocula were grown on nutrient broth.

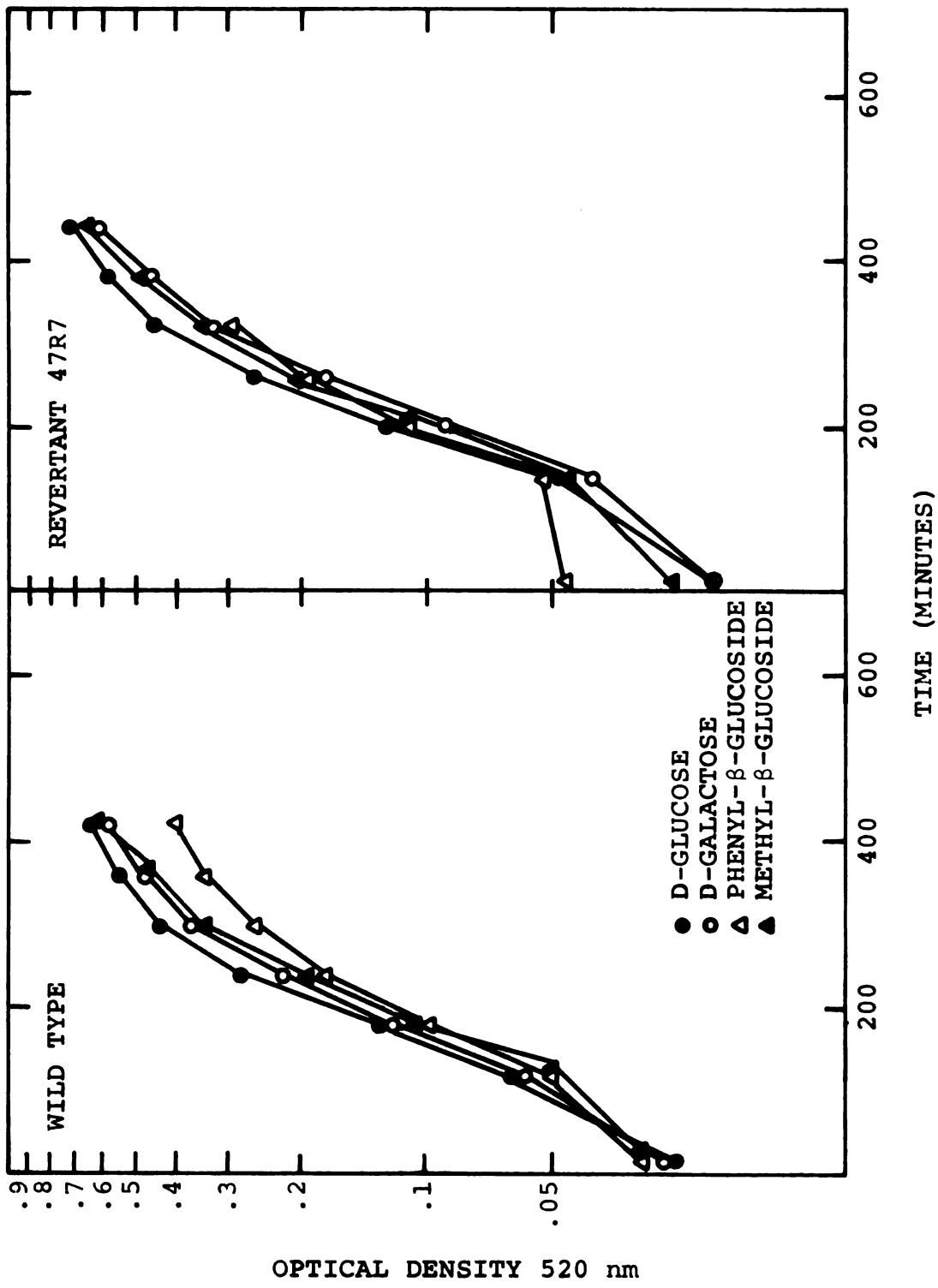

WILD TYPE
REVERTANT 47R7
D-GLUCOSE
D-GALACTOSE
PHENYL-β-GLUCOSIDE
METHYL-β-GLUCOSIDE
.9
.8
.7
.6
.5
.4
.3
.2
.1
.05
200
400
600
200
400
600
OPTICAL DENSITY 520 nm
TIME (MINUTES)

Figure 13.--A comparison of growth rates of the wild type and revertant 47R7, on cellobiose, cellobiitol, gentiobiose, salicin, and arbutin. All inocula were grown on nutrient broth.

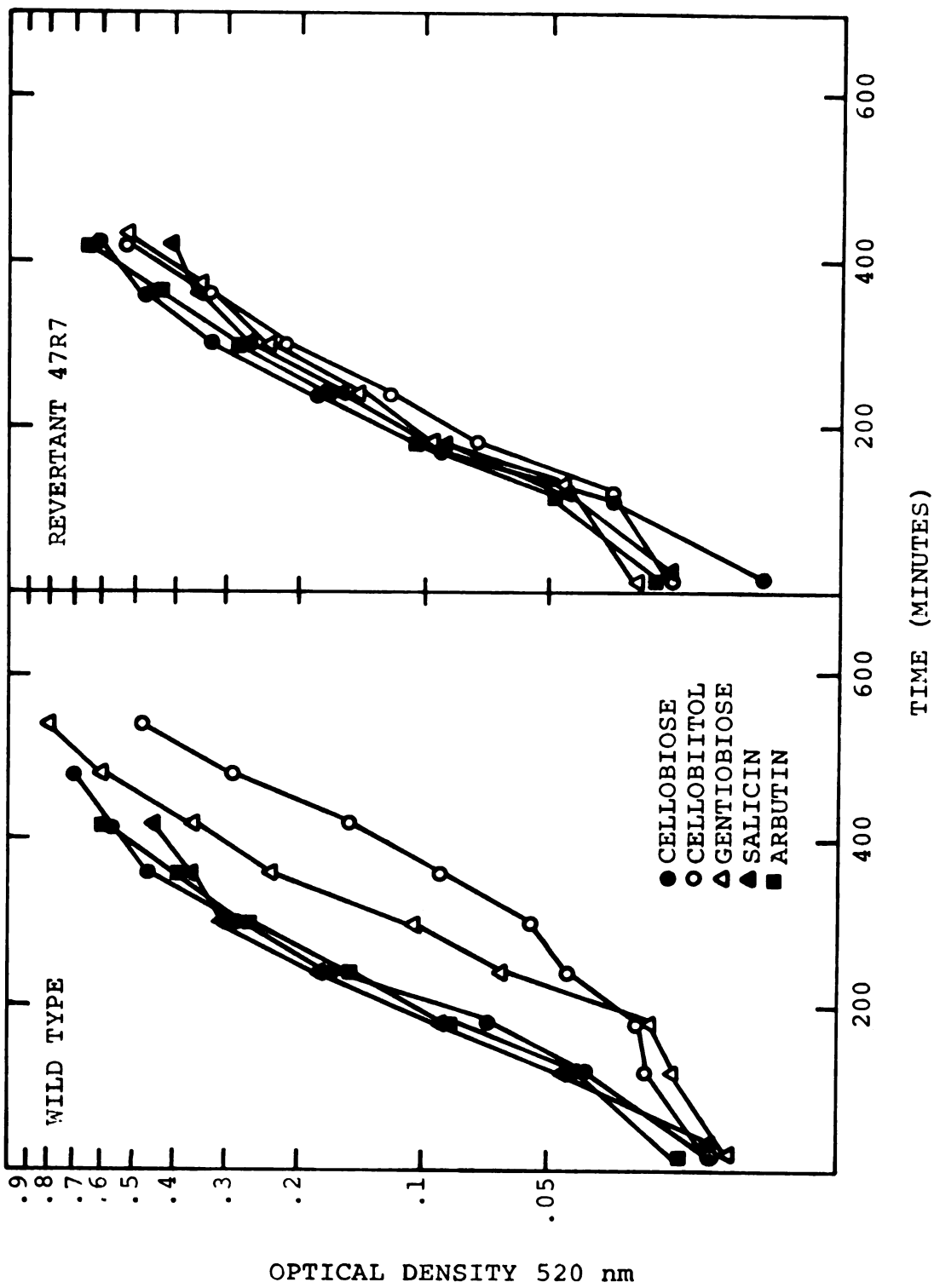

WILD TYPE
REVERTANT 47R7
CELLOBIOSE
CELLOBIITOL
GENTIOBIOSE
SALICIN
ARBUTIN
.9
.8
.7
.6
.5
.4
.3
.2
.1
.05
200
400
600
200
400
600
OPTICAL DENSITY 520 nm
TIME (MINUTES)

Figure 14.--A comparison of growth rates of revertants 47R13 and 41R4 on D-glucose, D-galactose, phenyl-β-glucoside, and methyl-β-glucoside. The inocula were grown on nutrient broth. The growth rates for these two revertants may be compared to those for the wild type depicted in Figure 12.

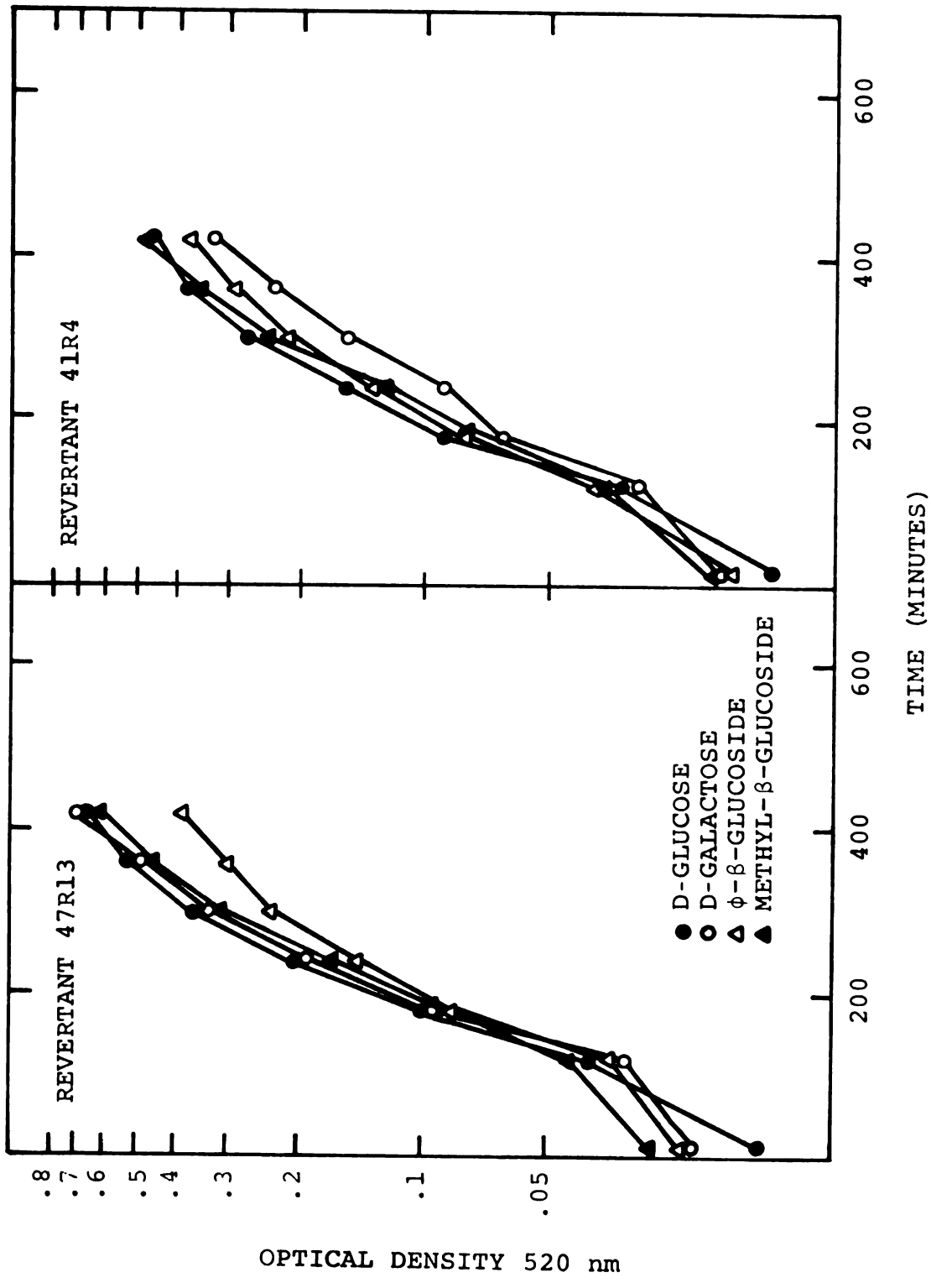
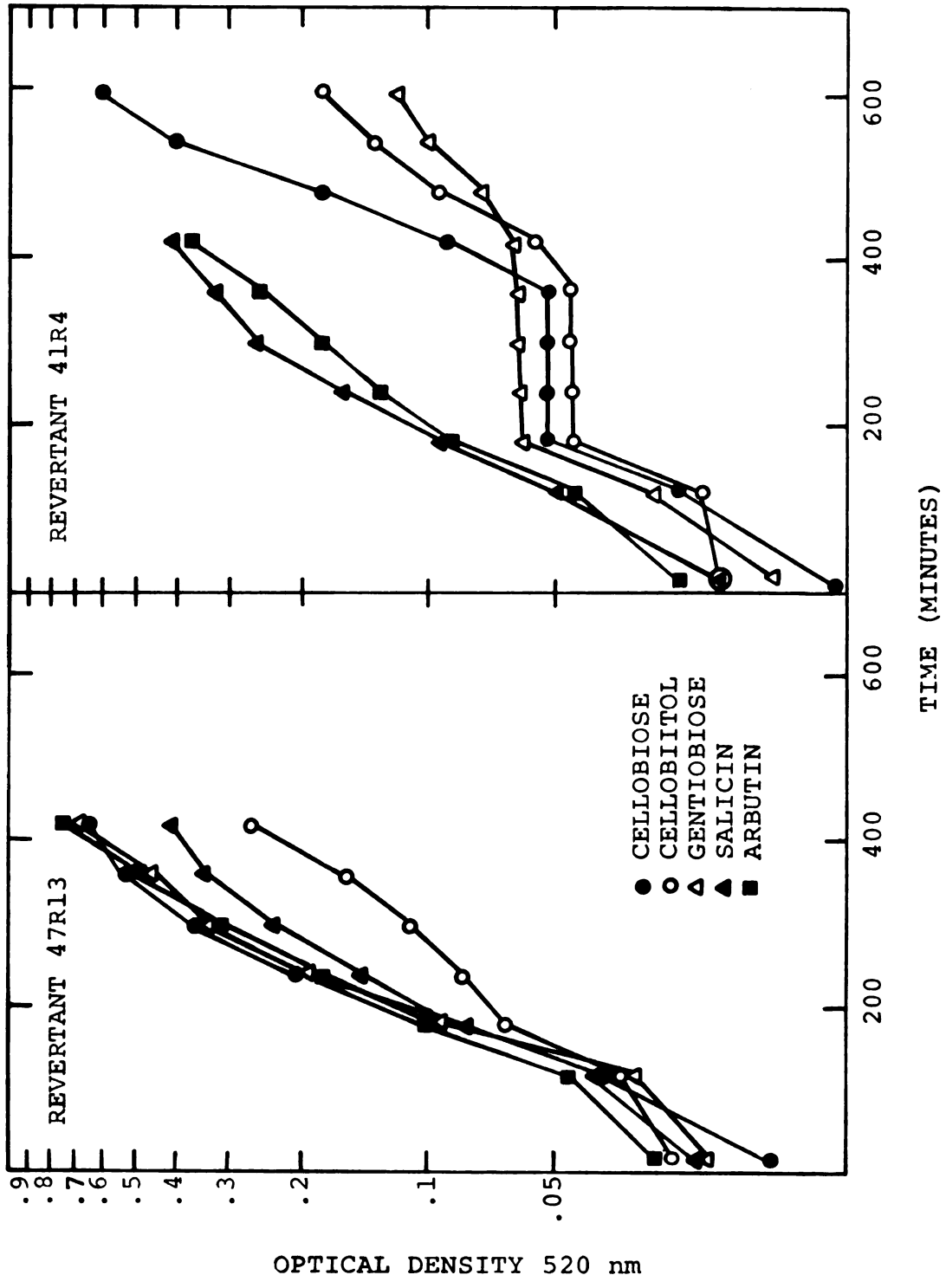

REVERTANT 41R4
REVERTANT 47R13
D-GLUCOSE
D-GALACTOSE
ϕ-β-GLUCOSIDE
METHYL-β-GLUCOSIDE
.8
.7
.6
.5
.4
.3
.2
.1
.05
200
400
600
200
400
600
OPTICAL DENSITY 520 nm
TIME (MINUTES)

Figure 15.--A comparison of growth rates of revertants 47R13 and 41R4 on cellobiose, cellobiitol, gentiobiose, salicin, and arbutin. The inocula were grown on nutrient broth. The growth rates for these two revertants may be compared to those for the wild type depicted in Figure 13.


REVERTANT 47R13
REVERTANT 41R4
CELLOBIOSE
CELLOBIITOL
GENTIOBIOSE
SALICIN
ARBUTIN
.9
.8
.7
.6
.5
.4
.3
.2
.1
.05
200
400
600
200
400
600
OPTICAL DENSITY 520 nm
TIME (MINUTES)

and 41R4 on D-glucose, D-galactose, phenyl-β-glucoside, methyl-β-glucoside, cellobiose, cellobiitol, gentiobiose, salicin, and arbutin. These data are tabulated in terms of generation times in Table XIII. The data suggest that revertants 47R7 and 47R13 have experienced complete reversion of the enzymatic lesion apparent in the mutants. Growth of 47R7 and 47R13 on all sugars tested was comparable to the growth of the wild type on these sugars. Generation times for growth on all sugars tested, with the exception of cellobiitol and arbutin, for the wild type, 47R7 and 47R13 differed by less than ten minutes. Both 47R7 and 47R13 grew faster on cellobiitol and arbutin than the wild type. These data suggest that revertants 47R7 and 47R13 have completely reverted and have reacquired all the wild-type characteristics. Revertant 41R4 mimics the wild type in growth characteristics on D-glucose, D-galactose, arbutin, salicin, phenyl-β-glucoside, and methyl-β-glucoside. However, a lag of approximately 100 minutes occurs when 41R4 is grown on cellobiose, gentiobiose, and cellobiitol. After this lag period ended, growth on these three sugars increases to give generation times of 62, 140, and 115 minutes respectively. One possible explanation for these data is that the revertant might not have been pure, but may have contained a significant number of the parental type. The generation time of 62 minutes for cellobiose approaches the wild-type rate, but those for gentiobiose and

TABLE XIII.--Generation times for growth studies of wild-type U^- parent and revertants 47R7, 47R13, and 41R4. [The generation times were computed from the growth curves shown in Figures 12 through 15.]

Sugar	Wild type	47R7	47R13	41R4
D-glucose	50	58	50	53
D-galactose	62	63	55	77
Cellobiose	48	59	60	62
Cellobiitol	90	71	85	115
Gentiobiose	65	66	70	140
Arbutin	68	62	50	62
Salicin	60	61	55	64
Phenyl-β-glucoside	63	63	65	64
Methyl-β-glucoside	64	65	55	68

Note:

Generation times are in minutes.

cellobiitol are considerably higher than the wild-type values. The indication here is that 41R4 is a partial revertant.

Enzymatic Analysis of Revertants 47R7, 47R13, and 41R4

The wild type and revertants 47R7, 47R13, and 41R4 were grown on a mineral medium (described in Methods section) with cellobiose as the sole carbon source. The cells were centrifuged, washed, recentrifuged, and crude extracts prepared as outlined in the Methods section. The crude extracts prepared from all four cell types were then analyzed for both the β-glucoside kinase and phospho-β-glucosidase. The phospho-β-glucosidase was induced to approximately the same extent in all four organisms (see Table XIV). The specific activities ranged from 0.043 to 0.034 μmoles/minute/mg of protein, indicating all three revertants possess the induced wild-type level of phospho-β-glucosidase. The β-glucoside kinase analysis showed a more divergent range of specific activity values; the important point, however, is that all three revertants possess β-glucoside kinase activity. The specific activities of the wild type, 47R7, 47R13, and 41R4 are 0.20, 0.12, 0.07 and 0.04 respectively. None of the revertants possess a specific activity as high as that for the wild type, but 47R7 and 47R13 do possess respectable specific activities. The low specific activity of 0.04 for 41R4 could explain

TABLE XIV.--Enzymatic analysis of wild-type cells and revertants 47R7, 47R13, and 41R4. [Wild-type, 41R4, 47R7, and 47R13 were grown separately in a mineral medium plus uracil and contained cellobiose as the sole carbon source. Crude extracts were made and were assayed for the β-glucoside kinase and phospho-β-glucosidase by the standard assay for each. The data is presented in terms of specific activity (μmoles/minute/mg of protein).]

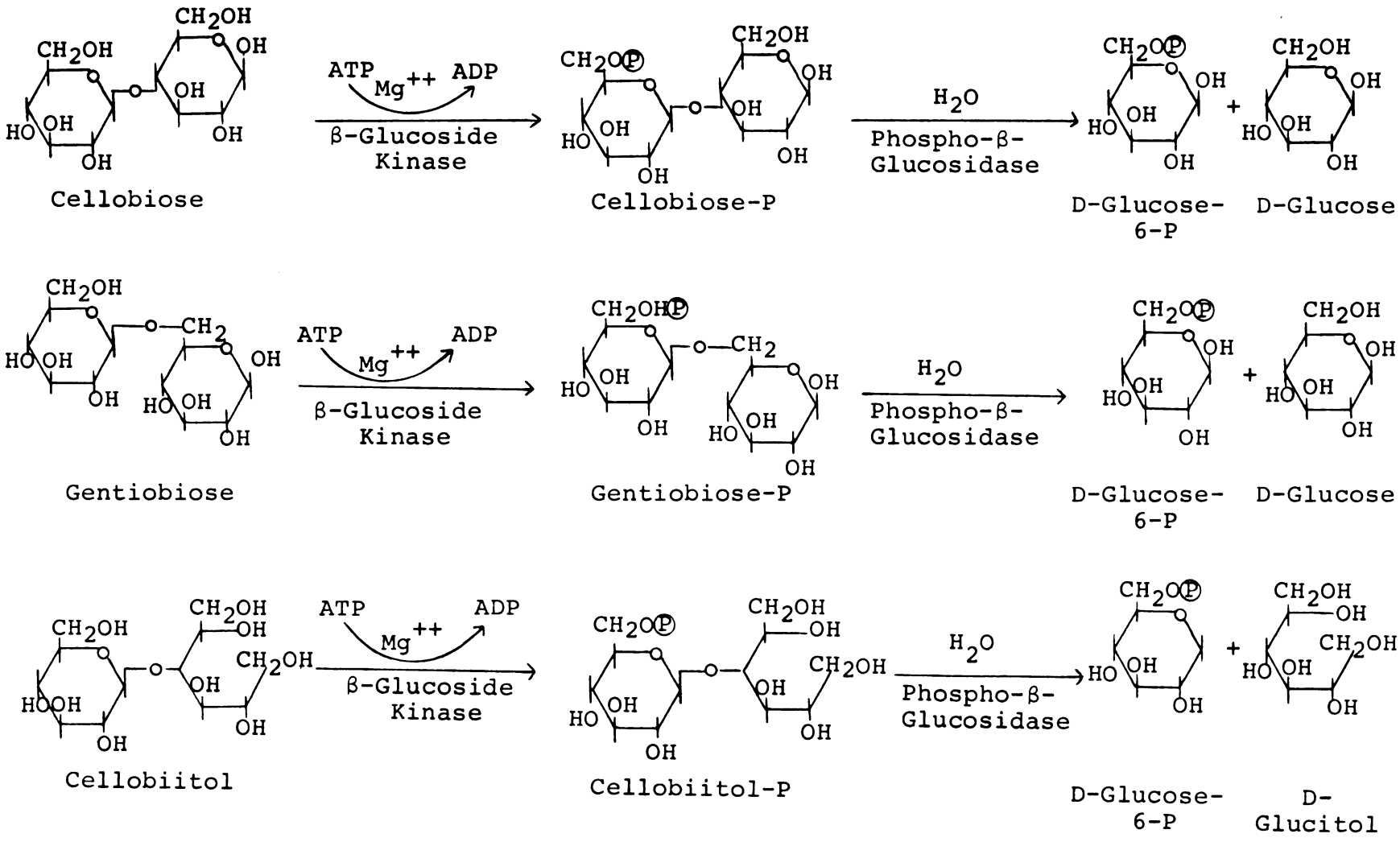
Organism	β-glucoside kinase	Phospho-β-glucosidase
Wild type	0.201	0.0414
47R7	0.115	0.0385
47R13	0.0695	0.0339
41R4	0.044	0.043

the lag observed for this organism for growth on cellobiose, gentiobiose, and cellobiitol. These results indicate that the metabolism of cellobiose, gentiobiose, and cellobiitol in A. aerogenes proceeds by only one mechanism, and that mechanism requires a functional β-glucoside kinase. In mutants 41 and 47 this activity has been lost along with the ability of these two organisms to metabolize the three disaccharides. Revertants of these two mutants have re-acquired the ability to metabolize cellobiose, gentiobiose, and cellobiitol, and concomitantly have regained the β-glucoside kinase activity.

Discussion

The pathways of cellobiose, gentiobiose, and cellobiitol metabolism in A. aerogenes as deduced from the experiments described in this section of the thesis are shown in Figure 16. An initial phosphorylation of the three disaccharides occurs with ATP as the phosphoryl donor mediated by a β-glucoside kinase to form the phosphate esters on the six position of the non-reducing moiety in cellobiose and gentiobiose, and on the six position of the glucose moiety in cellobiitol. The second reaction is a hydrolysis of the phosphorylated intermediate mediated by a phospho-β-glucosidase to yield a mole each of D-glucose-6-phosphate and D-glucose (glucitol in the case of cellobiitol). Both moieties of the disaccharides thus enter the carbohydrate pool and are freely metabolizable by the glycolytic pathway

Figure 16.--Pathways of cellobiose, gentiobiose, and cellobiitol metabolism in A. aerogenes.


Cellobiose
ATP
Mg++
ADP
β-Glucoside Kinase
Cellobiose-P
H2O
Phospho-β-Glucosidase
D-Glucose-6-P
D-Glucose
Gentiobiose
Gentiobiose-P
Cellobiitol
Cellobiitol-P
D-Glucitol

of metabolism. The pathway of D-glucitol metabolism in this organism has been described (93).

