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UNIVERSITY OF ALBERTA

KINETICS OF ALLOLACTOSE SYNTHESIS USING β–GALACTOSIDASE

by

GREG HOLLOWAY

A THESIS
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for my mother

Doris Faye Collins Holloway

October 17, 1937 – August 3, 1988

ABSTRACT

The use of enzymes in the synthesis of carbohydrates has been receiving increasing attention because of the important roles played by carbohydrates in biological systems and the difficulty of synthesizing carbohydrates (with high yield) by traditional organic synthesis methods. β–galactosidase from *Escherichia coli* was chosen as a model enzyme for studying the kinetics of enyme–catalyzed carbohydrate synthesis.

The β–galactosidase–catalyzed hydrolyses of lactose and *o*–nitrophenyl–β–D–galactopyranoside (ONPG) were performed in the presence and absence of added glucose. In the absence of added glucose, the catalytic rate constant (k_{cat}) for lactose hydrolysis was estimated to be 75.0 s^{-1} (based on glucose production). The k_{cat} for ONPG hydrolysis (based on *o*–nitrophenol (ONP) production) was determined to be 1115 ± 6 s^{-1}.

The addition of glucose to lactose and ONPG hydrolyses caused a decrease in the apparent k_{cat}. The observed decrease was greater than could be accounted for by competitive inhibition (by glucose) alone. The additional decrease in k_{cat}^{app} was attributed to an increase in the steady–state concentration of the enzyme intermediate complex involving glucose, thereby decreasing the steady–state concentration of free enzyme available for substrate hydrolysis. Apparent inhibition constants (K_i^{app}) were estimated to be 6.6 mM for ONPG hydrolysis and 2.4 mM for lactose hydrolysis. The difference in these two values reflects the different abilities of ONPG and lactose to compete with glucose for binding to free enzyme.

The effect of added glucose on the β–galactosidase–catalyzed production of allolactose was also studied. A maximum allolactose yield of 53% (based on ONPG) was achieved. An apparent k_{cat} of 352 s^{-1} was the maximum rate that could be obtained for allolactose synthesis. A comparison of the rates of galactose and allolactose production to the apparent rate of ONPG consumption indicated that both galactose and allolactose were involved in side reactions forming trisaccharides.

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NOMENCLATURE

k_a	first–order rate constant (s^{-1})
k_{-a}	second–order rate constant ($s^{-1}\,mM^{-1}$)
k_{cat}	overall rate constant (s^{-1})
$k_{cat,i}$	overall rate constant in the presence of inhibitor (s^{-1})
$k_{cat,o}$	overall rate constant in the absence of inhibitor (s^{-1})
k_{cat}^{allo}	overall rate constant for the production of allolactose (s^{-1})
k_{cat}^{app}	overall apparent rate constant (s^{-1})
k_{cat}^{corr}	overall rate constant corrected for inhibition due to glucose (s^{-1})
k_{cat}^{gal}	overall rate constant for galactose production (s^{-1})
k_{cat}^{onp}	overall rate constant for ONP production (s^{-1})
k_{cat}^{rel}	relative overall rate constant (ratio of the overall rate constant in the absence of glucose to the apparent overall rate constant in the presence of glucose) (dimensionless)
k_{pred}^{rel}	k_{cat}^{rel} predicted from a fit to Equation (4.3.4) (dimensionless)
K_i	competitive inhibiton constant for glucose (mM)
K_i^{app}	apparent inhibition constant for glucose (mM)
K_i''	apparent binding constant for glucose to the enzyme–galactose complex (mM)
K_m	Michaelis constant (mM)
$K_{m,i}$	Michaelis constant in the presence of inhibitor (mM)
$K_{m,o}$	Michaelis constant in the absence of inhibitor (mM)
K_m^{app}	apparent Michaelis constant (mM)
K_s	binding constant for substrate to free enzyme (mM)
k_2	first–order rate constant (s^{-1})
k_3'	pseudo–first–order rate constant (s^{-1})
k_4	first–order rate constant (s^{-1})