The findings presented here are interesting in light of the emphasis of the PEP-dependent phosphotransferase system of Kundig and Roseman (57), the work of Schaefler and coworkers (76, 77, 78) and that of Fox and Wilson (60). The latter two groups of workers have presented much evidence that the PEP-dependent phosphotransferase system functions in the transport and mobilization of all of the β-glucosides tested for the metabolic pathways of the organism. The evidence presented here shows that the metabolism of all three of the β-glucosides dealt with are notable exceptions to the current dogma. The PEP-dependent phosphotransferase system was tested directly for the phosphorylation of cellobiose and was found not to phosphorylate this disaccharide at appreciable rates. Mutant analysis of the proposed pathways in which the genetic lesion was determined to be the lack of a β-glucoside kinase which also only affected the metabolism of the three β-glucosides in question further substantiates these conclusions. Growth on salicin and arbutin, known to be metabolized by the phosphotransferase system, was not affected in the mutant organisms. Spontaneous revertants of the mutant organisms, when analyzed for enzyme activities, were found to possess somewhat less than wild-type levels of the β-glucoside kinase, but showed normal growth patterns for

cellobiose, gentiobiose, and cellobiitol as well as the other β-glucosides. Therefore, from the mutant analyses, it is clear that the mutant organisms possessed an intact phosphotransferase system, deduced from its normal growth patterns on sugars known to be metabolized via this system, but the lack of growth on cellobiose, gentiobiose, and cellobiitol is due to the lack of the first enzyme involved in this new pathway of metabolism, the β-glucoside kinase. The β-glucoside kinase is a soluble enzyme and not particulate, as deduced from the fact that the activity remains in the supernatant when the particulate fraction is removed by centrifugation. Therefore, it does not seem that the β-glucoside kinase functions in transport to trap the cellobiose as its phosphate derivative, but acts on free cellobiose which is transported into the interior of the cell and converts it to a freely metabolizable form, namely its phosphate derivative. How the cellobiose, gentiobiose, or cellobiitol is transported into the cell remains unclear, but possibly occurs by the type of energy-dependent mechanism of lactose transport in Escherichia coli (94, 95).

An attempt to obtain mutants for the enzyme phospho-β-glucosidase were unsuccessful, probably due mainly to the fact that there are two phospho-β-glucosidases present in crude extracts of cellobiose- and gentiobiose-grown cells. These data will be presented and discussed in Part III of the Results section.

Cellobiose contains three positions at which phosphorylation is likely to take place, the reducing carbon and two six position carbons. The reducing carbon was eliminated as a possible phosphorylation site when it was found that there was no acid-labile phosphate present in the biosynthesized cellobiose-phosphate. However, the two six positions are equivalent as far as chemical reactivity is concerned, and either could serve as the actual site of phosphorylation. Since gentiobiose is phosphorylated by the enzyme and contains only one free six position, that being on the non-reducing glucose moiety, indications were that the six position of cellobiose on the non-reducing ring was also the site of reaction. However, direct evidence that this, indeed, was the actual site of phosphorylation was obtained using cellobiitol-phosphate as a substrate for the phospho-β-glucosidase reaction. Since glucose-6-phosphate would be released only if the phosphate group were esterified to the glucose moiety, this experiment unequivocally established the six position of the non-reducing ring as the actual site of phosphorylation.

RESULTS--PART II

Induction, Purification, and Properties of the β-Glucoside Kinase

Part I of the Results section of this thesis established that in A. aerogenes the disaccharides cellobiose, gentiobiose, and cellobiitol were metabolized by the same mechanism, that is an initial phosphorylation at the hydroxymethyl carbon of the non-reducing moiety (the glucose moiety in the case of cellobiitol), using ATP as the phosphoryl donor, by a β-glucoside kinase. The second reaction consisted of a hydrolysis of the phosphorylated intermediate by a phospho-β-glucosidase yielding glucose-6-phosphate and glucose (glucitol in the case of cellobiitol). This section of the thesis describes the induction, purification, and properties of the β-glucoside kinase, and establishes that in A. aerogenes cellobiose, gentiobiose, and cellobiitol induce and are phosphorylated by the same β-glucoside kinase.

Lack of Induction of the β-Glucoside Kinase by Sugars other than β-Glucosides

Twenty-two sugars, not including β-glucosides, were tested for their ability to induce the β-glucoside kinase in wild-type A. aerogenes PRL-R3. Cells were grown in

mineral medium (composition described in Methods section) with the sugars listed below serving as the sole carbon source. The cells were grown until an optical density of approximately 0.8 was reached, centrifuged, washed, recentrifuged, and crude extracts prepared as described in the Methods section. Enzymatic analysis of the crude extracts was performed using the pyruvate kinase-lactate hydrogenase linked assay to detect the β-glucoside kinase activity. Induction of the β-glucoside kinase by the following compounds could not be detected (maximum of 2.0% of the induction by cellobiose): L-sorbose, D-mannitol, D-glucose, D-fructose, L-rhamnose, D-galactose, glycerol, D-ribose, L-arabinose, D-sorbitol, D-mannose, D-glucuronate, inulin, D-xylose, sucrose, maltose, trehalose, methyl-α-glucoside, turanose, melibiose, lactose, and raffinose. Also, β-glucoside kinase activity could not be detected in cells grown on nutrient broth or casamino acids. Wild-type A. aerogenes does not grow on PNP-β-glucoside, amygdalin, or melezitose. Induction experiments employing these three sugars as growth substrates may be accomplished by growing the cells in a casamino acid medium until an optical density of approximately 0.4 is reached. If the sugars are added at this time and the cells are grown for several hours in the presence of the growth substrate, it would be possible to test for enzyme induction after preparing the crude extracts. These experiments were not performed, however.

Induction of the β-Glucoside Kinase by β-Glucosides

Seven β-glucosides were tested for their ability to induce the β-glucoside kinase: cellobiose, gentiobiose, cellobiitol, salicin, arbutin, phenyl-β-glucoside, and methyl-β-glucoside. The cells were grown in mineral medium with each of these β-glucosides serving as the sole carbon source in separate experiments. Cells grown on nutrient broth were used as the uninduced control. After an optical density of approximately 0.8 had been reached, the cells were centrifuged, washed with water, recentrifuged, and crude extracts prepared as described in the Methods section. The β-glucoside kinase activity was measured with the standard pyruvate kinase-lactate dehydrogenase assay system. Only three of the β-glucosides--cellobiose, gentiobiose, and cellobiitol--induced the kinase. The specific activities in the crude extracts were 0.18, 0.15, and 0.14 μmoles/minute/mg of protein (see Table XV). None of the other four sugars--salicin, arbutin, phenyl-β-glucoside, or methyl-β-glucoside--induced the enzyme. These data strongly reinforce the genetic evidence presented in Part I of the Results section of this thesis. To recapitulate, the genetic experiments showed that a mutation in the wild-type organism causing the loss of the β-glucoside kinase activity prevented the mutant from growing on cellobiose, gentiobiose, and cellobiitol, but had no deleterious effect on growth on salicin, arbutin, phenyl-β-glucoside, or methyl-

TABLE XV.--Induction of the β-glucoside kinase by several β-glucosides. [Wild-type A. aerogenes was grown in mineral medium with the sole carbon sources being the inducers listed in the Table below. The cells were grown, collected, and crude extracts prepared as described in the Methods section. The level of β-glucoside kinase in the crude extracts was detected by using the standard lactate dehydrogenase-pyruvate kinase-linked assay. Nutrient broth-grown cells were used as the uninduced control.]

Growth Substrate	Specific Activity (μmoles NADH oxidized/min/mg of protein)
Nutrient broth	0.006
Cellobiose	0.18
Gentiobiose	0.15
Cellobiitol	0.14
Arbutin	0.001
Salicin	0.006
Methyl-β-glucoside	0.006
Phenyl-β-glucoside	0.001

β-glucoside. The mutants and revertants when tested for β-glucoside kinase activity were only induced with cellobiose; therefore, although the growth experiments suggested that gentiobiose and cellobiitol also induced the kinase, no direct enzymatic evidence was presented. The induction data presented here demonstrate directly that both gentiobiose and cellobiitol, as well as cellobiose, induce this enzyme. These data further support the observed genetic data by demonstrating that salicin, arbutin, phenyl-β-glucoside, and methyl-β-glucoside do not induce the β-glucoside kinase; and since the cells grow readily on these four β-glucosides, their pathways of metabolism must be independent of the β-glucoside kinase.

Evidence for the Common Identity of the β-Glucoside Kinase in Cells Grown on Cellobiose, Gentiobiose, or Cellobiitol

The induction data presented in the above section demonstrated that cells grown on cellobiose, gentiobiose, or cellobiitol contain the inducible β-glucoside kinase activity. Several possibilities exist as to the nature of the β-glucoside kinase activity: all three disaccharides may induce different enzymes, each being specific for the inducer molecule; all three disaccharides may induce the same enzyme which is active on all three inducer molecules; or, two of the disaccharides may induce one enzyme active on both inducer molecules and the third β-glucoside induces an

enzyme specific for this one molecule. Therefore, experiments were designed to determine whether one common enzyme with general specificity or several different species of enzymes possessing β-glucoside kinase activity were induced. Three types of experiments were performed with crude extracts prepared from cells grown on cellobiose, gentiobiose, and cellobiitol: first, relative activities of the crude extracts toward six β-glucosides were measured; second, K_m values for cellobiose and gentiobiose for all three extracts were determined; and third, thermal denaturation curves following the decrease of activity with time for cellobiose, gentiobiose, and cellobiitol were analyzed.

a. Relative Activity in Crude Extracts Prepared from Cells Grown on Cellobiose, Gentiobiose, and Cellobiitol

Wild-type A. aerogenes was grown in mineral medium containing cellobiose, gentiobiose, and cellobiitol as the sole carbon source in separate experiments. The cells were harvested and crude extracts prepared as described in the Methods section. β-Glucoside kinase activity toward cellobiose, gentiobiose, cellobiitol, salicin, arbutin, and phenyl-β-glucoside was measured by the pyruvate kinase-lactate dehydrogenase-linked assay, and the data are presented as specific activities normalized to cellobiose, 1.0, in Table XVI. These data show that the β-glucoside kinase activity induced by all three disaccharides exhibit approximately the same relative specific activity toward all six

TABLE XVI.--Activity of β-glucoside kinase in crude extracts from cells grown on cellobiose, gentiobiose, and cellobiitol. [Cells were grown in a mineral medium containing either cellobiose, gentiobiose, or cellobiitol as the sole carbon source. The cells were collected and crude extracts prepared as described in the Methods section. The standard pyruvate kinase-lactate dehydrogenase-linked assay was employed to detect activity on the substrates listed below. The data are reported as specific activities relative to that for cellobiose. Actual specific activities (μmoles NADH oxidized/minute/mg of protein) for cellobiose phosphorylation were 0.25, 0.075, and 0.079 for cells grown on cellobiose, gentiobiose, and cellobiitol respectively.]

Enzyme Substrate	Growth Substrate		
	Cellobiose	Gentiobiose	Cellobiitol
Cellobiose	1.00	1.00	1.00
Salicin	0.83	0.68	0.75
Phenyl-β-glucoside	0.64	0.65	0.66
Gentiobiose	0.55	0.55	0.53
Arbutin	0.54	0.48	0.45
Cellobiitol	0.15	0.15	0.18

substrates. A single β-glucoside kinase with general specificity toward β-glucosides induced by cellobiose, gentiobiose, or cellobiitol is suggested by these experiments. Of interest also is the fact that although the β-glucoside kinase is not induced by salicin, phenyl-β-glucoside, and arbutin (see previous section) these compounds do serve as substrates for the kinase.

b. K_m Values for Cellobiose and Gentiobiose from Crude Extracts from Cells Grown on Cellobiose, Gentiobiose, and Cellobiitol

The same crude extracts described in the previous section (a) were used in this set of experiments. If the same β-glucoside kinase is induced by growth on cellobiose, gentiobiose, or cellobiitol, the same relative K_m values for cellobiose and gentiobiose should be found in all three extracts of cells grown on the three disaccharides. The pyruvate kinase-lactate dehydrogenase-linked assay was employed to determine these K_m values by varying the concentration of substrate and maintaining a constant crude enzyme concentration. Figures 17 and 18 are Lineweaver-Burk plots from which K_m values for cellobiose and gentiobiose were determined to be 1.0 and 1.5 mM respectively for all three extracts. These data again suggest that a single, common β-glucoside kinase is induced by all three disaccharides. The K_m values for cellobiitol were not determined.

Figure 17.--Lineweaver-Burk plot relating β-glucoside kinase activity to cellobiose concentration in crude extracts from cells grown on cellobiose, gentiobiose, and cellobiitol. The standard pyruvate kinase-lactate dehydrogenase assay was employed except that the cellobiose concentration was varied, as indicated, with the β-glucoside kinase concentration constant from each extract.

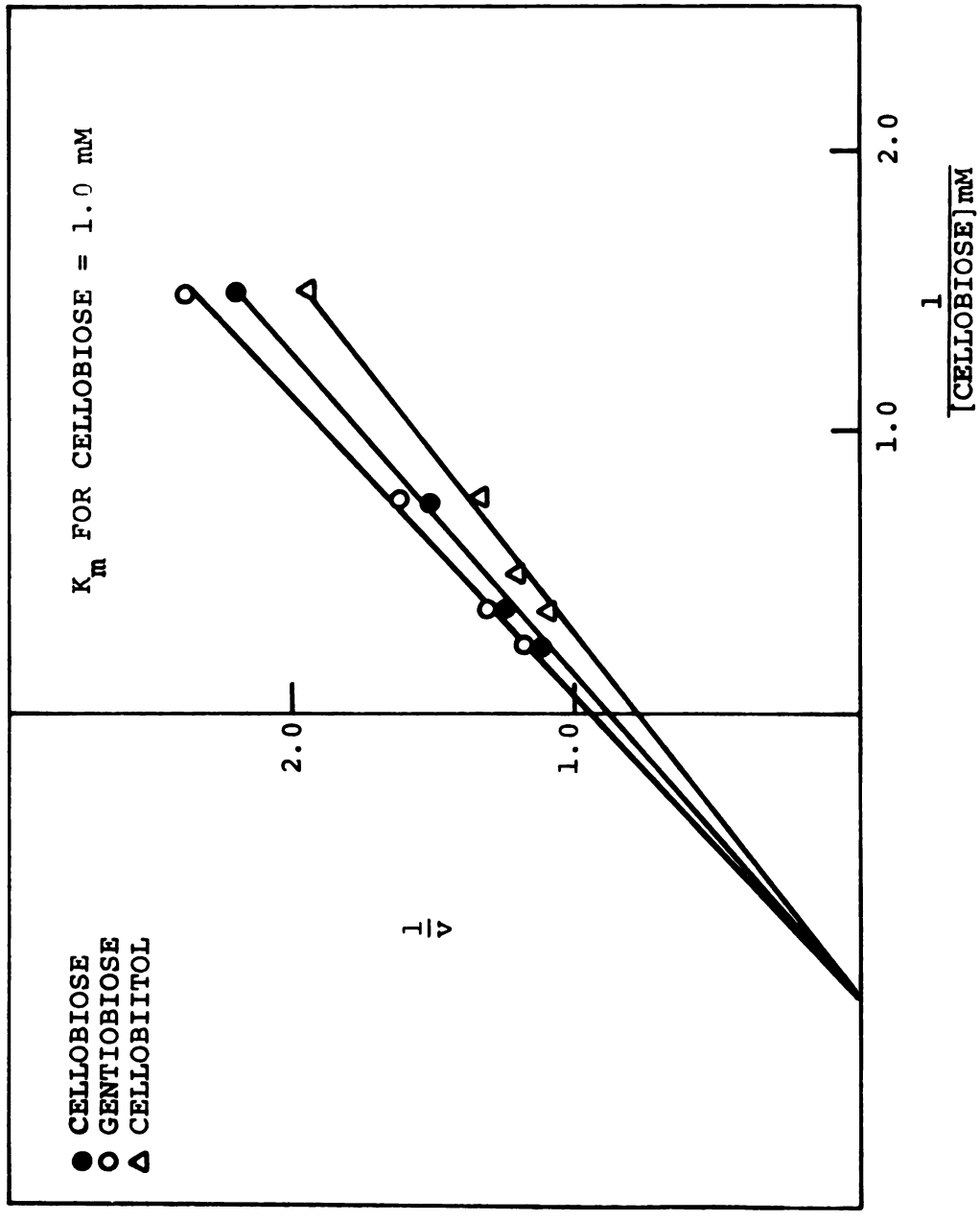
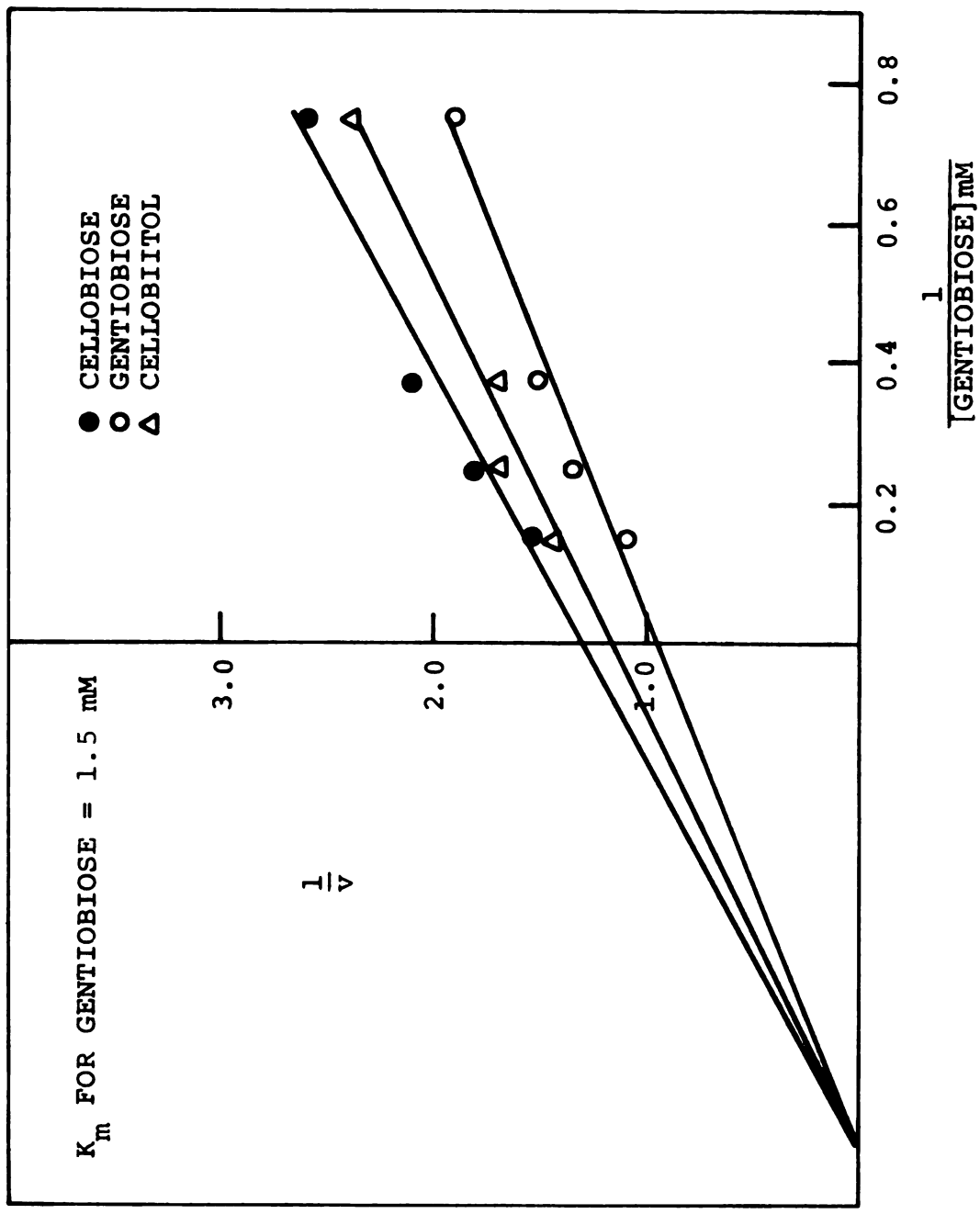

CELLOBIOSE
GENTIOBIOSE
CELLOBITOL
K_m FOR CELLOBIOSE = 1.0 mM
$\frac{1}{v}$
2.0
1.0
1.0
2.0
$\frac{1}{[CELLOBIOSE]mM}$

Figure 18.--Lineweaver-Burke plot relating β-glucoside kinase activity to gentiobiose concentration in crude extracts from cells grown on cellobiose, gentiobiose, and cellobiitol. The standard pyruvate kinase-lactate dehydrogenase assay was employed except that the gentiobiose concentration was varied, as indicated, with the β-glucoside concentration constant from each cell extract.


CELLOBIOSE
GENTIOBIOSE
CELLOBIITOL
K_m FOR GENTIOBIOSE = 1.5 mM
$\frac{1}{v}$
3.0
2.0
1.0
0.2
0.4
0.6
0.8
$\frac{1}{[GENTIOBIOSE]mM}$

c. Thermal Denaturation Studies

The crude extracts from cells grown on cellobiose, gentiobiose, and cellobiitol prepared as described in (a) were used to determine thermal denaturation curves measuring the rate of decrease of activity of β-glucoside kinase toward cellobiose, gentiobiose, and cellobiitol with time. The experiments were run at 47°C in a constant temperature bath. The β-glucoside kinase activity from the three crude extracts showed a parallel decrease in activity for all three substrates (Figure 19). A half-life of five minutes was observed for each extract. Having measured the decrease of three activities simultaneously and observed a parallel reduction of all three activities with time, these data support the previous observations about the relative activities and K_m values in the crude extracts: namely, that a single, common β-glucoside kinase is induced by growth on cellobiose, gentiobiose, or cellobiitol.

Purification of the β-Glucoside Kinase from Cellobiose-Grown Cells

Eighteen grams (net weight) of A. aerogenes PRL-R3 grown in a mineral medium with cellobiose as the sole carbon source were sonicated and the crude extracts prepared as described in the Methods section. The fractionation procedures were carried out at 0-4°C unless stated otherwise. A summary of the purification procedure is given in Tables XVII and XVIII.

Figure 19.--Thermal denaturation curves for crude extracts from cells grown on cellobiose, gentiobiose, and cellobiitol. All three extracts were heated in a constant temperature bath at 47°C. 0.2 ml of crude extract, containing approximately 1.0, 1.4, and 1.2 mg of protein from cellobiose-grown (curve A), gentiobiose-grown (curve B), and cellobiitol-grown (curve C) cells respectively, was diluted with 0.2 ml of 0.2 M glycylglycine buffer (pH 7.5). Aliquots were withdrawn at the indicated times, immediately immersed in an ice bath, and the enzyme tested for activity on cellobiose, gentiobiose, and cellobiitol. The standard pyruvate kinase-lactate dehydrogenase-linked assay was employed to detect kinase activity.

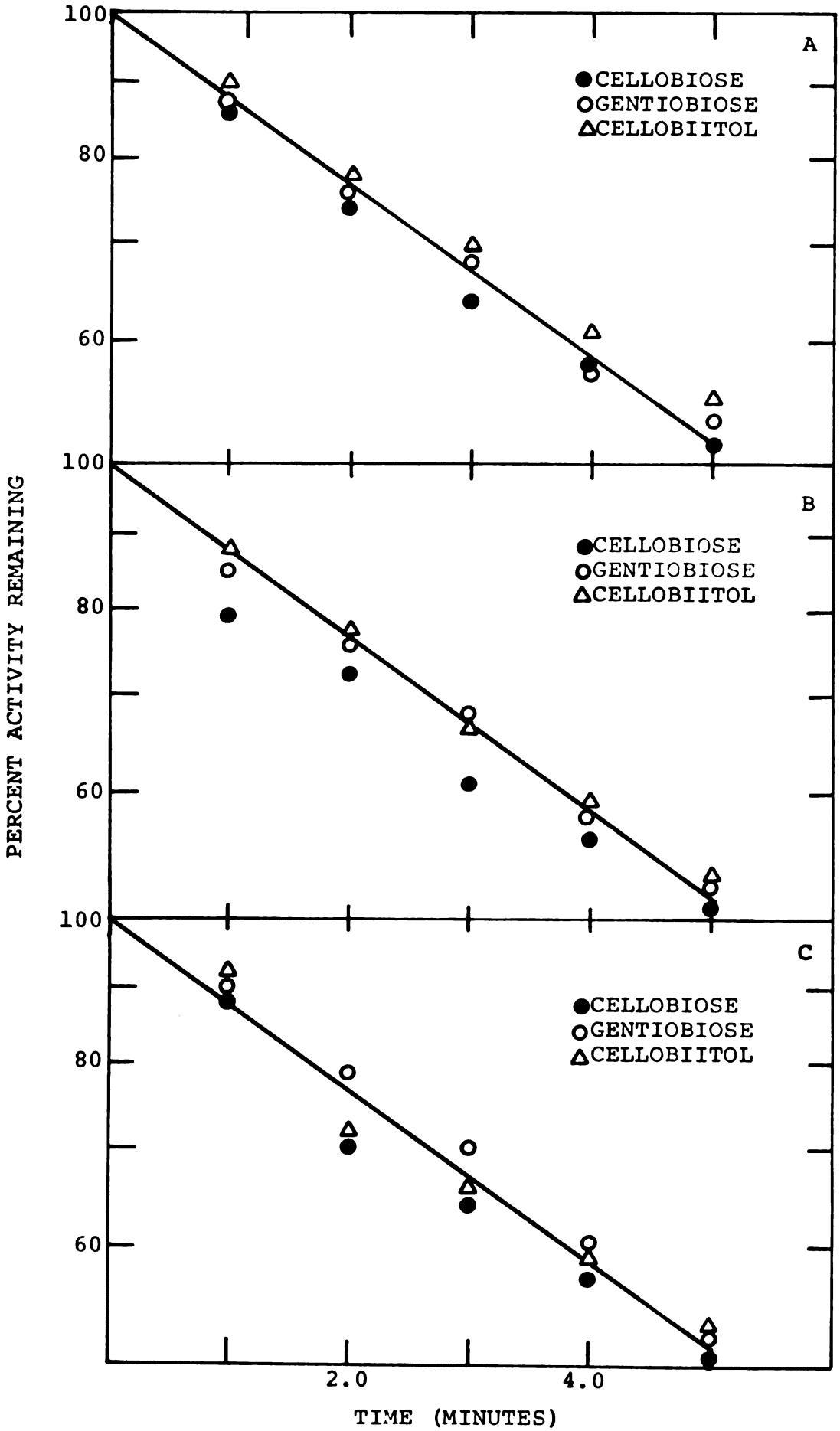

A
CELLOBIOSE
GENTIOBIOSE
CELLOBIITOL
B
CELLOBIOSE
GENTIOBIOSE
CELLOBIITOL
C
CELLOBIOSE
GENTIOBIOSE
CELLOBIITOL
100
80
60
100
80
60
100
80
60
2.0
4.0
PERCENT ACTIVITY REMAINING
TIME (MINUTES)

TABLE XVII.--Separation of β-glucoside kinase and phospho-β-glucosidase.

Fraction	Total Protein (mg)	A_{280}: A_{260}	Total Activity (units)[a]	Recovery (%)	Specific Activity (units/mg protein)	Fold Purified
Crude Extract	2620	0.6				
1. β-Glucoside Kinase			218	100	0.083	1
2. Phospho-β-glucoside			98.5	100	0.038	1
Protamine Sulfate	1800	0.7				
1. β-Glucoside Kinase			183	84	0.102	1.2
2. Phospho-β-glucosidase			77	78	0.051	1.3
Ammonium Sulfate						
a. 0-40% saturated	459	0.8				
1. β-Glucoside Kinase			164	75	0.36	4.3
2. Phospho-β-glucosidase			5.8	5.9	0.012	
b. 40-60% saturated	450	0.75				
1. β-Glucoside Kinase			2.6	1.2	0.006	
2. Phospho-β-glucosidase			53	54	0.12	3.2

[a]A unit is defined as μmoles NADP reduced/minute for the phospho-β-glucosidase and μmoles NADH oxidized/minute for the β-glucoside kinase.

TABLE XVIII.--Further purification of β-glucoside kinase.

Fraction	Total Protein (mg)		A_{280}: A_{260}	Total Activity (units)[a]		Recovery (%)		Specific Activity (units/mg protein)	Fold Purified
Ammonium Sulfate 0-40%	459		0.8	164		75		0.36	4.3
Combined Sephadex G-100 Fractions	108		1.0	114		64		1.06	12.8
Calcium Phosphate Gel[b]	23.8		1.05	50		23		2.10	25.0
DEAE-Cellulose Chromatography[c]									
Fractions 7	0.24		1.3	1.7		0.8		7.1	86
8	0.38		1.3	3.0		1.4		7.7	88
9	1.50	3.12	1.4	9.1	22.4	4.3	10.6	6.1	74
10	0.55		1.5	5.1		2.3		9.2	110
11	0.44		1.2	3.3		1.5		7.5	84

[a]A unit is defined as μmoles NADH oxidized/minute.

[b]Values are for the combined 0.05 and 0.08M phosphate eluants.

[c]One-half the calcium phosphate gel purified enzyme was subjected to DEAE-cellulose chromatography.

Protamine Sulfate Fractionation

A 5 percent stock solution of protamine sulfate (Sigma) was prepared by dissolving 1 g in 20 ml of water, and then adjusting the pH to 7.0 with 1.0 N NaOH. To 154 ml of crude extract containing 17 mg of protein per ml were added 12.3 ml of cold protamine sulfate solution over a period of fifteen minutes. The solution was stirred an additional fifteen minutes, and then the precipitate, obtained by centrifugation at 27,000 x g for 10 minutes, was discarded.

Ammonium Sulfate Fractionation

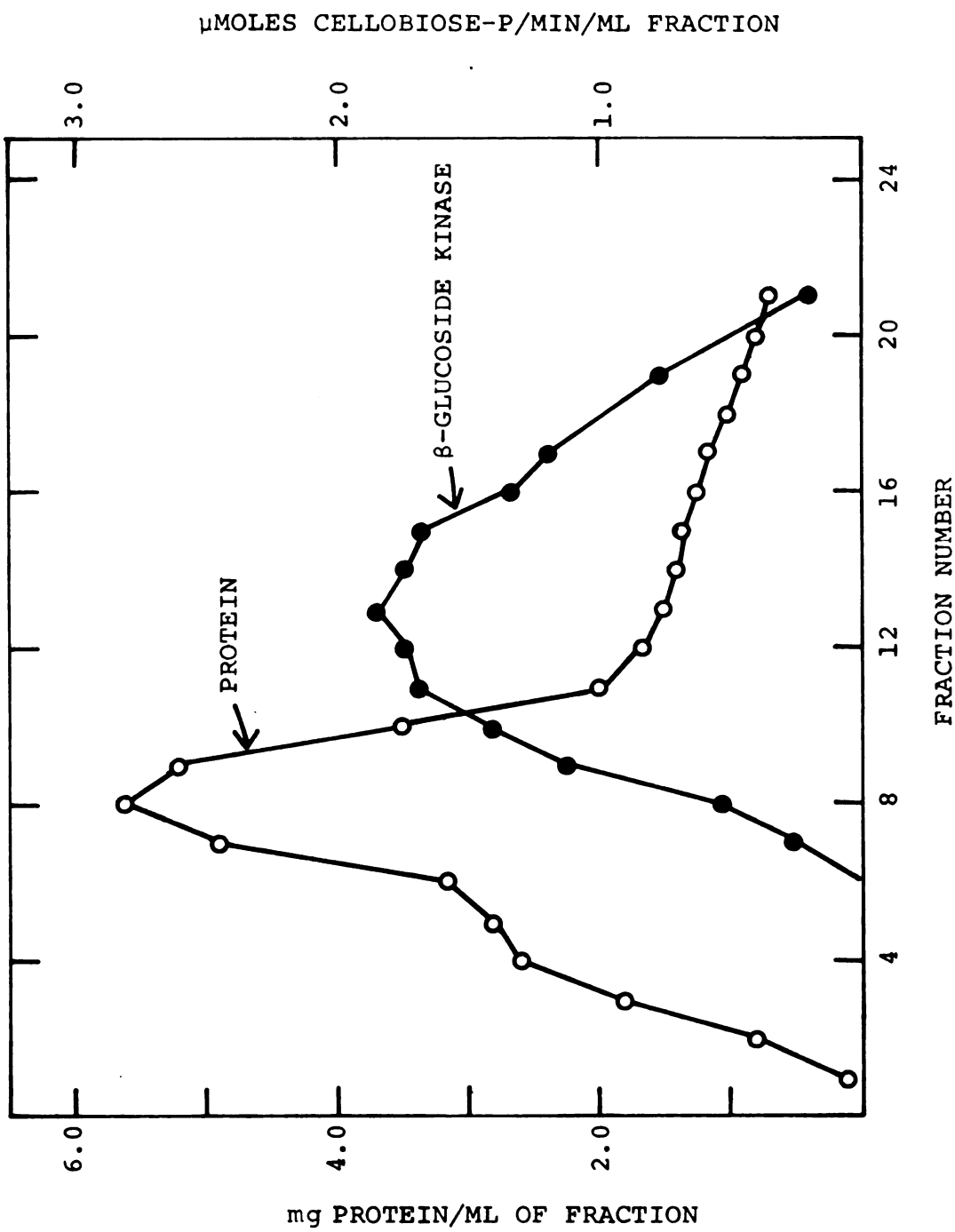
Two fractions were taken from the protamine sulfate supernatant by ammonium sulfate precipitation. A 0-to-40 percent saturated cut was precipitated from the protamine sulfate supernatant containing 12 mg of protein per ml by adding 42.2 g of solid ammonium sulfate to 154 ml of the solution over a period of thirty minutes. The solution was stirred an additional fifteen minutes and then centrifuged at 48,200 x g for ten minutes. This constituted precipitate a. A 40-to-60 percent saturated cut then was made by adding 24.5 g of ammonium sulfate to the supernatant over a period of thirty minutes. The solution was stirred an additional fifteen minutes and then centrifuged at 48,200 x g for ten minutes. This constituted precipitate b. Both precipitates were dissolved in water--precipitate a in 17 ml and precipitate b in 25 ml. Table XVII contains a summary

of the purification to this point. By just two steps of purification, protamine sulfate and ammonium sulfate, a 95 percent separation of the two enzymes was achieved. The β-glucoside kinase precipitated almost entirely in the 0-to-40 percent fraction with only 1 percent overlap of activity into the 40-to-60 percent fraction. The phospho-β-glucosidase showed about 5 percent overlap into the 0-to-40 percent fraction. However, the vast majority of phospho-β-glucosidase activity was present in the 40-to-60 percent fraction. To this point we had achieved a 4.3- and 3.2-fold purification of the β-glucoside kinase and phospho-β-glucosidase, respectively. The further purification of β-glucoside kinase in the 0-to-40 percent fraction is described below.

Sephadex G100 Chromatography

A column of Sephadex G100 (2 x 50 cm) was equilibrated with 0.02 M glycylglycine buffer (pH 7.5) in the cold. Precipitate a from the ammonium sulfate fractionation, containing 459 mg of protein in a volume of 17 ml, was carefully layered on top of the column and chromatographed using the same buffer at a rate of 0.5 ml/minute. Ten-ml fractions were collected, and the peak fractions (10-17) were combined for further purification (see Figure 20 for the elution profile).

Figure 20.--Fractionation of β-glucoside kinase on Sephadex G100. Details are given in the text.


µMOLES CELLOBIOSE-P/MIN/ML FRACTION
3.0
2.0
1.0
β-GLUCOSIDE KINASE
PROTEIN
4
8
12
16
20
24
FRACTION NUMBER
6.0
4.0
2.0
mg PROTEIN/ML OF FRACTION

Calcium Phosphate Gel

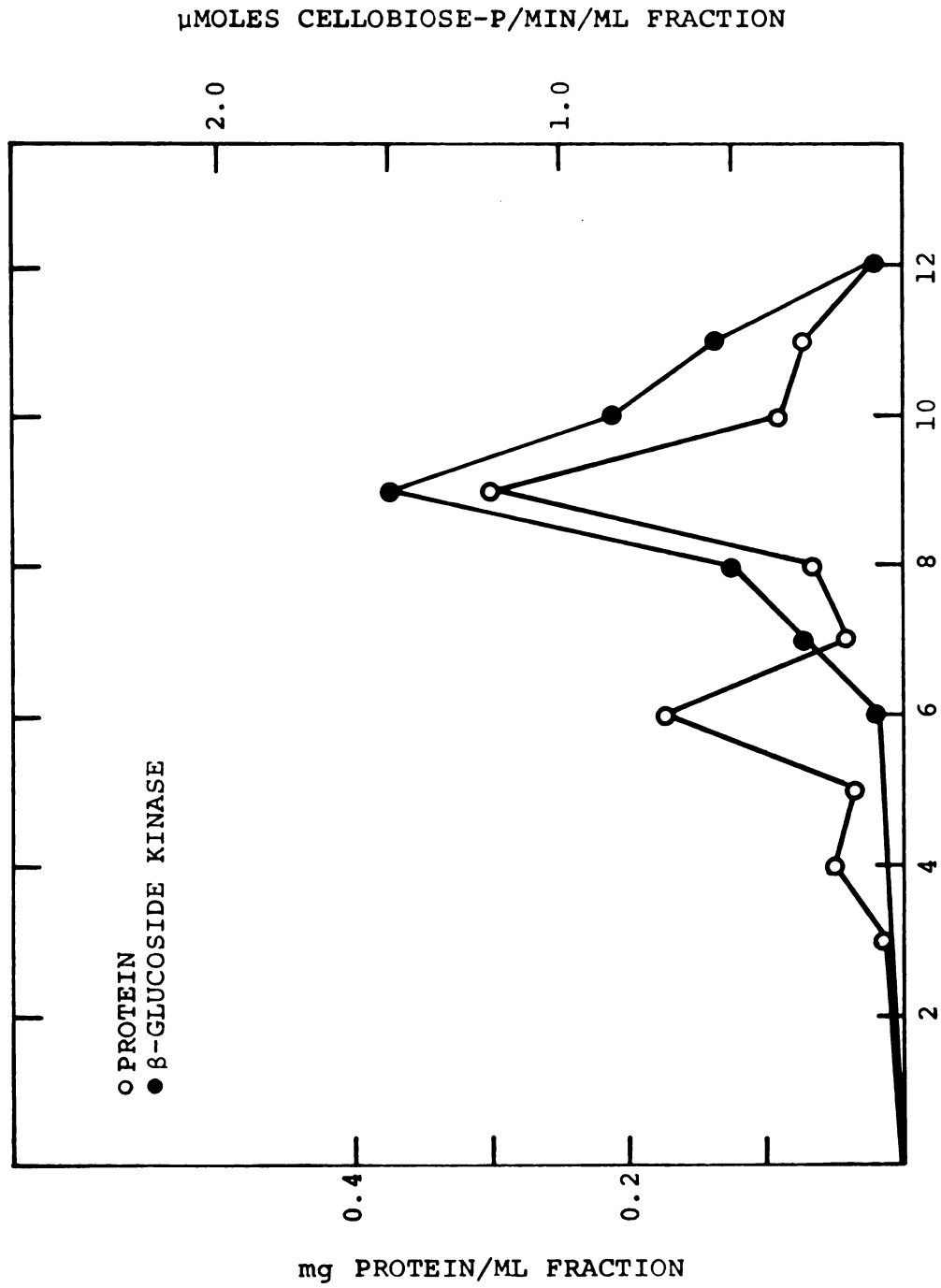
The combined peak fractions (70 ml) from the Sephadex G100 column (fractions 10-17) were treated with 20 ml of calcium phosphate gel (11% solids). The β-glucoside kinase was adsorbed, and the gel was then eluted with 0.05M, 0.08M, and 0.11M sodium phosphate buffer (pH 7.5) successively. Most of the activity was recovered in the 0.05M wash, but some overlap occurred with the 0.08M eluent. No activity was present in the 0.11M fraction.

DEAE-Cellulose Chromatography

A DEAE-cellulose (Sigma) column (1.2 x 7.0 cm) was equilibrated at room temperature first with 200 ml of 0.2M and second with 400 ml of 0.02M sodium phosphate buffer (pH 7.5). One-half of the enzyme previously fractionated by calcium phosphate gel treatment was placed on the column, which was then washed with 20 ml of 0.02M sodium phosphate buffer. The column was then developed by a stepwise elution with 0.1, 0.2, 0.3, and 0.4M NaCl solution in 0.02M sodium phosphate buffer. The enzyme eluted from the column between 0.3 and 0.4M NaCl concentration (see Figure 21).

Table XVIII gives a summary of the last three steps of purification of the β-glucoside kinase. The enzyme in the combined fractions after DEAE-cellulose chromatography was purified 85-fold. However, the most purified fraction from this step of the purification showed a specific activity of 9.2, representing a 110-fold purification.

Figure 21.--DEAE-cellulose chromatography of β-glucoside kinase. Details of procedure are given in the text. Linear NaCl concentration from 0.1 to 0.4 M was used for the elution.


µMOLES CELLOBIOSE-P/MIN/ML FRACTION
2.0
1.0
12
10
8
6
4
2
○ PROTEIN
● β-GLUCOSIDE KINASE
0.4
0.2
mg PROTEIN/ML FRACTION

This fraction was not combined with the other fractions, but was used as the source of enzyme for the studies describing the properties of the β-glucoside kinase.

Properties of Purified β-Glucoside Kinase

a. Ratios of Activities of Crude and Purified β-Glucoside Kinase

The ratios of activities on various substrates exhibited by the 110-fold purified β-glucoside kinase were compared with those found for the activity present in the crude extract. Constant ratios of activities in the crude and purified preparations of enzymes would provide evidence for the existence of a single enzyme with general substrate specificity rather than for several enzymes having rigid substrate specificity. The data presented in Table XIX show that the substrate activity ratios of cellobiose to gentiobiose, cellobiose to salicin, and cellobiose to phenyl-β-glucoside for both the crude and purified β-glucoside kinase remained constant throughout the purification procedure. These data suggest that a single enzyme is present in cells after induction by cellobiose, gentiobiose, or cellobiitol.

b. Effect of Mixing Substrates with Crude and Purified β-Glucoside Kinase

An enzyme preparation showing general substrate specificity should give non-additive rates when the activity is tested with an equimolar mixture of two different

TABLE XIX.--Ratio of activities of crude and purified β-glucoside kinase. [The substrate activities of 110-fold purified and crude β-glucoside kinase, obtained from cellobiose-grown cells, were compared as ratios for enzymes before and after purification. The ratios for the purified enzyme were obtained by using the V_{max} values listed in Table XXII determined from Lineweaver-Burk plots for each substrate. The ratios for the crude enzyme was obtained from rates using the substrates at a concentration of 33 mM. The standard pyruvate kinase-lactate dehydrogenase-linked assay was employed to determine all rates.]

Substrate Activity	Crude β-Glucoside Kinase	Purified β-Glucoside Kinase
$\frac{\text{Cellobiose}}{\text{Gentiobiose}}$	1.64	1.73
$\frac{\text{Cellobiose}}{\text{Salicin}}$	1.15	1.15
$\frac{\text{Cellobiose}}{\phi\text{-}\beta\text{-Glucoside}}$	1.53	1.43

substrates. The activities of both crude and purified β-glucoside kinase were measured using mixtures of the various substrates (see Tables XX and XXI). The crude enzyme was prepared by the same method as that described in section (a) above. The data presented in Tables XX and XXI show that in both the crude and purified preparations of β-glucoside kinase the rates for equimolar mixtures of substrates are neither additive nor inhibitory with either preparation. These data provide further evidence that the β-glucoside kinase activity is due to a single enzyme with multiple substrate activity.

c. Thermal Denaturation of Purified β-Glucoside Kinase

A thermal denaturation experiment was run on the purified β-glucoside kinase to provide additional data on this subject. One-hundred-ten-fold purified β-glucoside kinase was heated in a constant temperature bath at 45°C, and the decrease in activity of the kinase toward three disaccharides-cellobiose, gentiobiose, and cellobiitol--was measured simultaneously with time. Figure 22 shows a plot of the percent of activity remaining toward the three sugars with time. A straight line was obtained which showed a parallel decrease in activity toward all three disaccharides. These data are also consistent with the supposition that the enzymatic activity toward all three disaccharides is due to a single enzyme. These three different types of data--

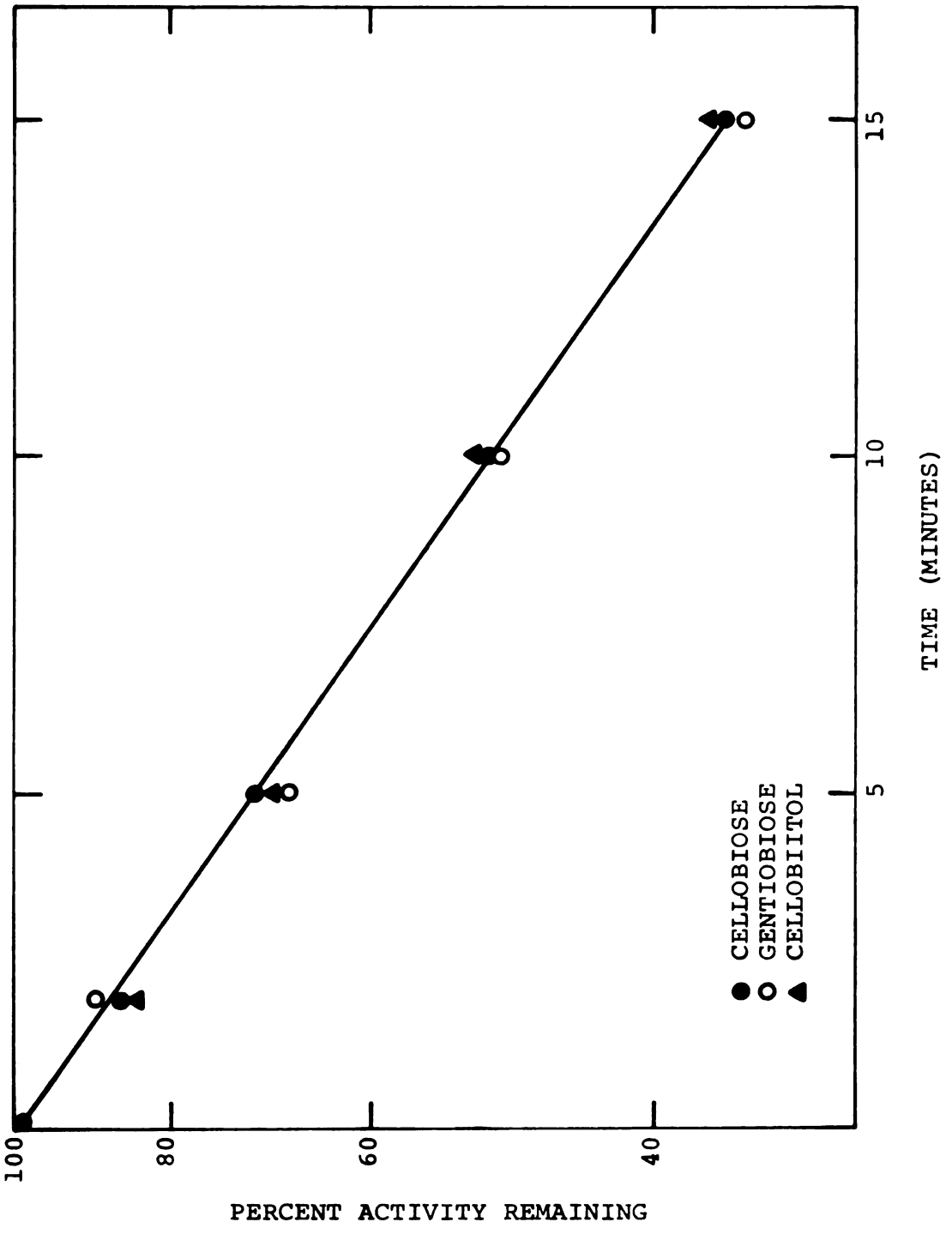
TABLE XX.--Effect of mixing substrates on β-glucoside kinase activity in the crude extract. [The activity of crude β-glucoside kinase, obtained from cellobiose-grown cells, was tested with mixed substrates, all at a concentration of 33 mM. The standard pyruvate kinase-lactate dehydrogenase-linked assay was employed.]

Substrate	Specific Activity (μmoles NADH oxidized/min/mg of protein
Cellobiose	0.476
Gentiobiose	0.350
Arbutin	0.241
Salicin	0.270
Cellobiose + Gentiobiose	0.414
Cellobiose + Arbutin	0.264
Cellobiose + Salicin	0.414
Gentiobiose + Arbutin	0.281
Gentiobiose + Salicin	0.321

TABLE XXI.--Effect of mixing substrates on activity of purified β-glucoside kinase. [The activity of 110-fold purified β-glucoside kinase was tested with mixed substrates, all at a concentration of 33 mM. The standard pyruvate kinase-lactate dehydrogenase-linked assay was employed.]

Substrate	Specific Activity (μmoles NADH oxidized/min/mg of protein)
Cellobiose	3.12
Gentiobiose	2.44
Arbutin	1.95
Salicin	2.78
Cellobiose + Gentiobiose	2.93
Cellobiose + Arbutin	2.10
Cellobiose + Salicin	2.93
Gentiobiose + Arbutin	2.24
Gentiobiose + Salicin	2.68

Figure 22.--Thermal denaturation of the β-glucoside kinase at 45°C. β-glucoside kinase was purified 110-fold from cellobiose-grown cells, and the enzyme used here was a DEAE-cellulose fraction. 0.02 mg of protein were diluted with 0.4 ml of 0.2 M glycylglycine buffer (pH 7.5), and aliquots were withdrawn and assayed for activity with cellobiose, gentiobiose, and cellobiitol at the times indicated. The pyruvate kinase-lactate dehydrogenase assay was used.


100
80
60
40
PERCENT ACTIVITY REMAINING
5
10
15
TIME (MINUTES)
CELLOBIOSE
GENTIOBIOSE
CELLOBIITOL

ratios of activities and effects of mixing substrates of crude and purified β-glucoside kinase, and thermal denaturation of purified kinase--are most reasonably interpreted to mean that the β-glucoside kinase activity is due to a single enzyme with multiple substrate specificity.

d. Phosphoryl Acceptor Specificity of Compounds, Excluding β-Glucosides

Nine monosaccharides, six disaccharides, three trisaccharides, and one polysaccharide were tested for phosphoryl acceptor activity with 110-fold purified β-glucoside kinase using cellobiose as the control. The concentration of all sugars in the reaction mixture was 33 mM. Only cellobiose was phosphorylated by the enzyme. With 0.003 unit of purified β-glucoside kinase in the pyruvate kinase-lactate dehydrogenase-linked assay, none of the following compounds served as substrates for the enzyme (< 0.5% of the rate on cellobiose): sorbitol, L-arabinose, D-mannitol, D-fructose, D-galactose, D-fucose, D-ribose, D-mannose, L-sorbose, sucrose, maltose, melibiose, methyl-α-glucoside, trehalose, lactose, turanose, melezitose, raffinose, and inulin. None of the above compounds (33 mM) inhibited the phosphorylation of cellobiose (33 mM). The significance of the lack of activity of the β-glucoside kinase on these compounds with regard to structural requirements for substrate activity will be deliberated in the Discussion at the end of this section of the thesis.

e. Phosphoryl Acceptor Activity of β-Glucosides

Twelve β-glucosides were tested for phosphoryl acceptor activity with 110-fold purified β-glucoside kinase. The compounds tested were cellobiose, salicin, phenyl-β-glucoside, gentiobiose, cellotriose, arbutin, cellotetraose, cellobiitol, amygdalin, sophorose, and methyl-β-glucoside. The pyruvate kinase-lactate dehydrogenase-linked assay was employed to measure the activity with these β-glucosides. All served as substrates for the enzyme; therefore, Lineweaver-Burk plots were employed to determine K_m and V_{max} values for each compound. The plots are shown in Figures 23 through 28. A summary of the K_m and V_{max} values determined from these data for each substrate is shown in Table XXII. The enzyme displayed the highest V_{max} for cellobiose; therefore, each V_{max} determined was normalized to cellobiose. The relative V_{max} values for the three disaccharides--cellobiose, gentiobiose, and cellobiitol--which induce the β-glucoside kinase in wild-type cells are 1.0, 0.58, and 0.25 respectively. Salicin (0.87) and phenyl-β-glucoside (0.70) show high V_{max} values also. The K_m values vary from 0.6 to 13.3 mM; the lowest value was obtained with arbutin and the highest with methyl-β-glucoside. The K_m values for cellobiose, gentiobiose, and cellobiitol were 1.0, 5.0, and 4.0 mM respectively. Of interest also is the fact that both cellotriose and cellotetraose serve as substrates for the enzyme and have K_m

Figure 23.--Lineweaver-Burk plot relating β-glucoside kinase activity to cellobiose concentration. The standard pyruvate kinase-lactate dehydrogenase assay was employed except that the concentration of cellobiose was varied, as indicated, with the concentration of β-glucoside kinase constant. One-hundred-ten-fold purified β-glucoside kinase was used. 1/v is expressed as $\Delta O.D._{340}/5$ minutes for this and subsequent Lineweaver-Burk plots.