ABBREVIATIONS

allo	allolactose
cer	ceramide
frc	fructose
fuc	fucose
gal	galactose
galNAc	N–acetylgalactosamine
gal–x	a β–galactoside
glc	glucose
glcNAc	N–acetylglucosamine
lac	lactose
ONP	*o*–nitrophenol
ONPG	*o*–nitrophenyl–β–D–galactopyranoside
neuAc	neuraminic (sialic) acid
PNPG	*p*–nitrophenyl–β–D–galactopyranoside
ser	serine
thr	threonine
tre	trehalose

Chapter 1: INTRODUCTION

Glycoconjugates are biological molecules composed of proteins or lipids containing oligosaccharides (chains of 3 or more monosaccharide units). Glycoconjugates play a number of biological roles, acting as modulators of cell growth, cell surface receptors for chemical signals from other cells, targets for invasion by viruses and bacteria, and as cell surface markers distinguishing different types of cells from one another. The carbohydrate portion of a glycoconjugate is often the major factor determining biological activity.

Recognition of the biological importance of complex carbohydrate structures has led to great interest in exploiting their biological activities for use in medical diagnosis and treatment. For example, cancer cells have specific alterations in some cell surface glycoconjugates. These changes in the cell surface distinguish cancerous cells from healthy cells and provide a potential means for tumor detection and location while the tumor is still only millimeters in diameter. Cancer cell surface markers may also provide a means for targeting drugs directly to tumors, and a basis for developing vaccines which boost the body's natural immunity against cancer.

Research into the medical applications of glycoconjugates will require at least gram and possibly kilogram quantities of appropriate carbohydrate structures. Methods of organic chemical synthesis are available, but in order to obtain the required specific linkages between monosaccharide units complicated reaction schemes involving many steps for protecting and deprotecting reactive hydroxyl groups are necessary. Consequently, these synthetic techniques are cumbersome, expensive, time–consuming and may give low yields in some cases.

Enzymatic synthesis of carbohydrates is an adjunct to chemical synthesis. Enzymatic reactions have the advantages of occurring under mild conditions and being easy to perform, often involving very few steps. Also, enzymes catalyze regio–specific and regio–selective reactions without requiring the protection of the hydroxyl groups. Although glycosyltransferases (the natural biosynthetic enzymes for oligosaccharides) are expensive and difficult to obtain, glycosidases (which normally hydrolyze oligosaccharides) can be used to perform the same types of reactions and are generally widely and cheaply available. Glycosidases have the

further advantage of using inexpensive monosaccharides as substrates, as opposed to the expensive activated sugar substrates required by glycosyltransferases.

Many researchers are now studying the enzymatic synthesis of disaccharides and oligosaccharides. Most of these studies have focused on whether or not a particular product was produced, or on the characterization of products produced in a given situation. Few studies have been directed toward the collection and interpretation of kinetic data. In this project, the hydrolytic enzyme β–galactosidase (E.C. 3.2.1.23) from *Escherichia coli* was used as a model system for studying the kinetics of disaccharide synthesis. β–galactosidase also has the ability to transfer a galactose moiety from a donor compound to an acceptor compound (a process called transglycosylation). If the acceptor is a monosaccharide, a disaccharide is formed. Glucose was used as an acceptor compound with two different donors in separate trials. The modelling of the reaction kinetics for each donor compound will be the focus of this thesis.