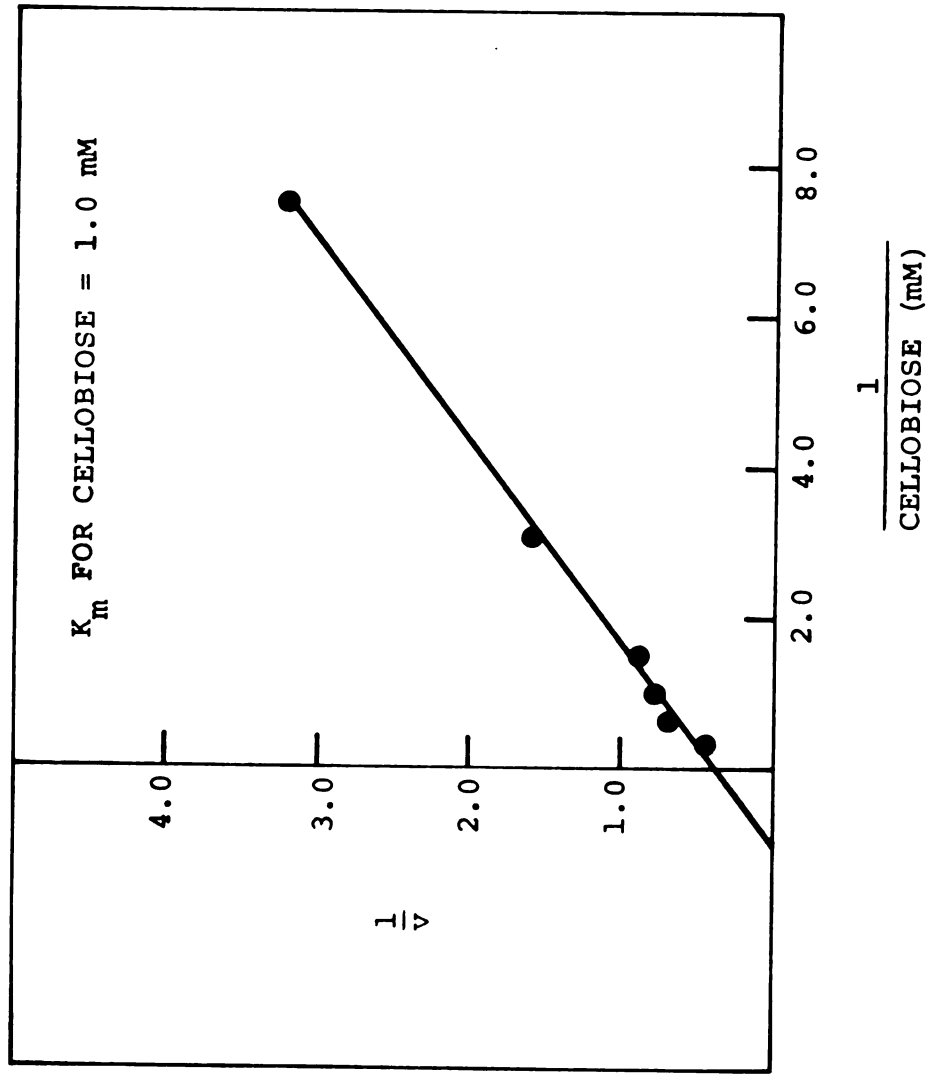
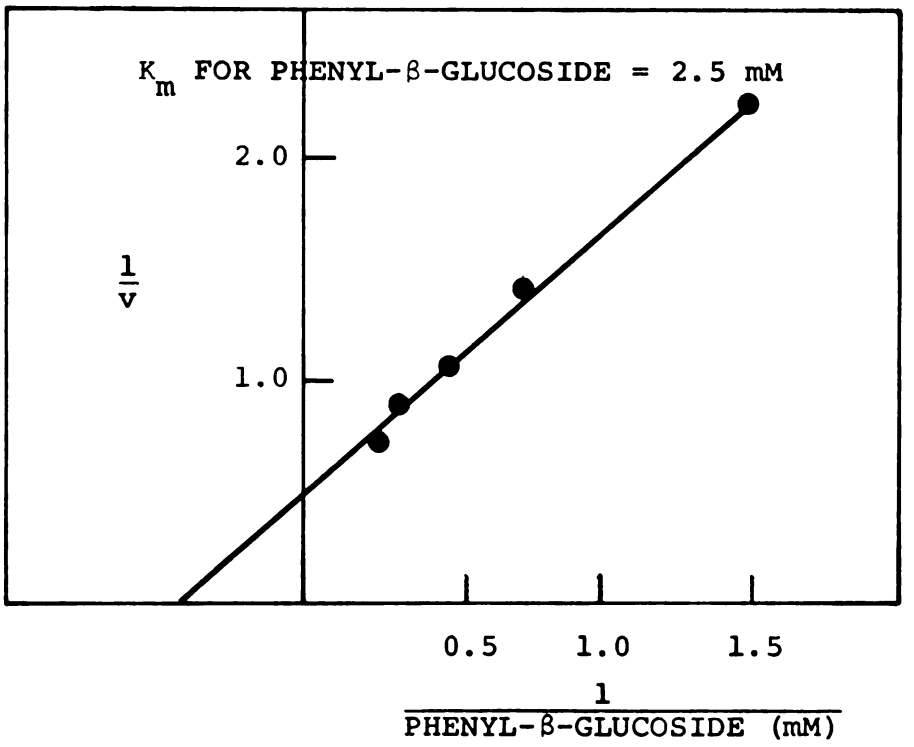

K_m FOR CELLOBIOSE = 1.0 mM
4.0
3.0
2.0
1.0
$\frac{1}{v}$
2.0
4.0
6.0
8.0
$\frac{1}{\text{CELLOBIOSE (mM)}}$

Figure 24.--Lineweaver-Burk plots relating β-glucoside kinase activity to phenyl-β-glucoside and salicin concentration. The standard pyruvate kinase-lactate dehydrogenase assay was employed except that the concentrations of phenyl-β-glucoside and salicin were varied, as indicated, with the concentration of β-glucoside kinase constant. One-hundred-ten-fold purified β-glucoside kinase was used.


K_m FOR PHENYL-β-GLUCOSIDE = 2.5 mM
2.0
1.0
$\frac{1}{v}$
0.5
1.0
1.5
$\frac{1}{\text{PHENYL-β-GLUCOSIDE (mM)}}$

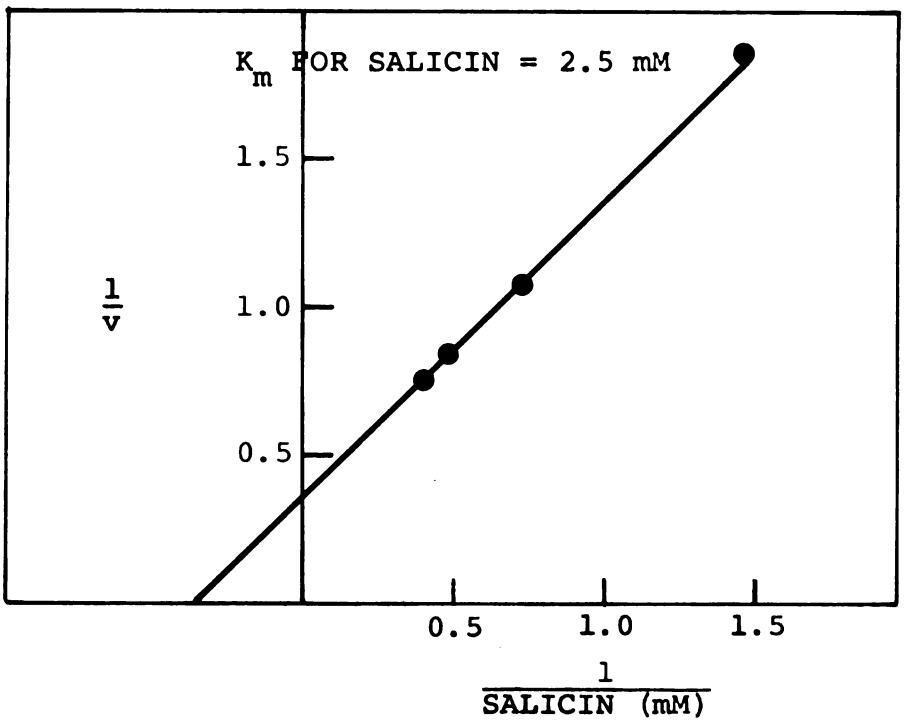
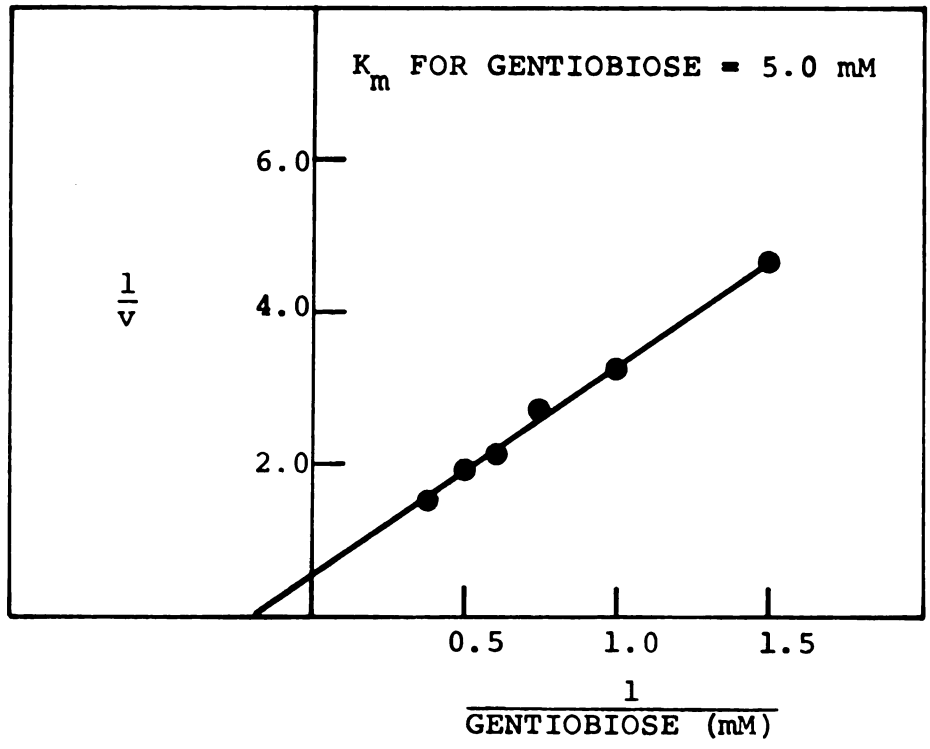

K_m FOR SALICIN = 2.5 mM
1.5
1.0
0.5
$\frac{1}{v}$
0.5
1.0
1.5
$\frac{1}{\text{SALICIN (mM)}}$

Figure 25.--Lineweaver-Burk plots relating β-glucoside kinase activity to gentiobiose and arbutin concentrations. The standard pyruvate kinase-lactate dehydrogenase assay was employed except that the concentrations of gentiobiose and arbutin were varied, as indicated, with the concentration of β-glucoside kinase constant. One-hundred-ten-fold purified β-glucoside kinase was used.


K_m FOR GENTIOBIOSE = 5.0 mM
6.0
4.0
2.0
$\frac{1}{v}$
0.5
1.0
1.5
$\frac{1}{\text{GENTIOBIOSE (mM)}}$

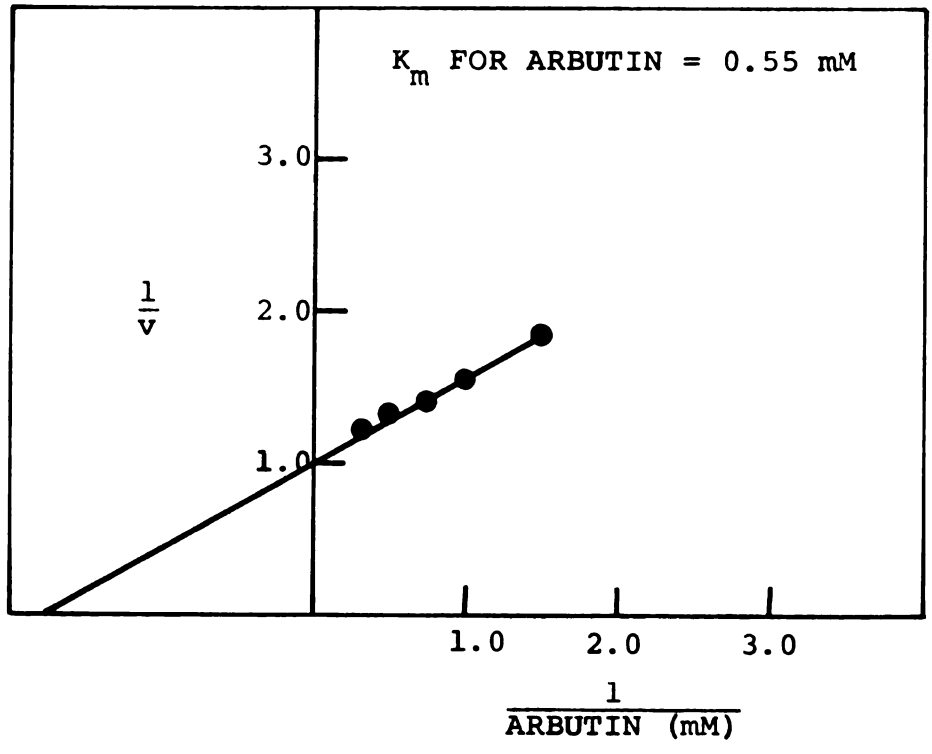
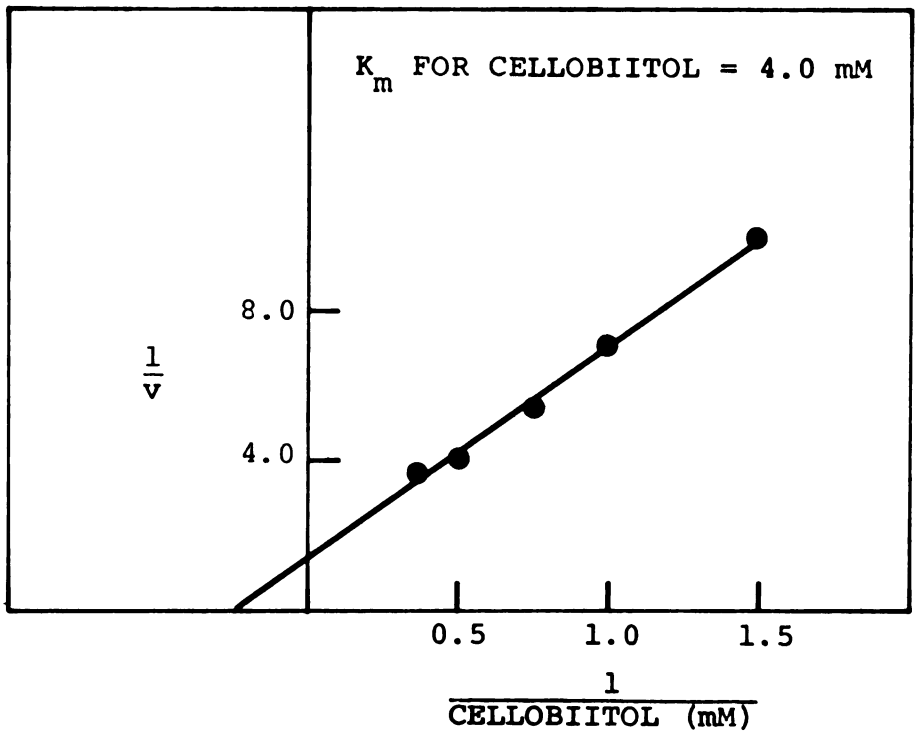

K_m FOR ARBUTIN = 0.55 mM
3.0
2.0
1.0
$\frac{1}{v}$
1.0
2.0
3.0
$\frac{1}{\text{ARBUTIN (mM)}}$

Figure 26.--Lineweaver-Burk plots relating β-glucoside kinase activity to cellobiitol and cellotetraose concentration. The standard pyruvate kinase-lactate dehydrogenase assay was employed except that the concentrations of cellobiitol and cellotetraose were varied, as indicated, with the concentration of β-glucoside kinase constant. One-hundred-ten-fold purified β-glucoside kinase was used.


K_m FOR CELLOBIITOL = 4.0 mM
8.0
4.0
$\frac{1}{v}$
0.5
1.0
1.5
$\frac{1}{\text{CELLOBIITOL (mM)}}$

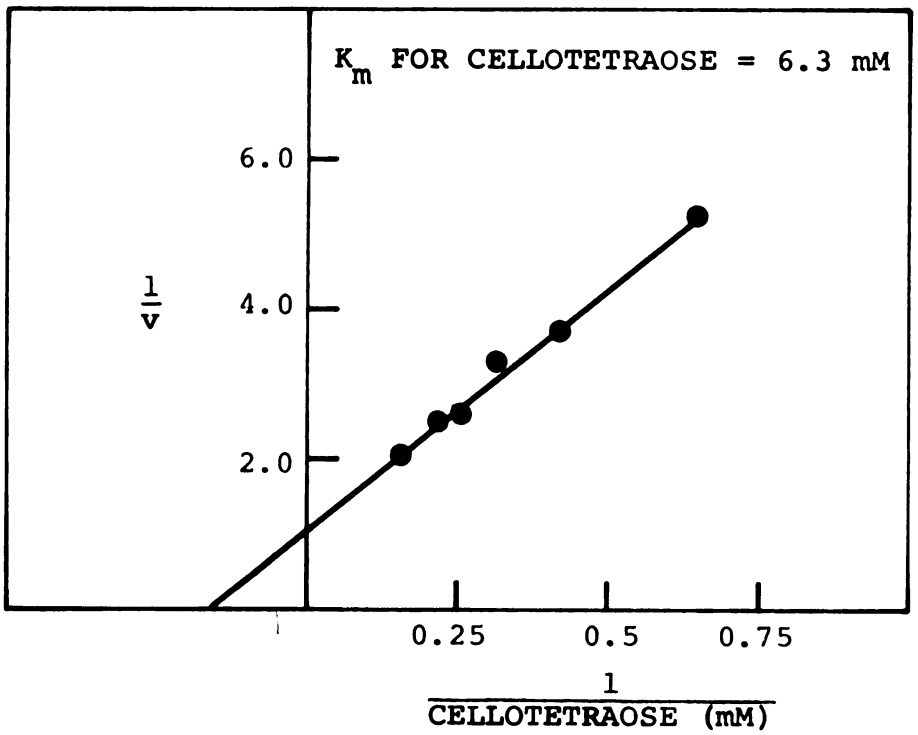
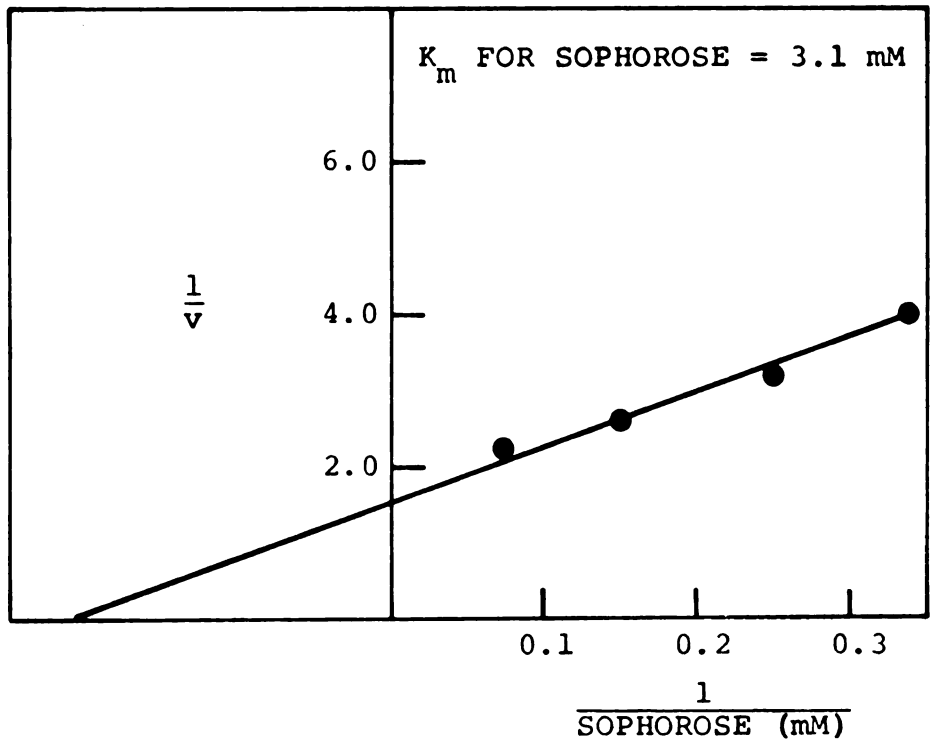

K_m FOR CELLOTETRAOSE = 6.3 mM
6.0
4.0
2.0
$\frac{1}{v}$
0.25
0.5
0.75
$\frac{1}{\text{CELLOTETRAOSE (mM)}}$

Figure 27.--Lineweaver-Burk plots relating β-glucoside kinase activity to sophorose and amygdalin concentration. The standard pyruvate kinase-lactate dehydrogenase assay was employed except that the concentrations of sophorose and amygdalin were varied, as indicated, with the concentration of β-glucoside kinase constant. One-hundred-ten-fold purified β-glucoside kinase was used.


K_m FOR SOPHOROSE = 3.1 mM
6.0
4.0
2.0
$\frac{1}{v}$
0.1
0.2
0.3
$\frac{1}{\text{SOPHOROSE (mM)}}$

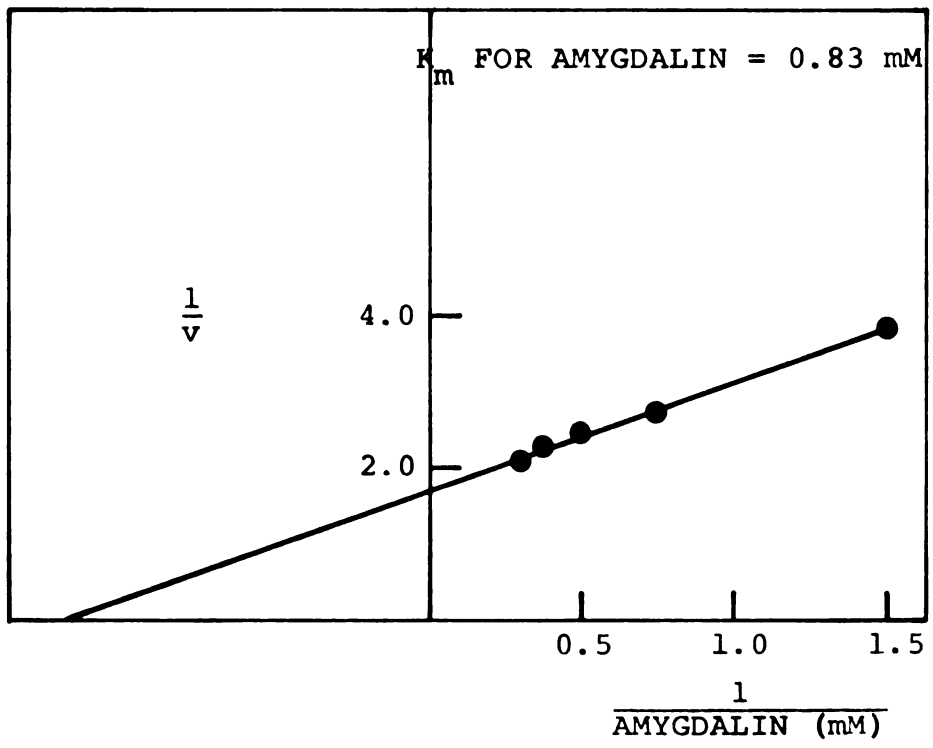
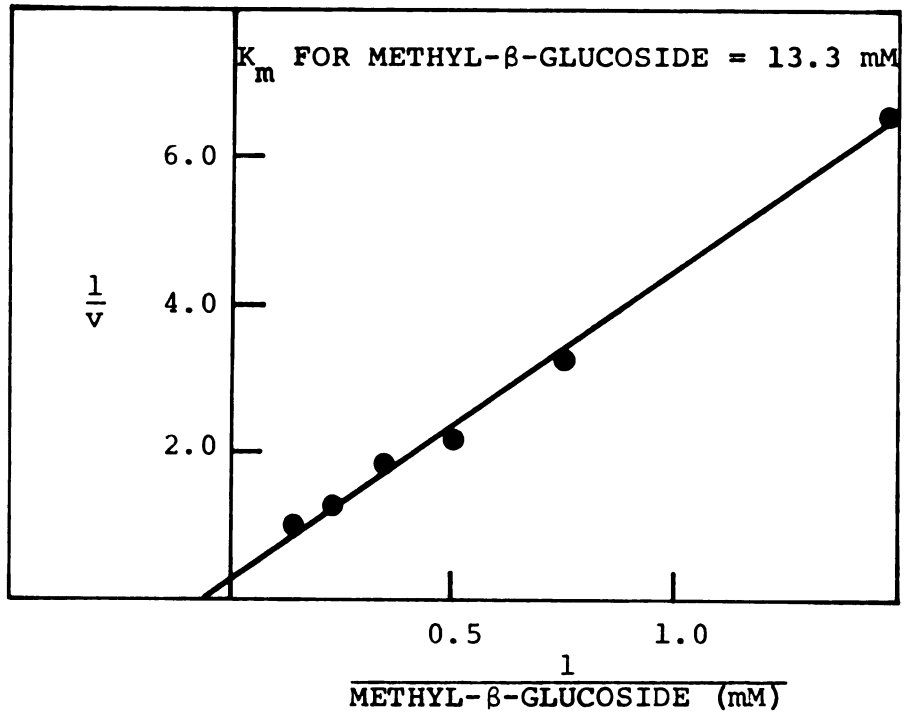

K_m FOR AMYGDALIN = 0.83 mM
4.0
2.0
$\frac{1}{v}$
0.5
1.0
1.5
$\frac{1}{\text{AMYGDALIN (mM)}}$

Figure 28.--Lineweaver-Burk plots relating β-glucoside kinase activity to methyl-β-glucoside and cellotriose concentration. The standard pyruvate kinase-lactate dehydrogenase assay was employed except that the concentrations of methyl-β-glucoside and cellotriose were varied, as indicated, with the concentration of β-glucoside kinase constant. One-hundred-ten-fold purified β-glucoside kinase was used.


K_m FOR METHYL-β-GLUCOSIDE = 13.3 mM
6.0
4.0
2.0
$\frac{1}{v}$
0.5
1.0
$\frac{1}{\text{METHYL-β-GLUCOSIDE (mM)}}$

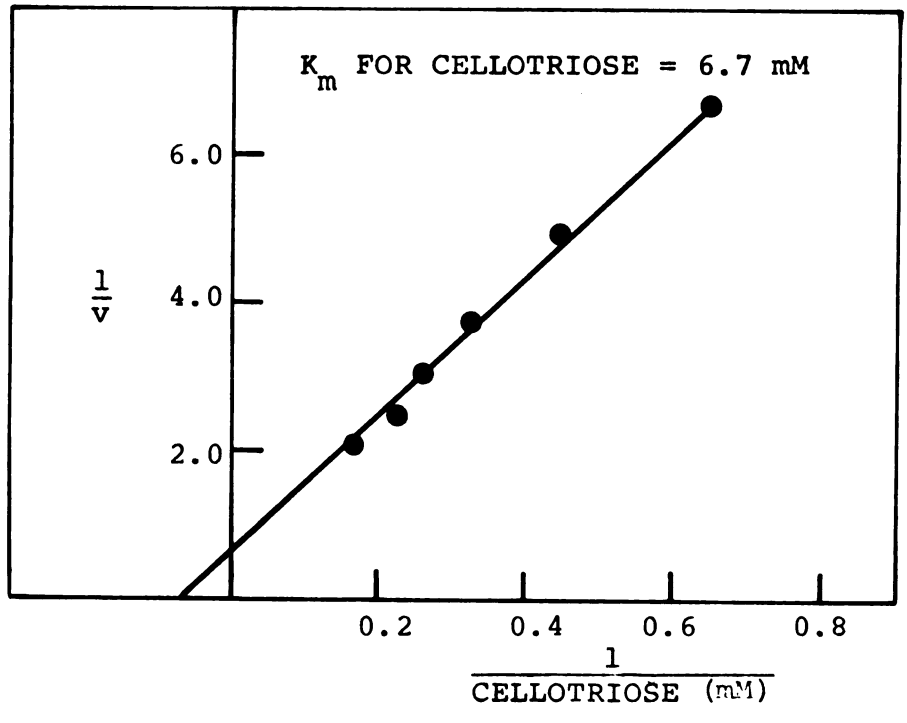

K_m FOR CELLOTRIOSE = 6.7 mM
6.0
4.0
2.0
$\frac{1}{v}$
0.2
0.4
0.6
0.8
$\frac{1}{\text{CELLOTRIOSE (mM)}}$

TABLE XXII.--A summary of the kinetic constants obtained for the β-glucoside kinase. [The data presented in the Table were derived from the Lineweaver-Burk plots given in Figures 23 through 28. The V_{max} values for all substrates were calculated relative to the V_{max} for cellobiose.]

Substrate	Lineweaver-Burk Plot	
	K_m (mM)	Relative V_{max}
Cellobiose	1.0	1.00
Salicin	2.5	0.87
Phenyl-β-glucoside	2.5	0.70
Gentiobiose	5.0	0.58
Cellotriose	6.7	0.47
Arbutin	0.6	0.36
Cellotetraose	6.3	0.33
Cellobiitol	4.0	0.25
Amygdalin	0.8	0.21
Sophorose	3.1	0.19
Methyl-β-glucoside	13.3	0.11
PNP-β-glucoside		<0.0001

values in the millimolar range also. PNP-β-glucoside was the only β-glucoside tested which had no substrate activity with the β-glucoside kinase.

f. Phosphoryl Donor Specificity

The following potential phosphoryl donor compounds were tested at 3.3 mM concentration with 110-fold purified β-glucoside kinase, and none served as a phosphoryl donor in the reaction (< 0.5% of the activity with ATP): acetyl-phosphate, α-glycerophosphate, fructose diphosphate, phosphoramidate, choline-phosphate, creatine-phosphate, 3-phosphoglyceric acid, phenylphosphate, and phosphoenol-pyruvate. The assay employed to detect phosphorylation of cellobiose by these compounds consisted of measuring cellobiose-phosphate formation. Cellobiose-phosphate was measured by including purified phospho-β-glucosidase in the reaction mixture, and then linking this reaction to the standard glucose-6-phosphate dehydrogenase detection system. Five nucleoside triphosphates and one nucleoside diphosphate were also tested. These included ATP, GTP, CTP, ITP, UTP, and ADP (see Table XXIII). All values were normalized to ATP 100. These data indicate a very high specificity of the β-glucoside kinase for ATP as the phosphoryl donor. Some activity was also obtained using the four other nucleoside triphosphates (GTP > CTP > ITP > UTP); however, the highest value, obtained for GTP, was only 15 percent of the rate with ATP. Hence, it seems that ATP is the only

TABLE XXIII.--Phosphoryl donor specificity of β-glucoside kinase. [Each nucleotide was tested at a concentration of 3.3 mM using the standard glucose-6-phosphate dehydrogenase-linked assay. Since the detection of activity was dependent on cellobiose-phosphate formation, excess purified phospho-β-glucosidase was included in the reaction mixture.]

Nucleotide	Relative Activity
ATP	100
GTP	15
CTP	12
ITP	7
UTP	3
ADP	< 0.5

significant phosphoryl donor utilized by the β-glucoside kinase. The nucleoside diphosphate ADP gave no activity with the kinase (< 0.5% of the activity with ATP). One other important observation is that phosphoenolpyruvate does not serve as a phosphoryl donor for the enzyme, and seems to have no link to cellobiose metabolism whatsoever.

g. Determination of the K_m for ATP

The K_m of 110-fold purified β-glucoside kinase was determined for ATP with cellobiose as the substrate using the same assay as that employed for the phosphoryl donor studies. The K_m value was 1.2 mM (Figure 29).

h. Approximate Molecular Weight Determination

Sucrose density gradient centrifugation, employing horseradish peroxidase as the standard, was used to obtain an approximate molecular weight of the β-glucoside kinase. Using the method of Martin and Ames (98), the sedimentation coefficient of the β-glucoside kinase was calculated by the equation $S_1 \times \text{distance}_2 = S_2 \times \text{distance}_1$. Taking 3.55 as the sedimentation coefficient of horseradish peroxidase (97) the sedimentation coefficient of the β-glucoside kinase was determined to be 8.03 S (see Figure 30). The molecular weight of the kinase was calculated from a plot of log S vs log of the molecular weight using the data of Tanford (99). This method gave a molecular weight of approximately 150,000 for the experimentally determined S value of 8.03.

Figure 29.--Lineweaver-Burk plot relating β-glucoside kinase activity to ATP concentration using cellobiose as the substrate. The glucose-6-phosphate dehydrogenase-linked assay was used with excess purified phospho-β-glucosidase to detect cellobiose-phosphate formation. Cellobiose and β-glucoside kinase concentrations were maintained constant as the ATP concentration was varied, as indicated. The $MgCl_2$ concentration was maintained at twice the ATP concentration for all ATP concentrations used. One-hundred-ten-fold purified β-glucoside kinase was employed.

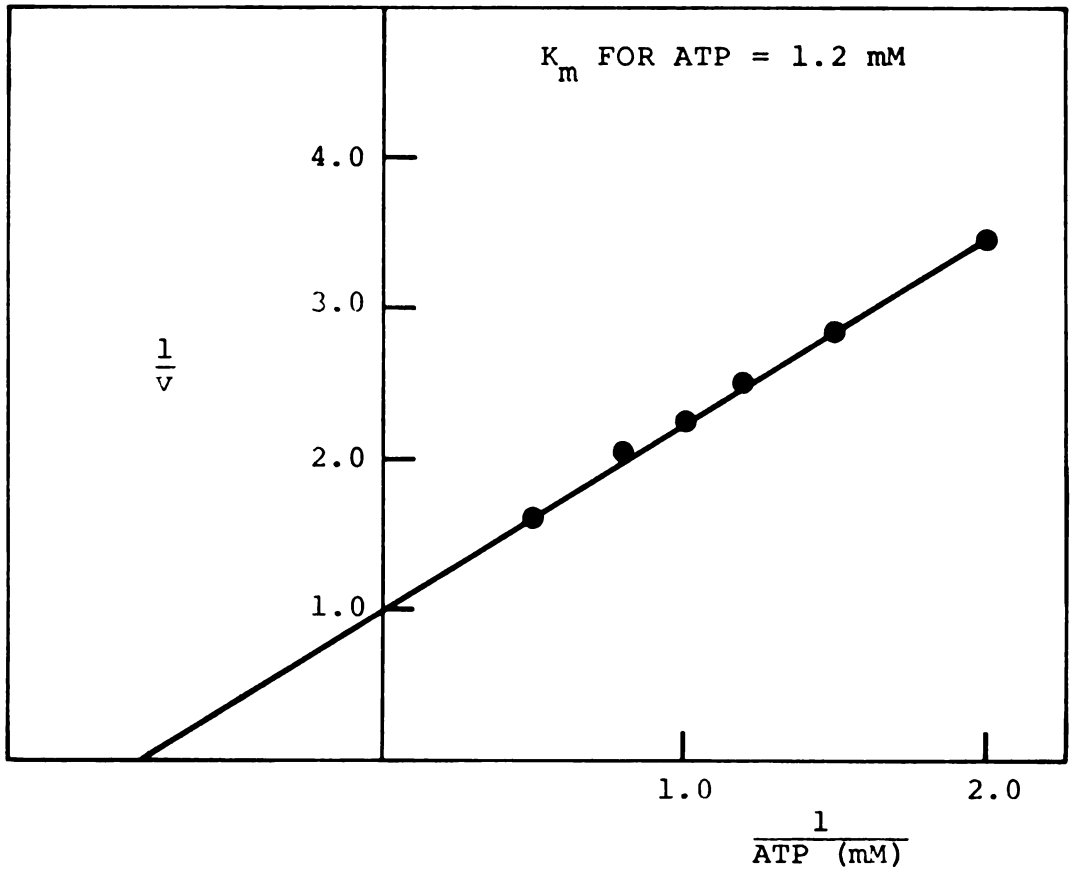
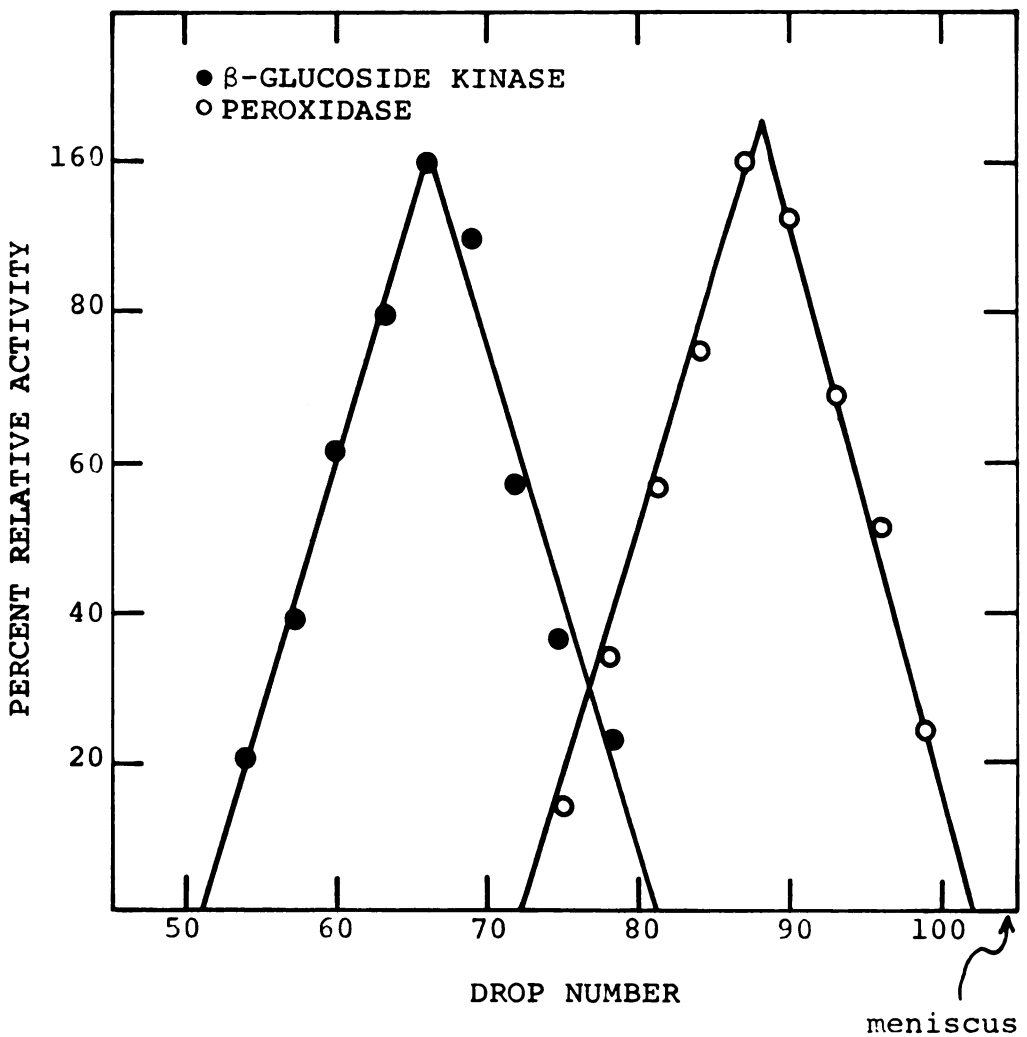

K_m FOR ATP = 1.2 mM
4.0
3.0
2.0
1.0
$\frac{1}{v}$
1.0
2.0
$\frac{1}{\text{ATP (mM)}}$

Figure 30.--Sucrose density gradient centrifugation of β-glucoside kinase and horseradish peroxidase marker. Details are given in the text.


● β-GLUCOSIDE KINASE
○ PEROXIDASE
PERCENT RELATIVE ACTIVITY
160
80
60
40
20
50
60
70
80
90
100
DROP NUMBER
meniscus

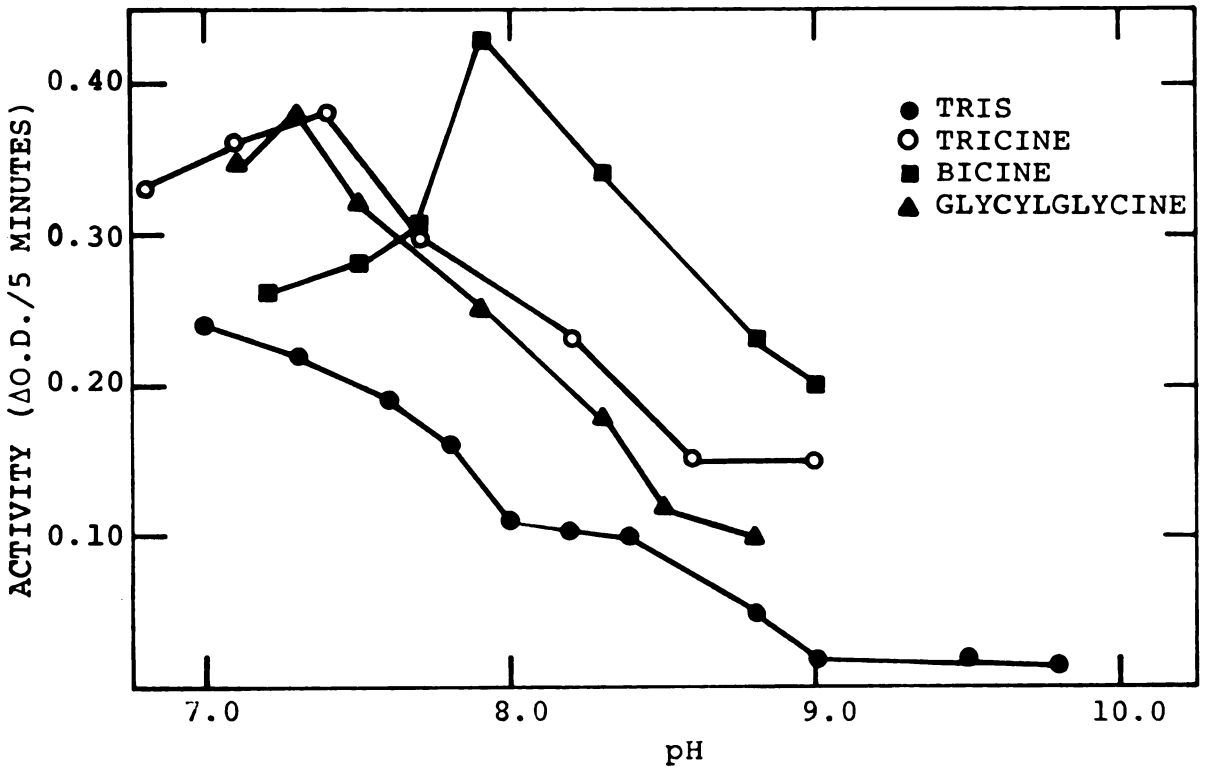
i. pH Optima

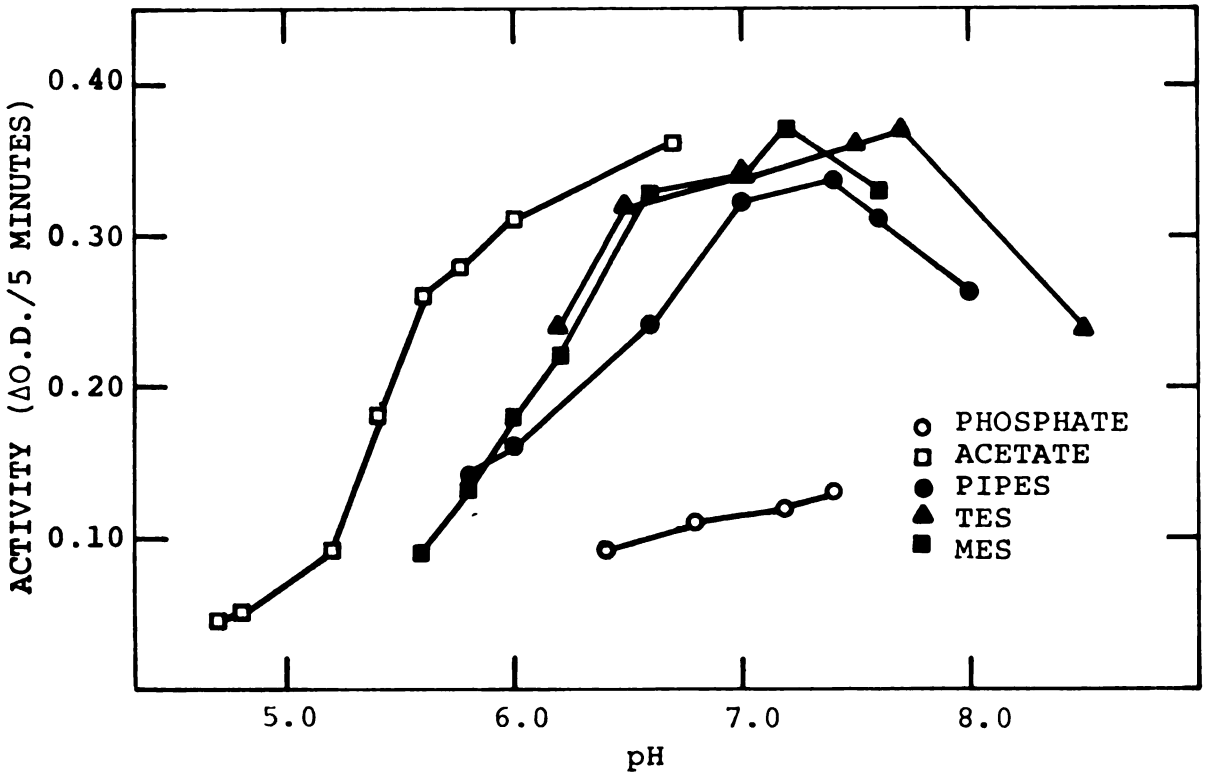
The pH optimum of the β-glucoside kinase was determined for nine different buffers (glycylglycine, Tricine, Bicine, Mes, Tes, Pipes, Tris-HCl, acetate, and phosphate) covering a pH range from 4.7 to 9.7. The detection system consisted of the measurement of cellobiose-phosphate formation by the standard glucose-6-phosphate dehydrogenase system including a 20-fold excess of phospho-β-glucosidase. The pH optima were between a pH of 7.0 to 7.4 for all buffers tested except for Bicine, which showed a shift to an optimum of pH 7.9 (see Figure 31).

Discussion

The data presented in this section of the thesis show that a single β-glucoside kinase is induced and is essential for the metabolism of cellobiose, gentiobiose, and cellobiitol. All three sugars induce the enzyme to approximately the same specific activity in crude extracts, while salicin, arbutin, phenyl-β-glucoside, and methyl-β-glucoside do not induce the enzyme. These data reaffirm the mutant data presented in the previous section in which a genetic lesion causing the loss of β-glucoside kinase only affects the organism when grown on cellobiose, gentiobiose, or cellobiitol; normal growth occurs on salicin, arbutin, phenyl-β-glucoside, and methyl-β-glucoside. The evidence presented also shows that the β-glucoside kinase induced by cellobiose, gentiobiose, and cellobiitol is the

Figure 31.--pH optima of the β-glucoside kinase in nine different buffers. The standard glucose-6-phosphate dehydrogenase assay was employed as described in the Methods section except that in addition to the regular components a 20-fold excess of phospho-β-glucosidase was included to insure that the pH effect was due only to the β-glucoside kinase.


ACTIVITY (ΔO.D./5 MINUTES)
0.40
0.30
0.20
0.10
7.0
8.0
9.0
10.0
pH
TRIS
TRICINE
BICINE
GLYCYLGLYCINE


ACTIVITY (ΔO.D./5 MINUTES)
0.40
0.30
0.20
0.10
5.0
6.0
7.0
8.0
pH
PHOSPHATE
ACETATE
PIPES
TES
MES

same enzyme as judged by activities in crude extracts, K_m values for cellobiose and gentiobiose in crude extracts, and thermal denaturation activities.

The β-glucoside kinase is an enzyme exhibiting general substrate specificity within the class of β-glucosidic compounds. When substrates were mixed with both crude and 110-fold purified enzyme, the specific activities were found to be neither additive nor inhibitory. Thermal denaturation data with both crude and purified enzyme showed a parallel loss of activity on cellobiose, gentiobiose, and cellobiitol. Ratios of specific activities (cellobiose/gentiobiose, cellobiose/salicin, and cellobiose/phenyl-β-glucoside) using both crude and purified enzyme yielded the same values. Therefore, the data strongly indicates that the multiple activities are due to a single enzyme.

Several general structural requirements for kinase activity may be deduced from the substrate studies presented in this section. The β-linkage is a prerequisite for activity since maltose, methyl-α-glucoside, and trehalose show no substrate activity with the enzyme. The non-reducing moiety must be glucose since the β-galactoside lactose shows no activity with the enzyme. Therefore, the hydroxyl group at position four in the non-reducing moiety must be in the axial position. Melibiose is an α-galactoside and presumably both of these deviations from

the structural requirements accounts for the lack of activity on this compound. No other compounds with different hydroxyl configurations in the non-reducing ring (such as a β-mannoside) were tested; therefore, little else can be said about the structural requirements of hydroxyl groups in this ring. The substituent constituting the aglycon moiety has very lax structural requirements. Aliphatic groups in β-linkage, such as methyl-β-glucoside and cellobiitol, showed activity as well as aromatic groups, such as p-hydroquinone (arbutin), o-hydroxymethylphenol (salicin), and phenyl group (phenyl-β-glucoside). However, p-nitrophenyl-β-glucoside gave no activity with the kinase, probably due to steric hindrance caused by the bulky nitro group in the para position of the aromatic ring. The enzyme was active with a hexose (D-glucose) in the reducing moiety, but not with fructose in this position, possibly because fructose is in the furanoside and not the pyranoside conformation. As a result no activity was obtained with sucrose. The β-glucoside kinase was active with compounds containing a one-two linkage (sophorose), one-four (cellobiose), and one-six (gentiobiose); therefore, the main requirement here was the β-linkage and not the position of the β-linkage. Laminaribiose (β-1→3), due to its limited availability, was not tested, but from all indications it would have been active also. Interesting substrates for the enzyme include cellotriose, cellotetraose, and amygdalin.

All three of these compounds contain extra substituents at the reducing end--cellotriose, an extra glucose moiety; cellotetraose, two extra glucose moieties; and amygdalin, a mandelonitrile group substituted on a gentiobiose base. None of these extra groups inhibits or prevents activity even though large, presumably because in the conformation of the molecules they are out of the way to minimize steric interaction. Due to the limited quantity of cellotriose, cellotetraose, and sophorose available, no induction experiments were performed to see if these substrates induced the β-glucoside kinase. Wild-type A. aerogenes does not grow on amygdalin; no induction studies were performed with this substrate although experiments of this type are possible.

Several compounds were tested for phosphoryl donor activity, but only ATP was found to possess significant phosphoryl donor activity with the enzyme. PEP showed < 0.5 percent of the activity with ATP, further indicating that this high energy compound is not involved in cellobiose metabolism. The kinase is a fairly large enzyme as indicated by an S value of 8.03 (molecular weight of approximately 150,000).

RESULTS--PART III

Induction, Purification, and Properties of the Phospho-β-Glucosidase

Part I of the Results section of this thesis demonstrated that a phospho-β-glucosidase is instrumental in the metabolism of cellobiose, gentiobiose, and cellobiitol. The metabolic function of this phospho-β-glucosidase is to hydrolyze the phosphorylated disaccharide yielding equimolar quantities of D-glucose-6-phosphate and D-glucose (glucitol in the case of cellobiitol). This section of the Results provides preliminary evidence for the existence of two different phospho-β-glucosidases (with different inducer specificities) in crude extracts from cellobiose-, gentiobiose-, salicin-, arbutin-, and nutrient broth-grown cells, describes the purification of a phospho-β-glucosidase isolated from cells grown on cellobiose, and describes the properties and multiple specificity of this enzyme from cellobiose-grown cells toward cellobiose-phosphate, gentiobiose-phosphate, cellobiitol-phosphate, salicin-phosphate, arbutin-phosphate, phenyl-β-glucoside-phosphate, and methyl-β-glucoside-phosphate.

Constitutive Phospho-β-Glucosidase Activity

Wild-type A. aerogenes was grown on sugars, excluding β-glucosides, to determine whether the phospho-β-glucosidase activity was constitutive in cells in which the enzyme was not required for the metabolism of the particular inducer. Nutrient broth-grown cells served as the control for the determination since no carbohydrate was present to serve as an inducer or repressor. In addition to nutrient broth, cells were grown in mineral medium containing the following sugars as the sole source of carbon and energy: lactose, D-fructose, glycerol, sucrose, D-glucose, and D-mannitol. The cells were harvested, washed, and crude extracts prepared as described in the Methods section. Phospho-β-glucosidase activity was determined using the standard D-glucose-6-phosphate dehydrogenase-linked assay with cellobiose-phosphate as the substrate. These data (Table XXIV) show that in nutrient broth-grown cells there is a low level (0.0048 μmoles/minute/mg of protein) of phospho-β-glucosidase activity. The specific activities for the enzyme varied by 10-fold--from 0.0048 in nutrient broth-grown to 0.044 μmoles/minute/mg of protein in glycerol-grown cells. The experiments indicate that there is a low constitutive level of phospho-β-glucosidase in cells grown on nutrient broth or sugars other than β-glucosides.

TABLE XXIV.--Constitutive activity of phospho-β-glucosidase in cells grown on sugars other than β-glucosides. [Wild-type A. aerogenes was grown on mineral medium (except in the case of nutrient broth-grown cells) and the sole carbon sources were the sugars listed below. The cells were grown, harvested, crude extracts prepared, and the enzyme assayed as outlined in the Methods section using cellobiose-phosphate as the substrate.]

Growth Substrate	Specific Activity (μmoles NADP reduced/ min/mg of protein)
Nutrient Broth	0.0048
D-Fructose	0.0242
Glycerol	0.044
Sucrose	0.0166
D-Glucose	0.0193
D-Mannitol	0.0036
Lactose	0.0053

Induction of Phospho-β-Glucosidase Activity by Growth on β-Glucosides

Cells were grown on β-glucosides to determine whether higher levels of phospho-β-glucosidase induction could be achieved after growth on β-glucosides. Cellobiose, gentiobiose, cellobiitol, salicin, arbutin, phenyl-β-glucoside, and methyl-β-glucoside were used in these induction experiments. The level of phospho-β-glucosidase in nutrient broth-grown cells was again taken as the uninduced, constitutive level. The cells were harvested, washed, and crude extracts prepared as described in the Methods section. Phospho-β-glucosidase activity was detected using the standard D-glucose-6-phosphate dehydrogenase assay with cellobiose-phosphate as the substrate. The data presented in Table XXV show that, again, a range of specific activities, differing by 10-fold, was obtained. Growth on cellobiose induced the activity to its highest level, 0.049 μmoles/minute/mg of protein. High levels of induction were obtained by growth on cellobiitol, gentiobiose, and methyl-β-glucoside, while low induction was obtained by growth on phenyl-β-glucoside, arbutin, and salicin. These data suggest that four β-glucosides--cellobiose, gentiobiose, cellobiitol, and methyl-β-glucoside--induce a phospho-β-glucosidase with high activity on cellobiose-phosphate; however, the other three inducers--phenyl-β-glucoside, salicin, and arbutin--cause a very low level of activity for cellobiose-phosphate as the substrate. Other workers

TABLE XXV.--Induction levels of phospho-β-glucosidase in cells grown on β-glucosides. [Wild-type cells were grown in mineral medium and the growth substrate at 0.5% concentration. Nutrient broth-grown cells were used as the control for the constitutive level of enzyme activity. The cells were grown, harvested, crude extracts prepared, and the enzyme assayed as described in the Methods section, using cellobiose-phosphate as the substrate.]