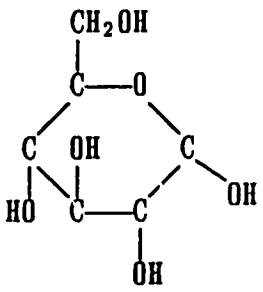
Chapter 2: LITERATURE SURVEY

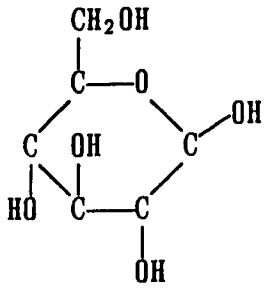
An extensive amount of literature concerning the enzymatic synthesis of carbohydrates has been published, therefore only a representative fraction of the available literature will be presented focusing on research most relevant to the model system selected for this study. This survey will begin with a brief review of carbohydrate structures (Section 2.1). A discussion of the role of carbohydrates as tumor antigens (an antigen is any molecule that causes an immune response) will follow (Section 2.2). The problems inherent in the chemical synthesis of carbohydrates will be mentioned (Section 2.3), followed by a discussion of the available methods for the enzymatic synthesis of carbohydrates (Sections 2.4, 2.4.1, & 2.4.2). Then the model system for this study will be introduced (Section 2.4.3), and the two types of reactions occurring in the model system will be discussed (Sections 2.5 & 2.6). Finally, the kinetics of the model system will be reviewed (Section 2.7).

2.1 General Background on Carbohydrates

A carbohydrate which cannot be hydrolyzed into simpler, smaller carbohydrates is called a monosaccharide. Some monosaccharides form ring structures in solution, as shown in Figure 1. The –OH group bound to carbon 1 (the anomeric carbon) in Figure 1 may be in either the axial (α) or equatorial (β) position. These two forms (anomers) exist in equilibrium with each other in solution. If the monosaccharide ring is six–membered, the sugar is known as a pyranose.

Monosaccharides can be joined together to form di–, oligo–, or polysaccharides. Disaccharides contain two monosaccharide units, oligosaccharides contain three to ten monosaccharide units, and polysaccharides contain more than ten monosaccharide units. If the hydroxyl group of the anomeric carbon is involved in the linkage, the bond is now fixed in either the α or the β position. The linkage is designated by stating the position of the bond and the numbers of the carbon atoms involved. For example, Figure 2 depicts the disaccharide β–lactose, which is 4–O–(β–D–galactopyranosyl)–β–D–glucopyranose (galactose $\beta(1\rightarrow4)$ linked to glucose, abbreviated as βGal(1→4)glc).





α-D-glucopyranose *β*-D-glucopyranose

Figure 1. *The anomers of glucose.*
(Hydrogens on ring carbons omitted for clarity)

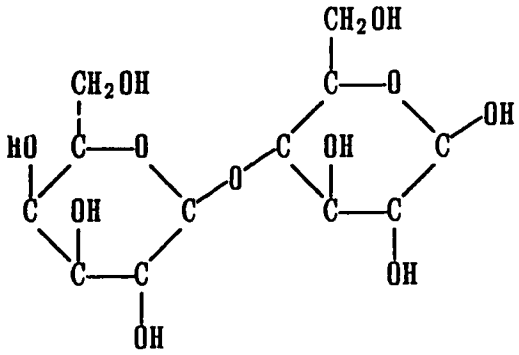


Figure 2. *β–lactose*
(Hydrogens on ring carbons omitted for clarity)

The hydroxyl group on the anomeric carbon of the glucose moiety is still free to mutarotate (change) between the α and β positions, thus giving rise to α– and β–lactose. Joining the same two monosaccharides at a different ring position results in a different disaccharide. A representation of galactose $\beta(1\rightarrow6)$ linked to glucose (βGal(1→6)glc) is shown in Figure 3. This disaccharide is known as allolactose.

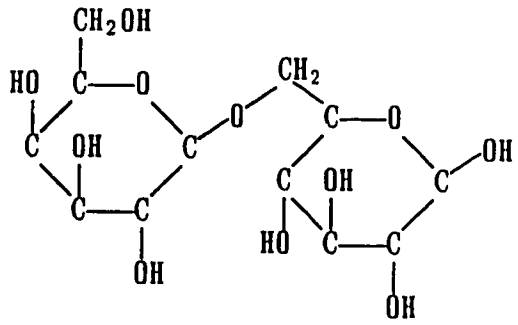


Figure 3. *β–allolactose*
(Hydrogens on ring carbons omitted for clarity)

Many monosaccharides can be joined together to form higher saccharides (oligo– and polysaccharides). Because of the many ways in which monosaccharides can be linked together, oligo– and polysaccharides have very complex tertiary structures. For example, joining of the 1–carbon of galactose to glucose can potentially form ten different disaccharides.