Growth Substrate	Specific Activity (μmoles NADP reduced/ min/mg of protein)
Nutrient Broth	0.0048
Cellobiose	0.048
Cellobiitol	0.034
Gentiobiose	0.036
Methyl-β-glucoside	0.030
Phenyl-β-glucoside	0.003
Arbutin	0.009
Salicin	0.017

(see Literature Review) have presented evidence that salicin and arbutin are transported via the PEP-dependent phosphotransferase system, thereby forming the phosphate derivatives of both sugars. In order to enter the metabolic pathways, these phosphate derivatives of salicin and arbutin have to be hydrolyzed, probably with a phospho-β-glucosidase. Since the induction level of phospho-β-glucosidase activity is low in salicin-, arbutin-, and phenyl-β-glucoside-grown cells determined by using cellobiose-phosphate as the substrate, the possibility existed that a different enzyme might be induced by these three β-glucosides with low activity on cellobiose-phosphate, explaining the observed data, but, with high activity on salicin-phosphate, arbutin-phosphate, and phenyl-β-glucoside-phosphate. This hypothesis was tested and the results are given in the following section.

Evidence for the Existence of Two Species of Phospho-β-Glucosidase in Crude Extracts from Cells Grown on Nutrient Broth, Cellobiose, Gentiobiose, Salicin, and Arbutin

a. Activity in Crude Extracts

Cells were grown on a mineral medium with either cellobiose, gentiobiose, salicin, or arbutin serving as the sole carbon source. Nutrient broth-grown cells were used as the uninduced control. The cells were then harvested and crude extracts prepared as described in the Methods section. Since the object of these experiments was to

determine the activity of the phospho-β-glucosidase induced by the four β-glucosides, the synthesis of seven β-glucoside-phosphates was necessitated. One-hundred-ten-fold purified β-glucoside kinase was incubated, in a constant temperature bath at 30°C, with 5.0 μmoles of β-glucoside (gentiobiose, cellobiitol, salicin, arbutin, phenyl-β-glucoside, or methyl-β-glucoside), 5.0 μmoles of ATP, and 10 μmoles of $MgCl_2$ (total volume 2.0 ml) for 1.5 hours. The reaction was terminated by heating the mixtures in a boiling water-bath for five minutes, and the denatured protein was removed by centrifugation at 12,100 x g for 10 minutes. The preparations were deionized with Dowex 50(H^+), and the β-glucoside phosphates were quantitated by end-point analyses using purified phospho-β-glucosidase from cellobiose-grown cells. The standard D-glucose-6-phosphate dehydrogenase-linked assay was employed for the analyses.

The crude extracts from nutrient broth-, cellobiose-, gentiobiose-, salicin-, and arbutin-grown cells were incubated in the D-glucose-6-phosphate dehydrogenase assay system modified only by varying the particular β-glucoside-phosphate used as the substrate. The specific activities of the phospho-β-glucosidase activity toward the seven β-glucoside phosphates vary with the growth sugar (Table XXVI, A). The activity in nutrient broth-grown cells was used as the constitutive level of enzyme activity toward the seven β-glucoside phosphates, and these data were

TABLE XXVI.--Activity of phospho-β-glucosidase in crude extracts from cells grown on nutrient broth, cellobiose, gentiobiose, salicin, and arbutin on seven β-glucoside-phosphates. [Cells were grown on nutrient broth and mineral medium containing cellobiose, gentiobiose, salicin, and arbutin as the sole carbon source. The cells were harvested and crude extracts prepared as described in the Methods section. The standard D-glucose-6-phosphate dehydrogenase assay was used except the β-glucoside phosphates listed below were used as substrates. The data is presented as specific activities (μmoles NADP reduced/min/mg of protein). Table A shows specific activities of the crude extracts for the various β-glucoside-phosphates. Table B shows the fold increase in specific activity over the nutrient broth level. Table C shows the fold increase in specific activity relative to that for cellobiose-P.]

Sugar-Phosphate	Crude Extracts from Cells Grown on Nutrient Broth and Sugars Listed Below				
	Nutrient Broth	Cellobiose	Gentiobiose	Salicin	Arbutin
A					
Cellobiose-P	0.0035	0.031	0.020	0.0003	0.0028
Salicin-P	0.014	0.049	0.042	0.10	0.095
Gentiobiose-P	0.0096	0.031	0.045	0.0045	0.0025
Phenyl-β-glucoside-P	0.017	0.038	0.070	0.074	0.055
Arbutin-P	0.007	0.020	0.057	0.088	0.025
Cellobiitol-P	--[a]	0.015	0.010	--[a]	--[a]
Methyl-β-glucoside-P	0.0035	--[a]	--[a]	0.029	0.0033

B					
Cellobiose-P	1	10	6	1	1
Salicin-P	1	3.5	3	10	7
Gentiobiose-P	1	3	4.5	0.5	0.26
Phenyl-β-glucoside-P	1	2	4.1	4	3
Arbutin-P	1	3	8	12	4
Methyl-β-glucoside-P	1	--[a]	--[a]	10	1
C					
Cellobiose-P	1	1	1	1	1
Salicin-P	4	1.6	2	33	34
Gentiobiose-P	2.7	1	2.2	1.5	1
Phenyl-β-glucoside-P	5	1.2	3.5	25	20
Arbutin-P	2	0.65	2.8	29	10
Cellobiitol-P	--[a]	0.49	0.5	--[a]	--[a]
Methyl-β-glucoside-P	1	--[a]	--[a]	10	--[a]

[a]--Signifies not determined.

recalculated in terms of the fold increase in activity over the nutrient broth level (Table XXVI, B). These data indicate that growth on cellobiose or gentiobiose increased phospho-β-glucosidase activity toward all substrates from 2- to 10-fold. Growth on salicin or arbutin, however, increased the activity only for salicin-phosphate, phenyl-β-glucoside-phosphate, and arbutin-phosphate from 3- to 10-fold; activity on cellobiose-phosphate remained at the uninduced level, while that on gentiobiose-phosphate decreased. One difference between growth on salicin and arbutin was that salicin caused a 10-fold increase in activity on methyl-β-glucoside-phosphate, while growth on arbutin resulted in no increase in activity toward this substrate over the uninduced level. These data were rearranged for analysis in another form; activity on all substrates was normalized to the activity on cellobiose-phosphate (Table XXVI, C). Induction by salicin and arbutin caused a great increase (10- to 34-fold) in activity toward salicin-phosphate, phenyl-β-glucoside-phosphate, and arbutin-phosphate, while activity on both cellobiose- and gentiobiose-phosphate remained constant. Enzyme activity, after growth on cellobiose, showed a constant level (< 1-fold difference in activity) for all substrates tested. Growth on gentiobiose caused a maximum of 3.5-fold difference in rates on the various substrates. The results from these experiments are not entirely clear, but do suggest that there are two phospho-β-glucosidases in crude extracts of A. aerogenes.

The most convincing argument for this hypothesis is the high increase in activity on salicin-, phenyl-β-glucoside-, and arbutin-phosphates with constant low activity on cellobiose- and gentiobiose-phosphates in cells grown on salicin or arbutin (Table XXVI, C), and the 10-fold increase in activity on cellobiose-phosphate with low activity on salicin-, phenyl-β-glucoside-, and arbutin-phosphates after growth on cellobiose (Table XXVI, B). However, these data do not indicate whether both species of phospho-β-glucosidase are present at constitutive levels in nutrient broth-grown cells, and hence, do not indicate whether both species are present after induction by salicin, arbutin, or cellobiose. The apparent distinguishing characteristics of the proposed phospho-β-glucosidases are given in Table XXVII. Ratios of activities for the various substrates determined for crude extracts from nutrient broth-, cellobiose-, gentiobiose-, salicin-, and arbutin-grown cells (Table XXVIII) also indicated that the activity on the β-glucoside-phosphate substrates was dependent on the inducer.

b. Thermal Denaturation

Crude extracts from salicin-, gentiobiose-, and cellobiose-grown cells were tested for their ability to catalyze the hydrolysis of five β-glucoside-phosphates after thermal denaturation. The experiment was designed to determine whether there were two patterns of loss of activity, as predicted by the data from (a) above, and if

TABLE XXVII.--Apparent distinguishing features of proposed phospho-β-glucosidases I and II as measured in crude extracts.

Phospho-β-Glucosidase	Inducers	Favored Substrates
I	Cellobiose	Cellobiose-P
	Gentiobiose	Gentiobiose-P
	Cellobiitol (?)	Cellobiitol-P (?)
II	Salicin	Salicin-P
	Arbutin	Arbutin-P
	Phenyl-β-glucoside (?)	Phenyl-β-glucoside-P

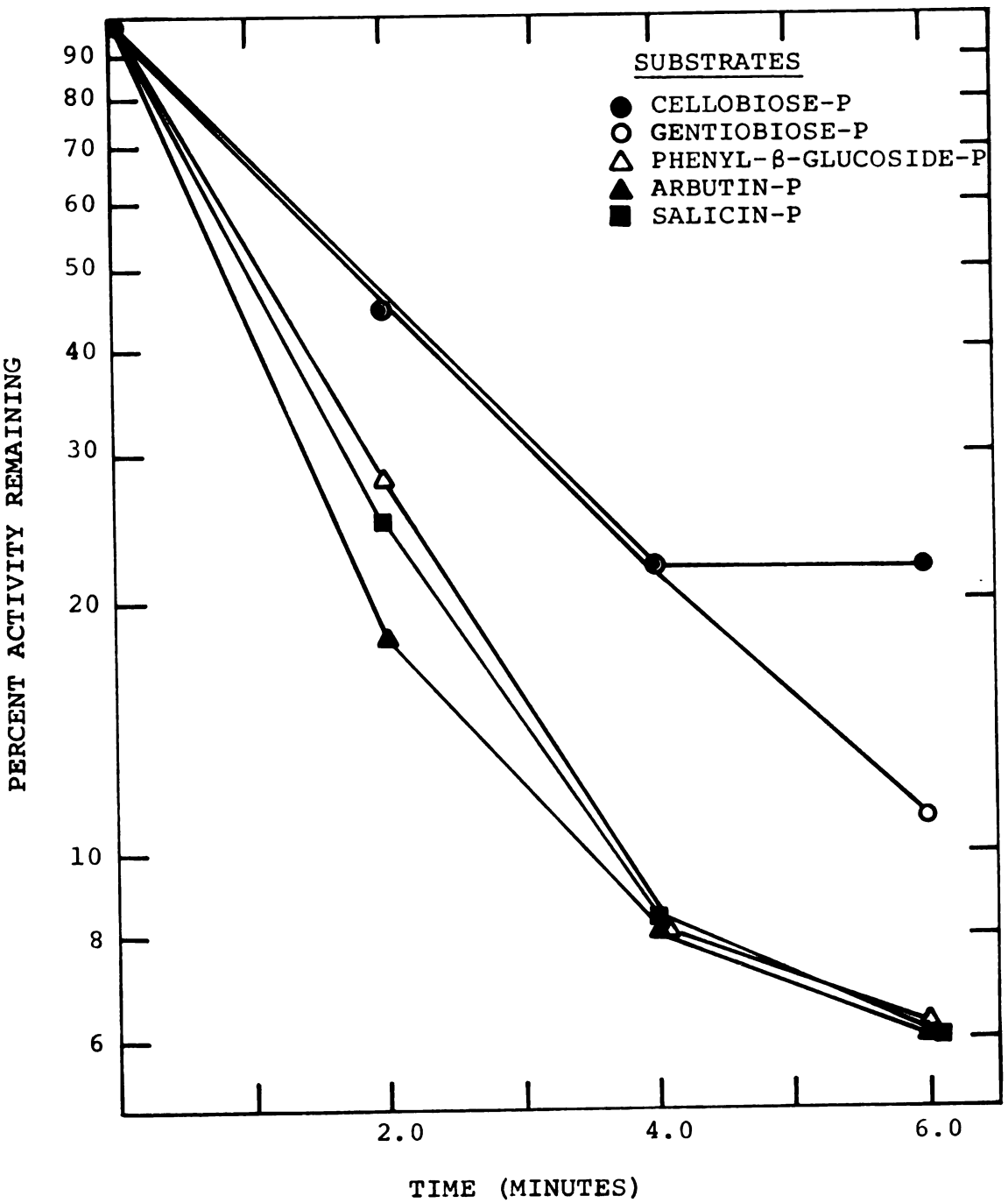
TABLE XXVIII.--Ratios of activities of phospho-β-glucosidase in crude extracts from cells grown on nutrient broth, salicin, cellobiose, gentiobiose, and arbutin. [The ratios of activities for the crude extracts listed below were determined from the specific activity data (A) of Table XXVI.]

Substrate Ratios	Crude Extracts from Cells Grown on Nutrient Broth and Sugars Listed Below				
	Nutrient Broth	Cellobiose	Gentiobiose	Salicin	Arbutin
$\frac{\text{Cellobiose-P}}{\text{Gentiobiose-P}}$	0.36	1.00	0.45	0.67	1.10
$\frac{\text{Cellobiose-P}}{\text{Salicin-P}}$	0.25	0.63	0.48	0.30	0.03
$\frac{\text{Cellobiose-P}}{\text{Phenyl-β-glucoside-P}}$	0.21	0.82	0.29	0.04	0.05
$\frac{\text{Cellobiose-P}}{\text{Arbutin-P}}$	0.50	1.55	0.35	0.03	0.11
$\frac{\text{Cellobiose-P}}{\text{Cellobiitol-P}}$	--[a]	2.10	--[a]	--[a]	--[a]

[a]--indicates ratio was not determined.

so, which activities were lost at the same rate. Concomitantly, the experiment might indicate if both enzymes are present in crude extracts--independent of the growth medium. Figure 32 shows the thermal denaturation data for crude extract from salicin-grown cells. Two patterns were evident, and each was biphasic. The activity for phenyl-β-glucoside-phosphate, arbutin-phosphate, and salicin-phosphate decreased at the same rate to the four minute mark (8% of the activity remaining), then decreased at a much slower rate for the last two minutes (to a final 6% of the activity remaining). The activity on cellobiose-phosphate and gentiobiose-phosphate was lost at the same rate to 22 percent of the activity remaining at the four minute mark, then the curves split. Gentiobiose-phosphate activity decreased to 12 percent remaining after six minutes, while cellobiose-phosphate remained constant at 22 percent at the end of the experiment. These data tend to support the hypothesis of two phospho-β-glucosidases having different substrate activities presented in (a) above. In addition the data indicate the presence of both phospho-β-glucosidases in crude extracts from salicin-grown cells. The biphasic nature of the curves, however, suggested that there was cross-reactivity between the two proposed enzymes, even though phospho-β-glucosidase I showed high activity for cellobiose- and gentiobiose-phosphates and phospho-β-glucosidase II had high activity for the aromatic β-

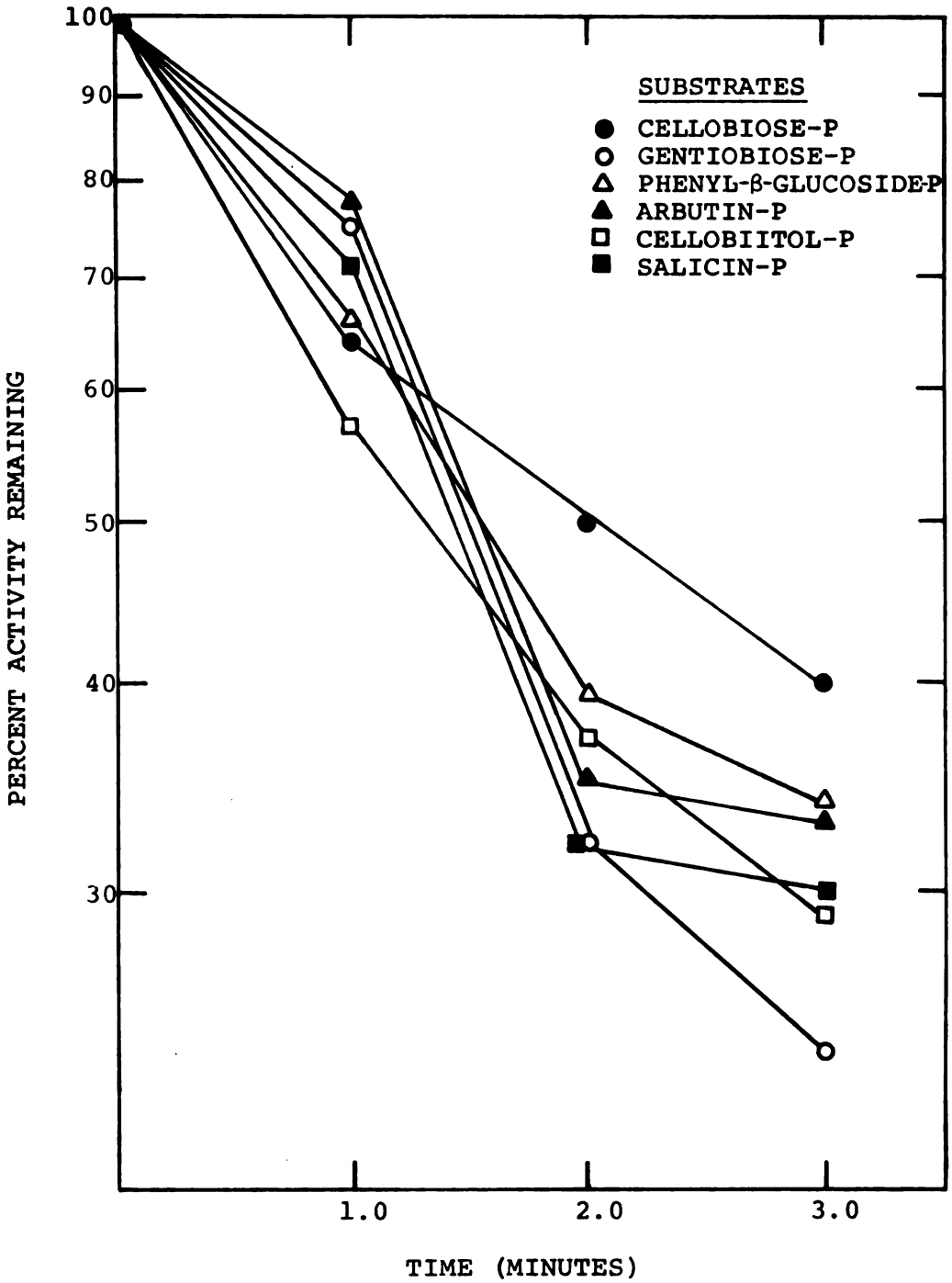
Figure 32.--Thermal denaturation of crude extract from salicin-grown cells. 0.4 ml of crude extract (approximately 3.4 mg of protein) was diluted with 0.4 ml of 0.2 M glycylglycine buffer (pH 7.5) and heated at 51°C in a constant temperature bath. Aliquots were withdrawn at the indicated times and assayed for activity on the six β-glucoside-phosphates indicated. The standard D-glucose-6-phosphate dehydrogenase-linked assay was used.


SUBSTRATES
CELLOBIOSE-P
GENTIOBIOSE-P
PHENYL-β-GLUCOSIDE-P
ARBUTIN-P
SALICIN-P
PERCENT ACTIVITY REMAINING
90
80
70
60
50
40
30
20
10
8
6
2.0
4.0
6.0
TIME (MINUTES)

glucoside-phosphates--salicin-, arbutin-, and phenyl-β-glucoside-phosphate.

Thermal denaturation data for cellobiose- and gentiobiose-grown cells were less clear. Data for gentiobiose-grown cells (Figure 33) showed that activity toward three substrates--arbutin-phosphate, gentiobiose-phosphate, and salicin-phosphate--decreased in parallel for all three β-glucoside-phosphates to the two minute mark (to approximately 34% of the activity remaining), at which time salicin-phosphate and arbutin-phosphate decreased to 30 and 33 percent of the activity remaining while the activity on gentiobiose-phosphate decreased to 24 percent remaining. The activity on cellobiose-phosphate decreased in one minute to 64 percent, and then decreased linearly to 40 percent after three minutes. The loss of activity on cellobiose-phosphate, phenyl-β-glucoside-phosphate, and cellobiitol-phosphate was intermediate to that obtained for salicin-phosphate, arbutin-phosphate, and gentiobiose-phosphate. The only pattern observed from these data is the parallel decrease in activity for salicin-phosphate, arbutin-phosphate, and gentiobiose-phosphate. From the induction data presented in (a) above, one would have predicted that the activity on phenyl-β-glucoside-phosphate should be grouped with that for salicin- and arbutin-phosphates, not gentiobiose-phosphate. This is one unexplained discrepancy in these data.

Figure 33.--Thermal denaturation of crude extract from gentiobiose-grown cells. 0.4 ml of crude extract (approximately 2.7 mg of protein) were diluted with 0.2 ml of 0.2 M glycylglycine buffer (pH 7.5) and heated at 51°C in a constant temperature bath. Aliquots were withdrawn at the indicated times and assayed for activity on the six β-glucoside-phosphates indicated. The standard D-glucose-6-phosphate dehydrogenase-linked assay was used.


100
90
80
70
60
50
40
30
PERCENT ACTIVITY REMAINING
SUBSTRATES
CELLOBIOSE-P
GENTIOBIOSE-P
PHENYL-β-GLUCOSIDE-P
ARBUTIN-P
CELLOBIITOL-P
SALICIN-P
1.0
2.0
3.0
TIME (MINUTES)

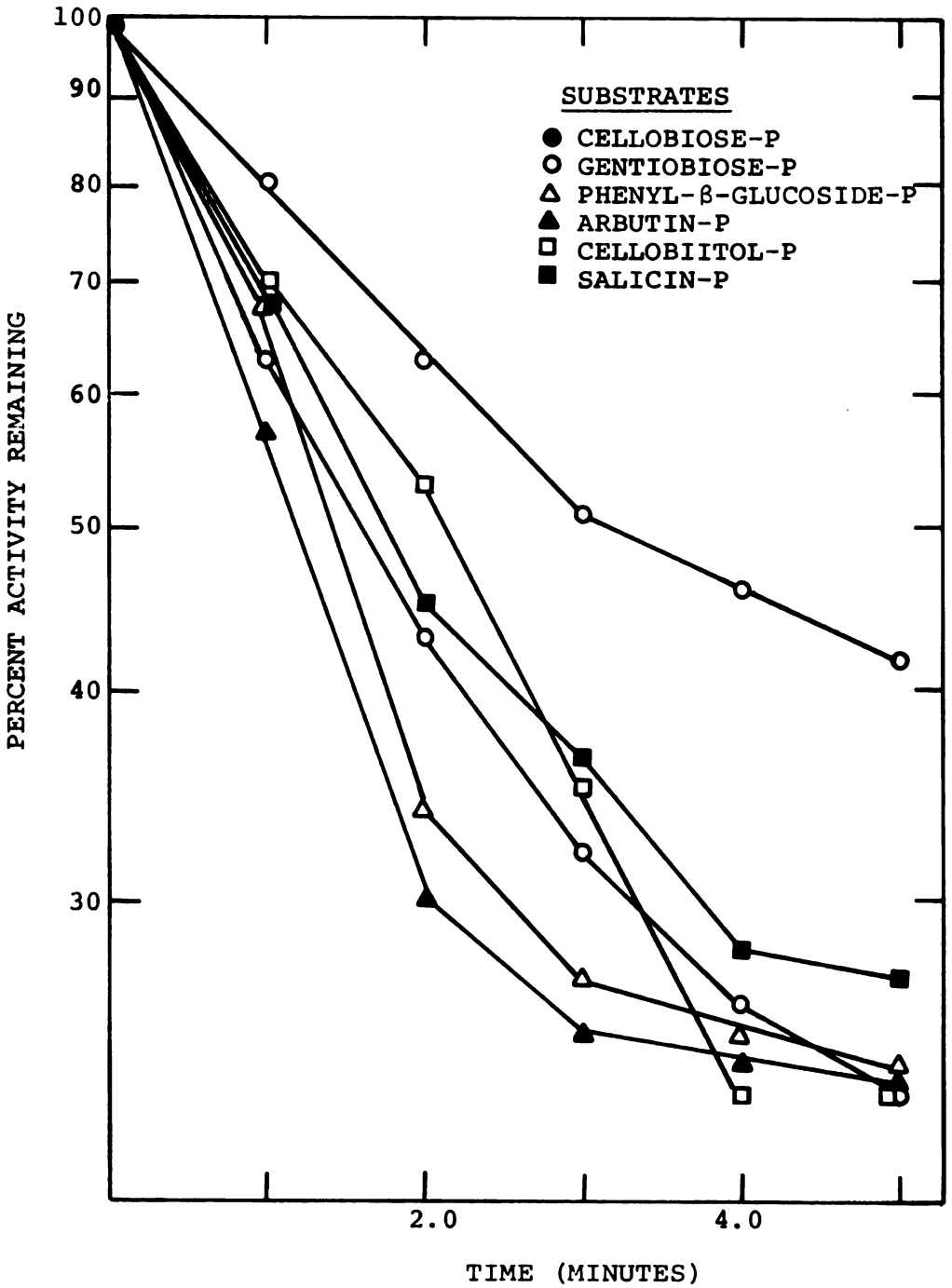
Thermal denaturation data obtained using crude extracts from cellobiose-grown cells (Figure 34) again showed no discernible patterns for substrate activity losses. Loss of activity on cellobiose-phosphate was linear to 50 percent remaining at 3 minutes, and then decreased linearly at a much slower rate for the final 2 minutes of the experiment to a final 41 percent of the activity remaining. Activity on the other five β-glucoside-phosphates (gentiobiose-, phenyl-β-glucoside-, arbutin-, cellobiitol-, and salicin-phosphates) showed a much higher rate of loss, but did not decrease to zero during the time of the experiment. All five yielded approximately 23.5 percent of activity remaining at the end of the experiment (cellobiitol-phosphate activity was slightly higher at 27 percent remaining).

Therefore, inconclusive results were obtained with crude extracts from cellobiose- and gentiobiose-grown cells concerning both the presence or absence of two distinct phospho-β-glucosidases and clear patterns of activity losses on the six β-glucoside-phosphate substrates.

Purification of Phospho-β-glucosidase from Cellobiose-Grown Cells

The separation from β-glucoside kinase and partial purification of the phospho-β-glucosidase through ammonium sulfate fractionation was described in Part II of the Results section of this thesis (see Table XVIII for a summary

Figure 34.--Thermal denaturation of crude extract from cellobiose-grown cells. 0.4 ml of crude extract (approximately 2.2 mg of protein) was diluted with 0.2 ml of 0.2 M glycylglycine buffer (pH 7.5) and heated at 51°C in a constant temperature bath. Aliquots were withdrawn at the indicated times and assayed for activity on the six β-glucoside-phosphates indicated. The standard D-glucose-6-phosphate dehydrogenase-linked assay was used.


100
90
80
70
60
50
40
30
PERCENT ACTIVITY REMAINING
SUBSTRATES
CELLOBIOSE-P
GENTIOBIOSE-P
PHENYL-β-GLUCOSIDE-P
ARBUTIN-P
CELLOBIITOL-P
SALICIN-P
2.0
4.0
TIME (MINUTES)

of the purification through the ammonium sulfate step). The phospho-β-glucosidase precipitated in the 40-60 percent saturated ammonium sulfate fraction (designated precipitate b), and was essentially free of β-glucoside kinase activity at this point (1.2% of the kinase activity remained in this fraction). This fraction was then further purified by chromatography on Sephadex G75. A column of Sephadex G75 (2.5 x 35.0 cm) was equilibrated with 0.02 M glycylglycine buffer (pH 7.5) in the cold, and after carefully layering the enzyme solution on the top of the column, elution with 0.02 M glycylglycine buffer (pH 7.5) was begun. The flow rate was adjusted to 1 ml/0.75 min, and five-ml fractions were collected. The elution profile is shown in Figure 35. A summary of the purification for the phospho-β-glucosidase is given in Table XXIX. The peak fractions (15-20) from the column were combined and showed a 14-fold purification, and contained no glucokinase or phosphoglucomutase activity (< 0.001 μmoles NADP reduced/minute/mg of protein). Since no other enzyme activity would interfere with the studies which follow, the enzyme was used without further purification.

Properties

a. Ratio of Activities of the Crude and Purified Phospho-β-glucosidase

Crude phospho-β-glucosidase was prepared from wild-type cells grown in mineral medium utilizing cellobiose as

Figure 35.--Sephadex G75 chromatography of phospho-β-glucosidase. Details of the procedure are given in the text.

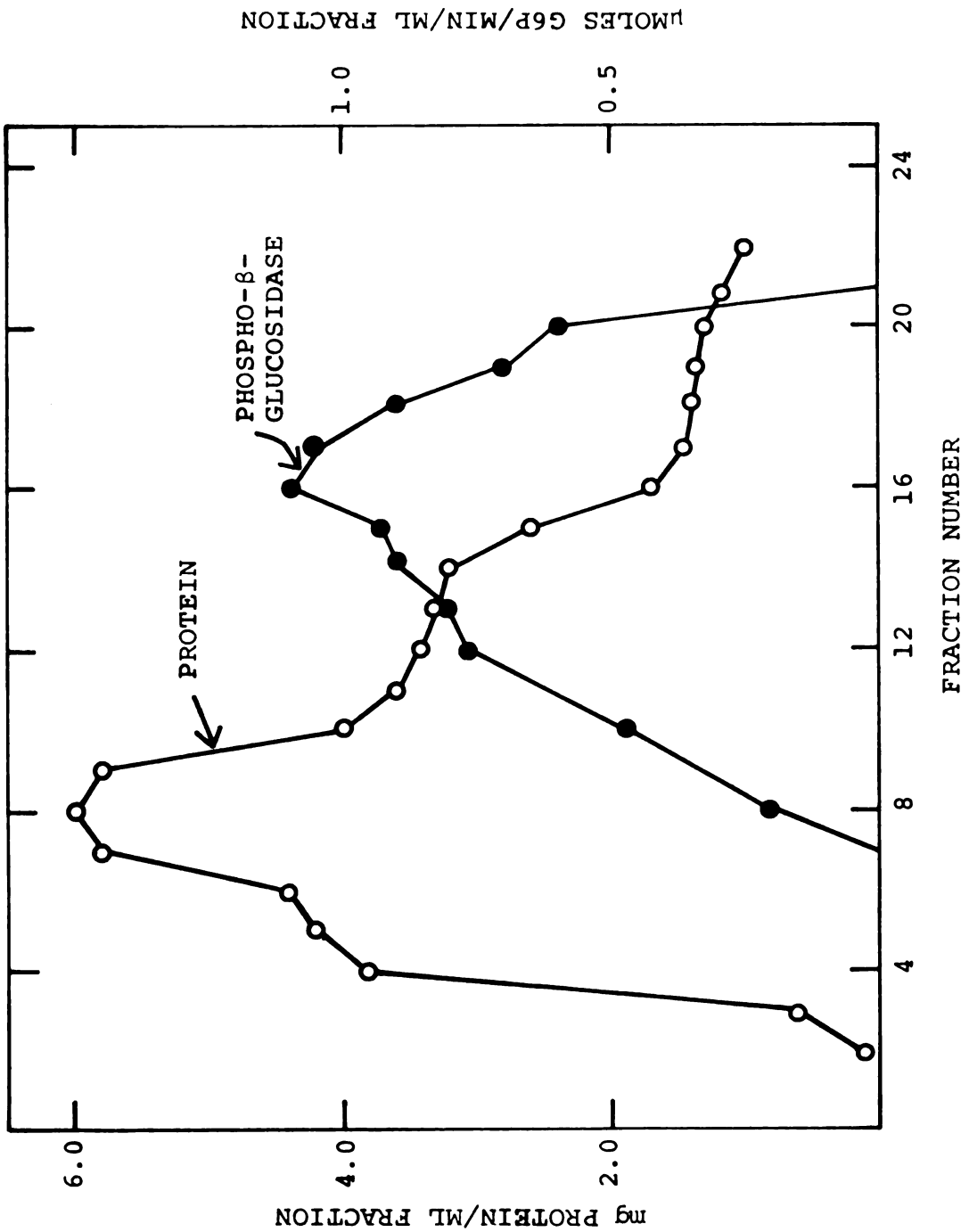

μMOLES G6P/MIN/ML FRACTION
1.0
0.5
PHOSPHO-β-
GLUCOSIDASE
PROTEIN
6.0
4.0
2.0
mg PROTEIN/ML FRACTION
4
8
12
16
20
24
FRACTION NUMBER

TABLE XXIX.--Further purification of phospho-β-glucosidase.

Fraction	Total Protein mg	Total Activity Units[a]	Specific Activity Units/mg Protein	Recovery %	Fold Purified
Ammonium Sulfate[b] 40-60% Saturated	450	53	0.12	54	3.2
Sephadex G75 Chromatography					
Fractions 15	13.0	4.6	0.36	4.7	9.4
16	8.5	5.5	0.65	5.6	17
17	8.0	5.3	0.66	6.7	17
18	7.5	4.5	0.60	6.1	16
19	7.0	3.5	0.50	5.1	13
20	6.5	3.0	0.46	4.7	12
	51	27	14	33	

[a]A unit is defined as μmoles NADP reduced/minute.

[b]These data are taken from Table XVII.

the sole carbon source as previously described in this thesis. This crude preparation and 14-fold purified phospho-β-glucosidase were tested to determine whether the ratios of activities on several substrates remained constant before and after purification, or whether the purification removed one of the two phospho-β-glucosidases, previously indicated to be present in crude extracts, causing a change in the substrate ratios. The data presented in Table XXX show that the substrate ratios changed very little after purification. The two ratios of cellobiose-phosphate to gentiobiose-phosphate and to salicin-phosphate remained constant after a 14-fold purification. However, the ratios of cellobiose-phosphate to phenyl-β-glucoside-phosphate and to arbutin-phosphate decreased from 0.82 and 1.24 to 0.41 and 0.93, respectively. These data suggest that only one phospho-β-glucosidase is present in cellobiose-grown cells which shows activity on both aromatic and aliphatic β-glucoside-phosphates. These data will be deliberated in detail in the Discussion section of this part of the thesis.

b. Thermal Denaturation of Purified Phospho-β-Glucosidase

Purified phospho-β-glucosidase was heated in a constant temperature bath at 51°C, and the decrease in

TABLE XXX.--Ratio of activities of crude and purified phospho-β-glucosidase from cellobiose-grown cells. [The data for determining the ratios of activities of the various substrates for the crude preparation of phospho-β-glucosidase from cellobiose-grown cells were derived from Table XXVI. The data for the purified phospho-β-glucosidase were taken from V_{max} values derived from the Lineweaver-Burk plots for each substrate, shown in Figures 37 through 40.]

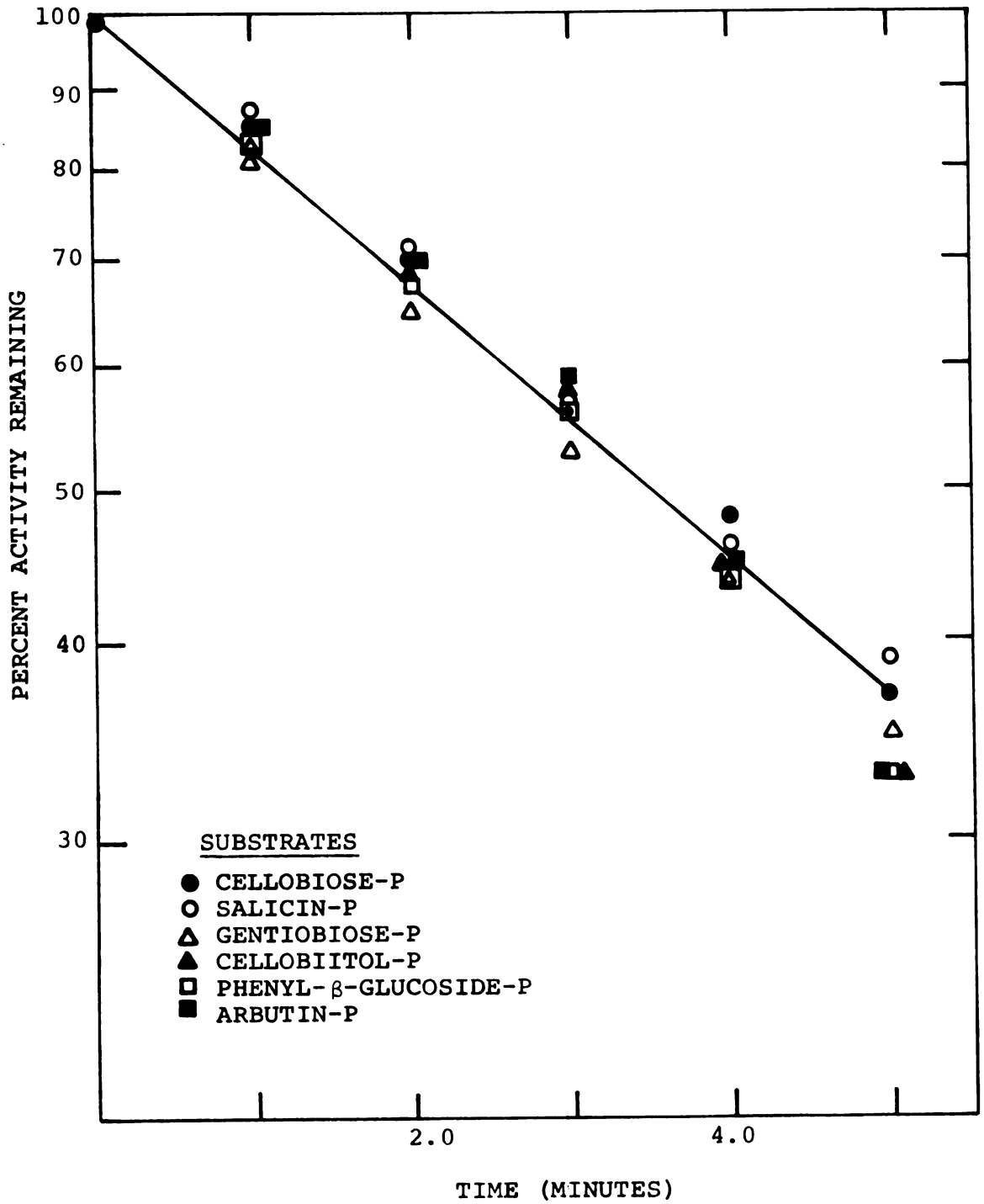
Substrate Ratios	Crude Enzyme	Purified Enzyme
$\frac{\text{Cellobiose-P}}{\text{Gentiobiose-P}}$	1.00	0.76
$\frac{\text{Cellobiose-P}}{\text{Salicin-P}}$	0.63	0.53
$\frac{\text{Cellobiose-P}}{\text{Phenyl-β-glucoside-P}}$	0.82	0.41
$\frac{\text{Cellobiose-P}}{\text{Arbutin-P}}$	1.55	0.93

activity toward cellobiose-phosphate, salicin-phosphate, gentiobiose-phosphate, cellobiitol-phosphate, phenyl-β-glucoside-phosphate, and arbutin-phosphate was measured and plotted as the percent of activity remaining for each substrate vs time of incubation (Figure 36). A single straight line was obtained showing a parallel decrease in activity for all six β-glucoside-phosphates tested. These data suggest that a single enzyme is present after purification which exhibits multiple substrate specificity.

c. Specificity for Compounds Other than β-Glucoside-Phosphates

No free sugar other than those previously described (cellobiose-phosphate, gentiobiose-phosphate, salicin-phosphate, arbutin-phosphate, phenyl-β-glucoside-phosphate, methyl-β-glucoside-phosphate, and cellobiitol-phosphate) served as substrates for the phospho-β-glucosidase. No activity was detected with the following sugars at a concentration of 6.6 mM (< 0.0001 μmole NADP reduced/min/mg of protein): cellobiose, gentiobiose, salicin, arbutin, phenyl-β-glucoside, methyl-β-glucoside, cellobiitol, D-glucose, trehalose, and maltose.

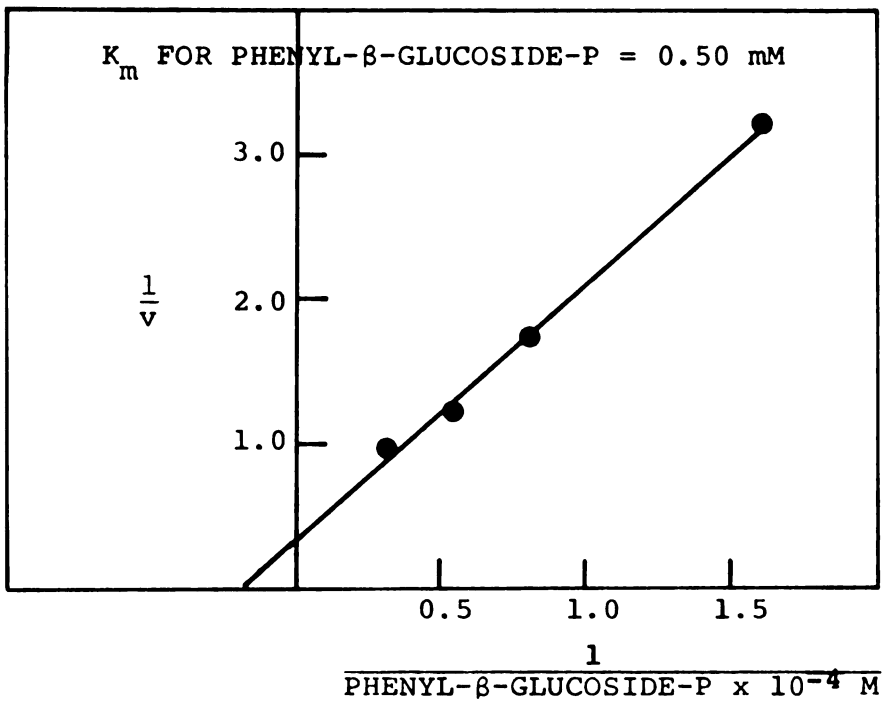
Figure 36.--Thermal denaturation of purified phospho-β-glucosidase at 51°C. Fourteen-fold purified enzyme was used. The protein solution was heated in 0.2 M glycylglycine buffer (pH 7.5). Aliquots were withdrawn at the times indicated and were analyzed with the standard D-glucose-6-phosphate dehydrogenase assay using cellobiose-P, gentiobiose-P, cellobiitol-P, salicin-P, arbutin-P, and phenyl-β-glucoside-P as substrates for the reaction.


100
90
80
70
60
50
40
30
PERCENT ACTIVITY REMAINING
SUBSTRATES
CELLOBIOSE-P
SALICIN-P
GENTIOBIOSE-P
CELLOBIITOL-P
PHENYL-β-GLUCOSIDE-P
ARBUTIN-P
2.0
4.0
TIME (MINUTES)

d. Substrate Activity of β-Glucoside-Phosphates

Seven β-glucoside-phosphates were tested for substrate activity with 14-fold purified phospho-β-glucosidase I. The compounds tested were cellobiose-phosphate, gentiobiose-phosphate, cellobiitol-phosphate, salicin-phosphate, arbutin-phosphate, phenyl-β-glucoside-phosphate, and methyl-β-glucoside-phosphate. The D-glucose-6-phosphate dehydrogenase assay was employed to measure the activity with all of the β-glucoside-phosphates. All served as substrates for the enzyme; therefore, Lineweaver-Burk plots were used to determine the K_m and V_{max} values for each compound. The plots are shown in Figures 37 through 40. A summary of the K_m and V_{max} values determined from these data for each substrate is shown in Table XXXI. The data show a range of K_m values from 0.23 mM for cellobiose to 0.5 mM for salicin- and phenyl-β-glucoside-phosphates. The greatest V_{max} value was obtained for phenyl-β-glucoside-phosphate; therefore, the V_{max} values were normalized relative to that of phenyl-β-glucoside-phosphate. Gentiobiose-phosphate, cellobiose-phosphate, and cellobiitol-phosphate have V_{max} values of 0.54, 0.41, and 0.39 relative to that of phenyl-β-glucoside-phosphate. No other α- or β-

Figure 37.--Lineweaver-Burk plots relating phospho-β-glucosidase activity to phenyl-β-glucoside-P and cellobiose-P concentration. The standard glucose-6-phosphate dehydrogenase assay was employed except that the concentrations of phenyl-β-glucoside-P and cellobiose-P were varied, as indicated, with the concentration of phospho-β-glucosidase constant. Fourteen-fold purified phospho-β-glucosidase was used.


K_m FOR PHENYL-β-GLUCOSIDE-P = 0.50 mM
3.0
2.0
1.0
$\frac{1}{v}$
0.5
1.0
1.5
$\frac{1}{\text{PHENYL-β-GLUCOSIDE-P x } 10^{-4} \text{ M}}$

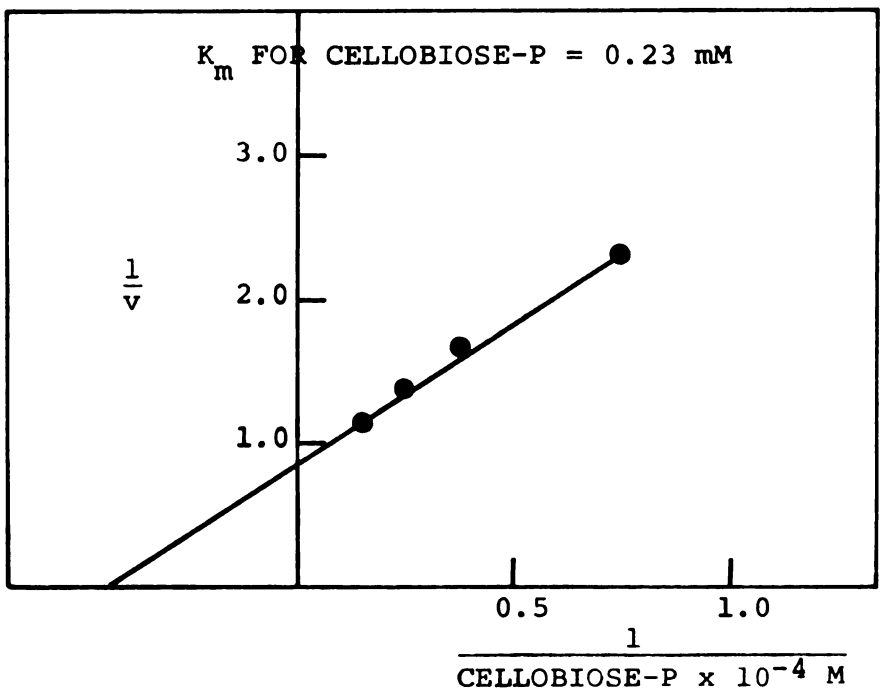
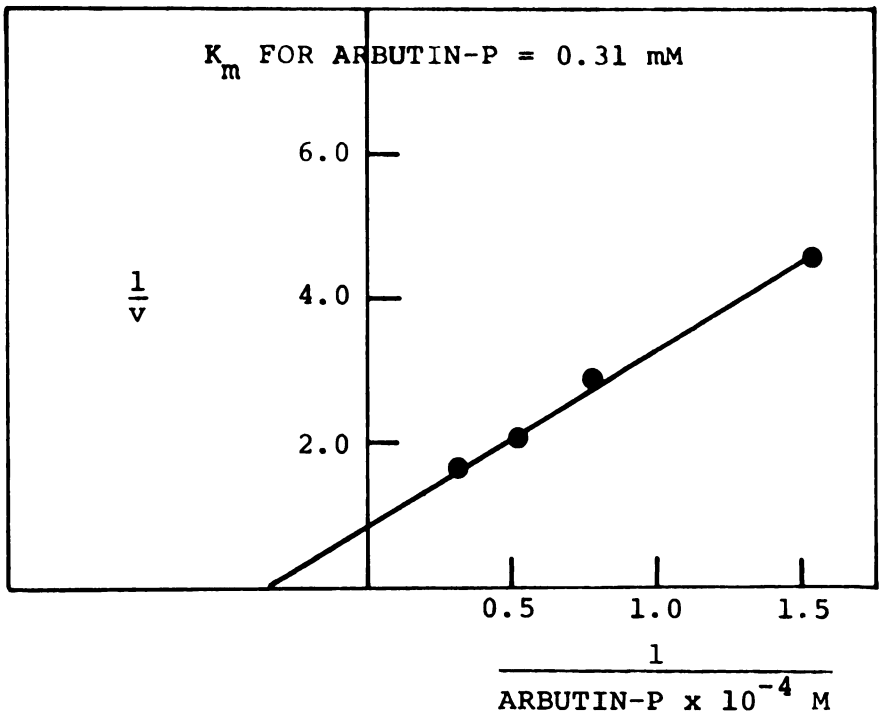

K_m FOR CELLOBIOSE-P = 0.23 mM
3.0
2.0
1.0
$\frac{1}{v}$
0.5
1.0
$\frac{1}{\text{CELLOBIOSE-P x } 10^{-4} \text{ M}}$

Figure 38.--Lineweaver-Burk plots relating phospho-β-glucosidase activity to arbutin-P and cellobiitol-P concentration. The standard glucose-6-phosphate dehydrogenase assay was employed except that the concentrations of arbutin-P and cellobiitol-P were varied, as indicated, with the concentration of phospho-β-glucosidase constant. Fourteen-fold purified phospho-β-glucosidase was used.


K_m FOR ARBUTIN-P = 0.31 mM
6.0
4.0
2.0
$\frac{1}{v}$
0.5
1.0
1.5
$\frac{1}{\text{ARBUTIN-P x } 10^{-4} \text{ M}}$

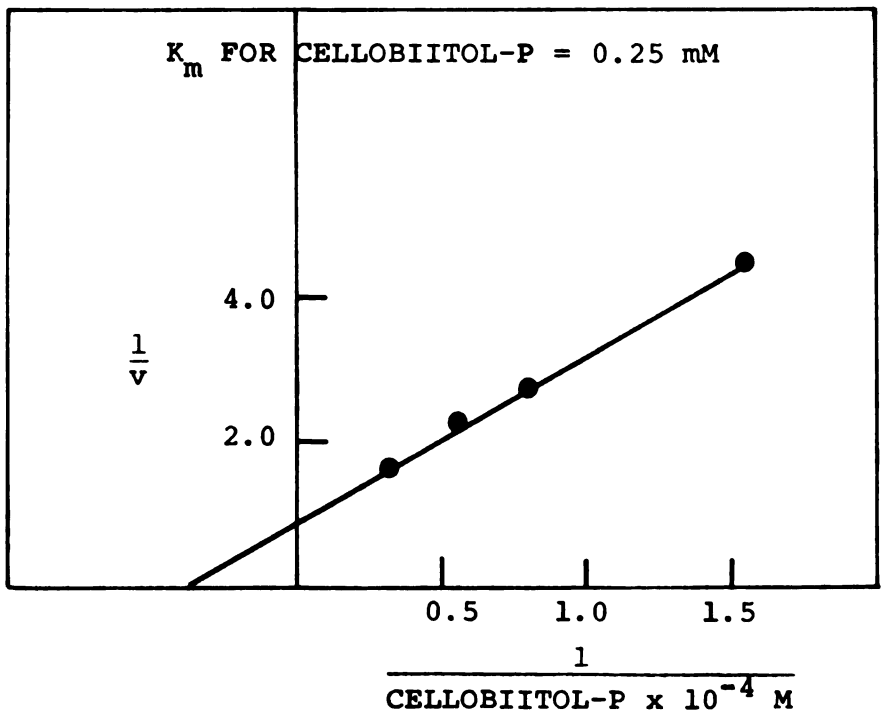
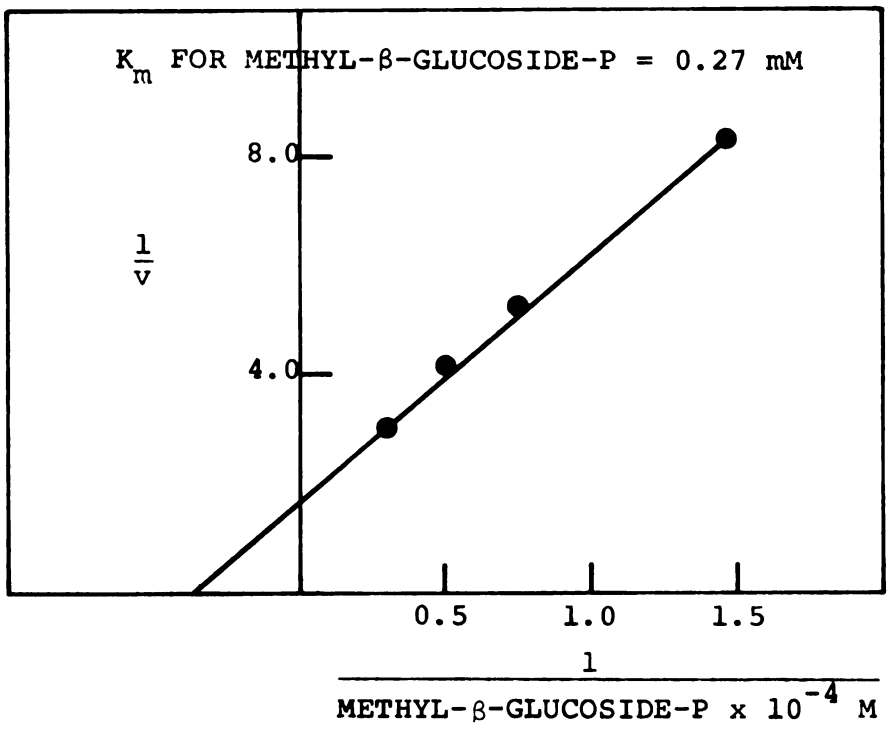

K_m FOR CELLOBIITOL-P = 0.25 mM
4.0
2.0
$\frac{1}{v}$
0.5
1.0
1.5
$\frac{1}{\text{CELLOBIITOL-P x } 10^{-4} \text{ M}}$

Figure 39.--Lineweaver-Burk plots relating phospho-β-glucosidase activity to methyl-β-glucoside-P and gentiobiose-P concentration. The standard glucose-6-phosphate dehydrogenase assay was employed except that the concentrations of methyl-β-glucoside-P and gentiobiose-P were varied, as indicated, with the concentration of phospho-β-glucosidase constant. Fourteen-fold purified phospho-β-glucosidase was used.