2.2 Tumor Antigens

The diagnosis, prognosis, and treatment of cancer all depend on the ability to distinguish between normal cells and cancer cells. Currently, the diagnosis of cancer hinges on the recognition of abnormal morphological features associated with the disease.

Research in the early 1900's indicated the possibility that animals could be immunized against cancer (Gold & Goldenberg, 1980). Inoculation of a transplantable cancer into a host conferred immunity against successive reinoculation of the cancer. The recognition of cancer cells by the immune system implied the existence of a specific biochemical difference between cancer cells and normal cells, and the development of a vaccine seemed promising. Subsequent experiments, however, produced contradictory results, causing interest in this line of research to wane.

Advances in transplantation immunity later revealed that the cause of the erratic results obtained in the early 1900's was genetic variation between both the transplanted tissue and the hosts. Experiments similiar to those done in the early 1900's were performed in the late 1950's, producing consistent results demonstrating that animals could be immunized against cancer. Further work has led to the discovery of many putative tumor antigens (see Table 1).

These tumor antigens are glycoconjugates, and very often the carbohydrate portion of the molecule contains the most important antigenic determinants. Exploitation of these carbohydrate antigens may lead to new methods for cancer diagnosis, prognosis, and therapy.

The TF and Tn antigens (see Figure 4), for example, are expressed on the surface of human breast, lung, and pancreatic adenocarcinomas. The serum levels of these antigens has been shown to provide a sensitive assay for early detection of tumors, and the ratio of TF to Tn is correlated with tumor behavior and aggressiveness (Springer, 1984). Also, radiolabelled monoclonal antibodies against TF have been produced and have been used to locate tumors by a technique known as radioimaging (Longenecker *et al.*, 1988). Monoclonal antibodies against tumor antigens could also be used (in theory) to target anti–cancer drugs specifically to tumors. In addition, researchers have demonstrated that the TF and Tn antigens stimulate effector T cells, which are known to play a vital role in anti–tumor immunity.

Promising applications such as these have generated intense interest in producing large quantities of carbohydrate tumor antigens.

Table 1. Representative Tumor Antigens

Reference	*Antigen*	*Associated cancer*
Yogeeswaran (1980)	βGal(1→4)Glc-Cer	Human epidermal carcinoma
	βGalNAc(1→3)-αGal(1→3)-βGlc(1→4)-Cer	Rat lymphosarcoma
Feizi (1985)	βGal(1→4)-βGlcNAc(1→6)Gal/GalNAc	Human gastric adenocarcinoma
	βGal(1→4)GlcNAc αFuc↑(1→3)	Human colorectal cancer
	αGal(1→3)-βGal(1→4)GlcNAc αFuc↑(1→2)	Pancreatic cancer
	αNeuAc(2→8)-αNeuAc(2→3)-βGal(1→4)Glc-Cer	Human melanoma
	αGal(1→4)-βGal(1→4)Glc-Cer	Burkitt's lymphoma
Springer (1984)	βGal(1→3)-αGalNAc-Ser/Thr (TF or T)	Human lung, pancreatic, & breast cancer
	αGalNAc-Ser/Thr (Tn)	Human lung, pancreatic, & breast cancer

Abbreviations: Gal = galactose, Glc = glucose, Cer = ceramide, GalNAc = N-acetylgalactosamine (2-deoxy-2-acetamido-galactose), GlcNAc = N-acetylglucosamine (2-deoxy-2-acetamido-glucose), Fuc = fucose, NeuAc = neuraminic acid (sialic acid), Ser = serine, Thr = threonine.