K_m FOR METHYL-β-GLUCOSIDE-P = 0.27 mM
8.0
4.0
$\frac{1}{v}$
0.5
1.0
1.5
$\frac{1}{\text{METHYL-β-GLUCOSIDE-P x } 10^{-4} \text{ M}}$

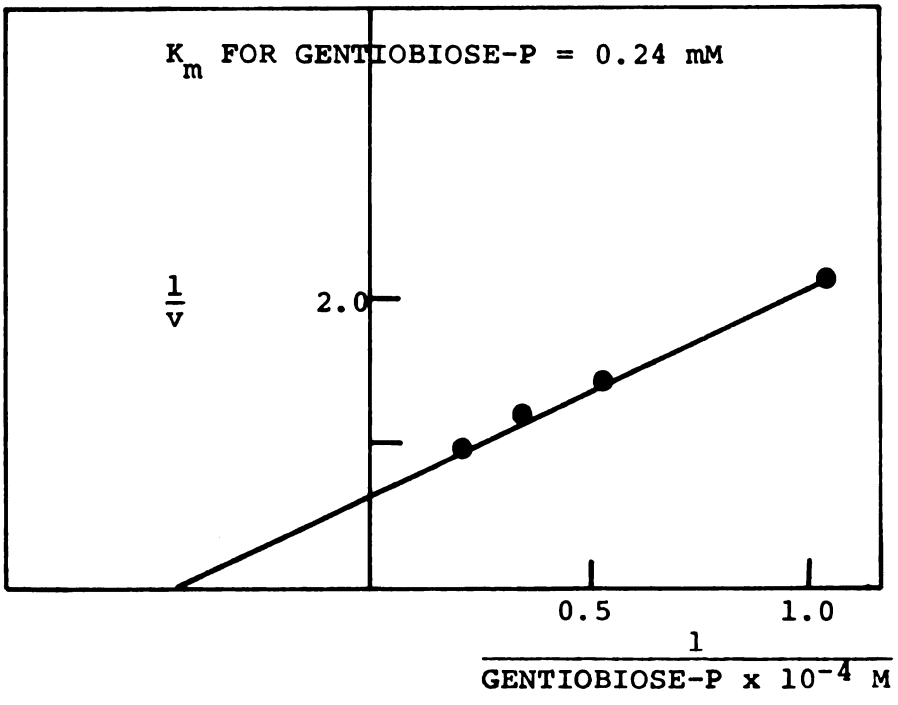

K_m FOR GENTIOBIOSE-P = 0.24 mM
$\frac{1}{v}$
2.0
0.5
1.0
$\frac{1}{\text{GENTIOBIOSE-P x } 10^{-4} \text{ M}}$

Figure 40.--Lineweaver-Burk plot relating phospho-β-glucosidase activity to salicin-P concentration. The standard glucose-6-phosphate dehydrogenase assay was employed except that the concentration of salicin-P was varied, as indicated, with the concentration of phospho-β-glucosidase constant. Fourteen-fold purified phospho-β-glucosidase was used.

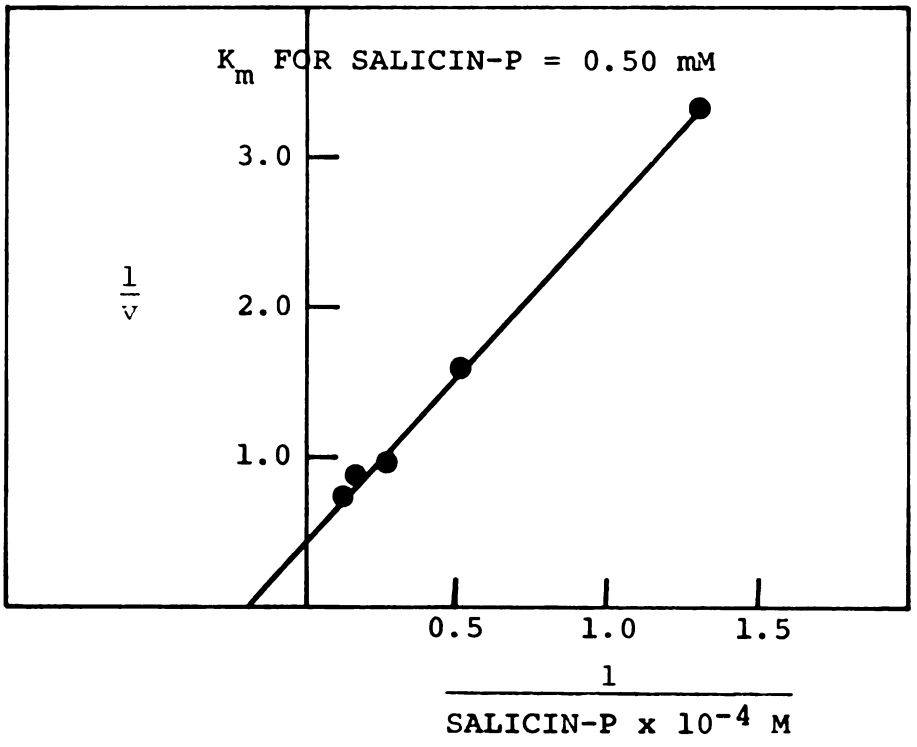

K_m FOR SALICIN-P = 0.50 mM
3.0
2.0
1.0
$\frac{1}{v}$
0.5
1.0
1.5
$\frac{1}{\text{SALICIN-P x } 10^{-4} \text{ M}}$

TABLE XXXI.--A summary of the kinetic constants obtained for 14-fold purified phospho-β-glucosidase. [The data presented in this table were derived from the Lineweaver-Burk plots given in Figures 37 through 40. The V_{max} values for all substrates were calculated relative to the V_{max} for phenyl-β-glucoside-phosphate.]

β-Glucoside-P	Lineweaver-Burk Plot	
	K_m (mM)	Relative V_{max}
Phenyl-β-glucoside-P	0.50	1.00
Salicin-P	0.50	0.78
Gentiobiose-P	0.24	0.54
Arbutin-P	0.31	0.44
Cellobiose-P	0.23	0.41
Cellobiitol-P	0.25	0.39
Methyl-β-glucoside-P	0.27	0.20

glucoside-phosphate is available for use in a substrate study of this type.

e. Effect of Mixing Substrates

The purified phospho-β-glucosidase was tested for activity using equimolar mixtures of two substrates to determine if the general specificity shown by the purified preparation were due to a single enzyme or more than one enzyme. A single enzyme should give non-additive rates on an equimolar mixture of two substrates. The data (Table XXXII) show the rates to be neither additive nor inhibitory. These data further suggest that the multiple activities detected for the phospho-β-glucosidase present after purification is due to one enzyme.

f. pH Optima

The pH optimum of the phospho-β-glucosidase in nine different buffers (glycylglycine, Tes, Mes, Tris-HCl, phosphate, Pipes, acetate, Bicine, and Tricine) was determined. The pH range covered was from 5.2 to 9.0. The standard D-glucose-6-phosphate dehydrogenase-linked assay was used to detect the activity. The pH optimum varied with the buffer, but all fell between 6.8 and 7.7 (Figure 41).

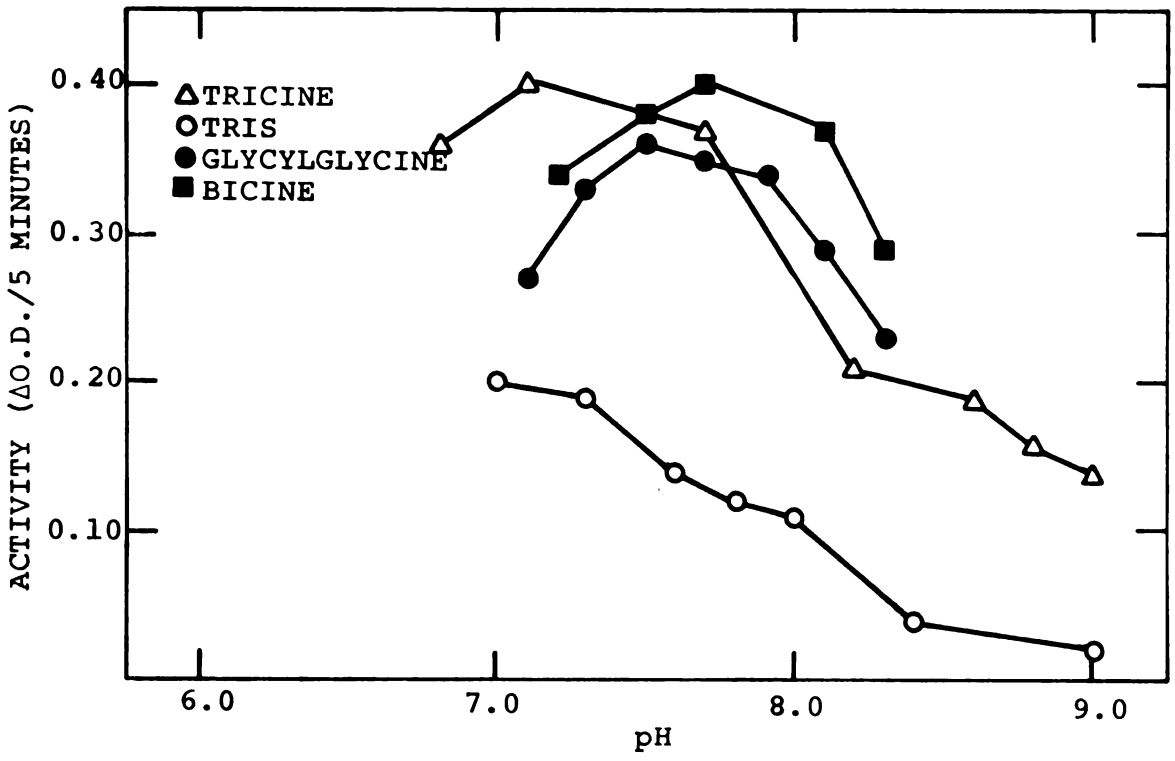
g. Approximate Molecular Weight Determination

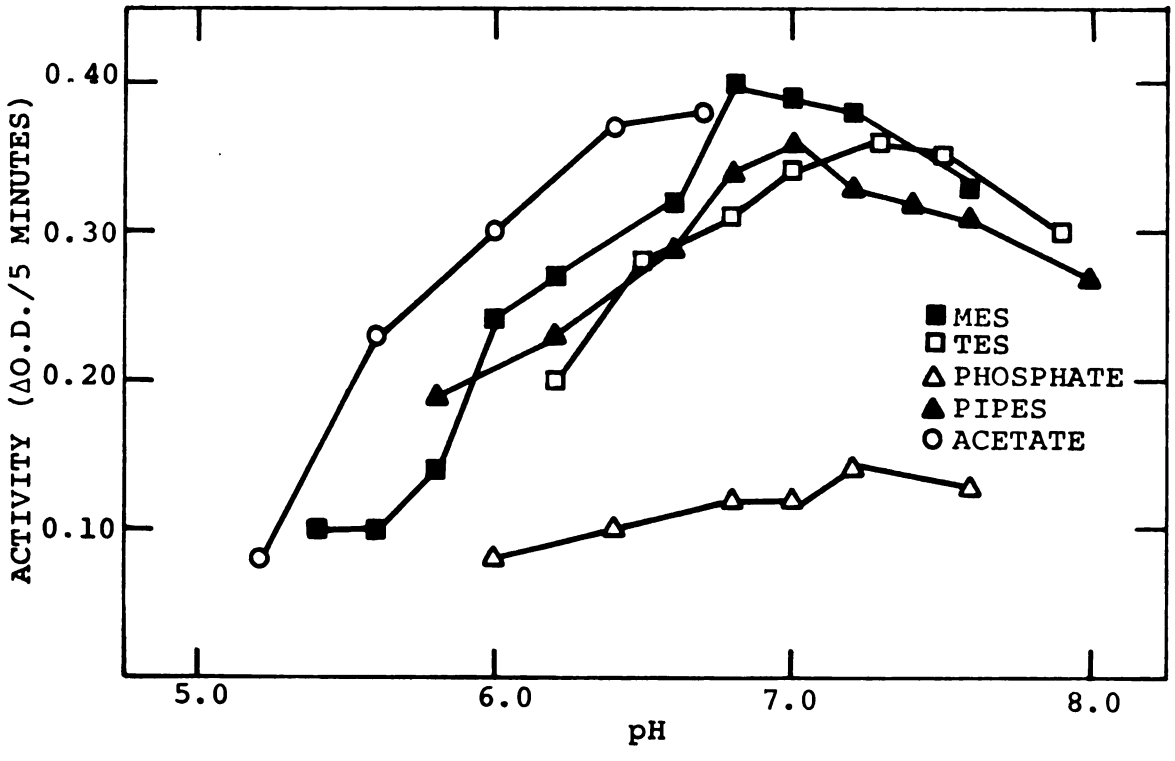
An approximate molecular weight was determined for the phospho-β-glucosidase using sucrose density gradient

TABLE XXXII.--Effect of mixing substrates on the activity of purified phospho-β-glucosidase.

Substrate	Specific Activity µmoles NADP reduced/min/ mg of protein
Cellobiose-P	0.38
Phenyl-β-glucoside-P	0.31
Salicin-P	0.63
Arbutin-P	0.15
Gentiobiose-P	0.40
Cellobiose-P + Phenyl-β-glucoside-P	0.33
Cellobiose-P + Salicin-P	0.49
Cellobiose-P + Arbutin-P	0.26
Cellobiose-P + Gentiobiose-P	0.38

Figure 41.--pH optima of phospho-β-glucosidase in nine different buffers. The glucose-6-phosphate dehydrogenase assay was employed. Details of the buffers and pH ranges used are given in the text. Fourteen-fold purified phospho-β-glucosidase was used.


ACTIVITY (ΔO.D./5 MINUTES)
0.40
0.30
0.20
0.10
△TRICINE
○TRIS
●GLYCYLGLYCINE
■BICINE
6.0
7.0
8.0
9.0
pH


ACTIVITY (ΔO.D./5 MINUTES)
0.40
0.30
0.20
0.10
■MES
□TES
△PHOSPHATE
▲PIPES
○ACETATE
5.0
6.0
7.0
8.0
pH

centrifugation. A 5-20 percent sucrose gradient was employed, and horseradish peroxidase served as the standard (see Figure 42). Taking 3.5 S as the sedimentation coefficient of the peroxidase (97), the sedimentation coefficient of phospho-β-glucosidase was calculated from the equation,

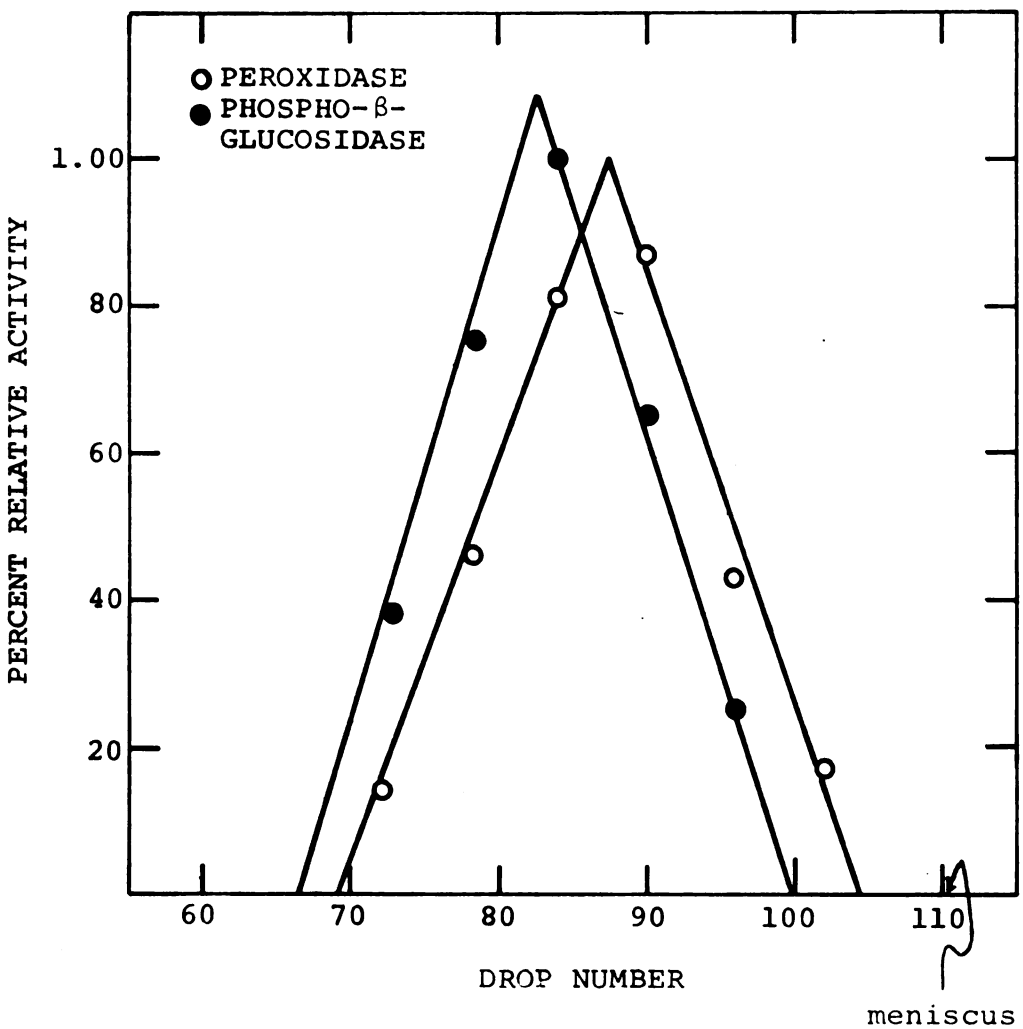
$$S_1 \times \text{distance}_2 = S_2 \times \text{distance}_1$$

(from Martin and Ames (98). Using this method an S value of 4.3 was obtained for the phospho-β-glucosidase. The approximate molecular weight was obtained from a plot of log S vs log of the molecular weight using the data of Tanford (99). A molecular weight of approximately 55,000 was obtained from the experimentally determined S value of 4.3.

h. Determination of K_i for D-Glucose

The ability of one of the products (D-glucose) of the hydrolysis of cellobiose-phosphate by the phospho-β-glucosidase to effect an inhibition of the reaction was tested by including high concentrations of D-glucose in reaction mixtures containing a constant amount of phospho-β-glucosidase and varying concentrations of cellobiose-phosphate near the K_m value. The standard D-glucose-6-phosphate dehydrogenase-linked assay was used to measure

Figure 42.--Sucrose density gradient centrifugation of phospho-β-glucosidase for the determination of an approximate molecular weight. Details are given in the text.

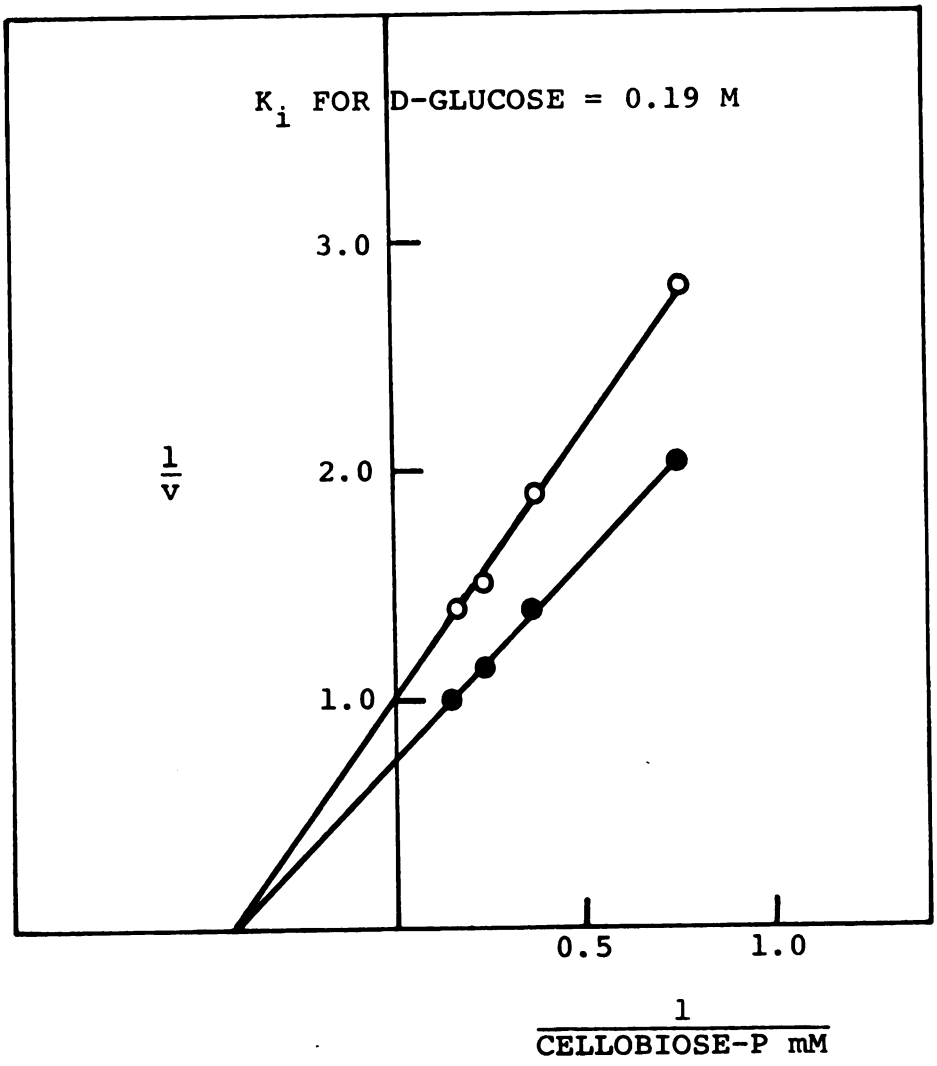

PEROXIDASE
PHOSPHO-β-GLUCOSIDASE
PERCENT RELATIVE ACTIVITY
1.00
80
60
40
20
60
70
80
90
100
110
DROP NUMBER
meniscus

enzyme activity. A non-competitive inhibition is observed for D-glucose, with a K_i of 0.2 M (Figure 43).

Discussion

The data presented in this section of the thesis suggests that not one but two phospho-β-glucosidases are present in crude extracts from cellobiose-, gentiobiose-, salicin-, arbutin-, and nutrient broth-grown cells. A low constitutive amount of phospho-β-glucosidase activity is present in nutrient broth-grown cells. Induction studies, using cellobiose, gentiobiose, salicin, and arbutin as inducers, employing seven β-glucoside-phosphates (cellobiose-, gentiobiose-, salicin-, arbutin-, phenyl-β-glucoside-, methyl-β-glucoside-, and cellobiitol-phosphates) as substrates suggested that the phospho-β-glucosidase induced was dependent on the inducer. Salicin and arbutin induced phospho-β-glucosidase activity on salicin-, phenyl-β-glucoside-, and arbutin-phosphates approximately 10-fold. The activity of these extracts on cellobiose- or gentiobiose-phosphates remained at approximately the uninduced constitutive level. One difference was noted between induction by salicin and arbutin, however, which was that salicin extracts also had high activity on methyl-β-glucoside-phosphate while that from arbutin did not. Induction by cellobiose and gentiobiose caused a maximum of a 10-fold increase in activity on cellobiose- and gentiobiose-phosphates. Activity on cellobiitol-phosphate was

Figure 42.--Determination of K_i for D-glucose for purified phospho-β-glucosidase. The standard D-glucose-6-phosphate dehydrogenase assay was employed, using cellobiose-phosphate as the substrate. Fourteen-fold purified phospho-β-glucosidase was used.


K_i FOR D-GLUCOSE = 0.19 M
3.0
2.0
1.0
$\frac{1}{v}$
0.5
1.0
$\frac{1}{\text{CELLOBIOSE-P mM}}$

intermediate between that on cellobiose- and gentiobiose-phosphates, while activity on salicin-, phenyl-β-glucoside-, arbutin-, and methyl-β-glucoside-phosphates remained at the uninduced levels. However, the specific activities on all β-glucoside-phosphates were approximately the same. On the basis of these data, it seemed that the activities could be formed into two groups; one group consisted of the aromatic β-glucoside-phosphates, and the other consisted of both aromatic- and disaccharide-phosphates.

Thermal denaturation studies with the same crude extracts discussed above should theoretically have reinforced the findings from the induction studies. Thermal inactivation studies with crude extract from salicin-grown cells did tend to support the induction data. Two curves were found in which activity on salicin-, arbutin-, and phenyl-β-glucoside-phosphates in one group and cellobiose- and gentiobiose-phosphates in the other were lost in parallel. These data also suggested that both phospho-β-glucosidases were present in the crude extract--one phospho-β-glucosidase with high activity on the aromatic substrates and the other at the constitutive level. The data for gentiobiose- and cellobiose-grown cells were inconclusive. No definite patterns for activity losses were evident and there was no evidence to indicate the presence of more than one phospho-β-glucosidase. However, from the induction data for salicin- and cellobiose-grown cells and the thermal

denaturation data for salicin-grown cells, two phospho-β-glucosidases (I and II), differing in both substrate specificity and induction, were suggested.

Substrate activity studies with purified phospho-β-glucosidase from cellobiose-grown cells indicated that no free α- or β-glucoside served as a substrate for the enzyme. However, when tested for activity with seven β-glucoside-phosphates, activity was observed on all. In addition to these observations, a thermal denaturation experiment in which the loss of activity on the same β-glucoside-phosphates was followed with time, a single linear line showing a parallel loss in activity was observed on all substrates. These thermal denaturation data indicate that one enzyme is responsible for all the activities.

Ratios of activities of the crude and purified phospho-β-glucosidase from cellobiose-grown cells have changed very little after the 14-fold purification. The ratios of activities on cellobiose-phosphate to salicin- and to gentiobiose-phosphates remain constant while those for cellobiose-phosphate to phenyl-β-glucoside- and arbutin-phosphates decrease from 0.82 and 1.55 to 0.41 and 0.93, respectively. These ratios have decreased less than 2-fold which seems to indicate that in cellobiose-grown cells one phospho-β-glucosidase is induced having activity on both the aromatic- and disaccharide-phosphates. Also, non-additive rates were observed with mixed substrates using

the purified enzyme. Schaefler and Schenkein have reported the presence of two phospho-β-glucosidases in A. aerogenes (78). These two enzymes, A and B, were differentiated on the basis of their reactivity on salicin. B has high activity on salicin-phosphate while A does not. Since they did not report further substrate studies with B, the activity data presented in this thesis cannot be compared to that enzyme.

SUMMARY

The biodegradative pathway of cellobiose, gentiobiose, and cellobiitol in A. aerogenes PRL-R3 has been elucidated. All three disaccharides are phosphorylated by a β-glucoside kinase using ATP as the phosphoryl donor. The disaccharide-phosphates are then hydrolyzed by a phospho-β-glucosidase to yield D-glucose-6-phosphate and D-glucose (glucitol in the case of cellobiitol). Cellobiose-phosphate, a new disaccharide-phosphate, was isolated and identified. The two enzymes of the pathway have been partially purified and characterized. Cellobiose, gentiobiose, and cellobiitol induce the β-glucoside kinase to approximately the same specific activity, and evidence was presented that all three sugars induce the same enzyme. Mutant strains of A. aerogenes were isolated which showed a lack of growth only on cellobiose, gentiobiose, and cellobiitol; the mutants were shown not to contain β-glucoside kinase activity. Phospho-β-glucosidase activity was constitutive, but was induced 10-fold after growth on cellobiose.

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