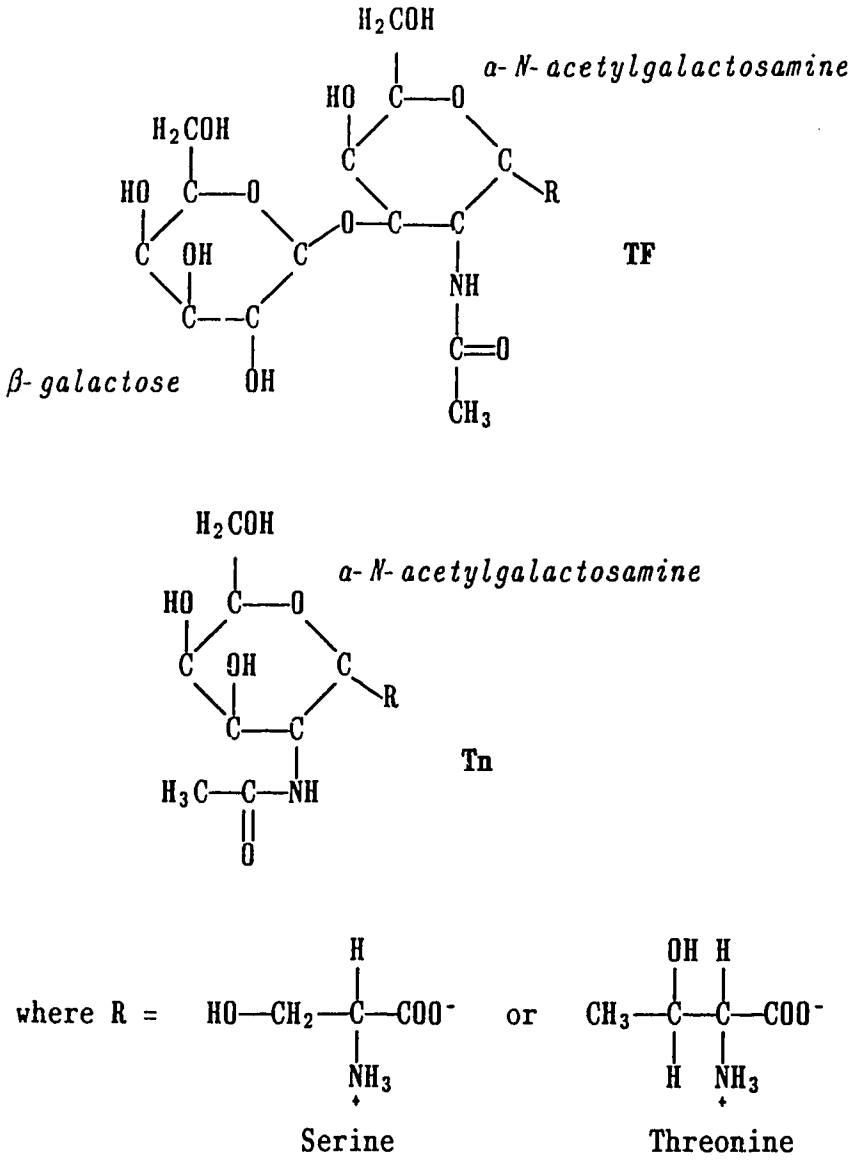


Figure 4. *The structures of the* TF (βGal(1→3)–αGalNAc–Ser/Thr) *and* Tn (αGalNAc–Ser/Thr) *antigens.*
(Hydrogens on ring carbons omitted for clarity)

2.3 Chemical Synthesis of Carbohydrates

Methods for the chemical synthesis of di– and oligosaccharides are well developed (*e.g.* Lemieux, 1978; Paulsen, 1984; Stinson, 1989; Kunz *et al.*, 1990; Kameyama *et al.*, 1991; & Defaye *et al.*, 1989). These methods, however, have several disadvantages. Sugars have several hydroxyl groups of similar reactivity, so chemical reaction schemes must consist of many blocking and unblocking steps. Total yields are often low, making large–scale synthesis impractical. Also, these reactions require organic solvents and complex reactants.

2.4 Enzymatic Synthesis of Carbohydrates

Enzymatic synthesis offers a convenient alternative to chemical synthesis. Enzymatic reactions have several advantages. The reactions take place under mild conditions (often at room temperature and pressure, and at neutral pH). Enzymes catalyze selective reactions without requiring the protection of reactive hydroxyl groups, which also means fewer reaction steps are involved. Enzymes can be immobilized to allow reuse, and many enzymes use simple, inexpensive mono– and disaccharides as substrates.

Two classes of enzymes have been used for the synthesis of carbohydrates: glycosyltransferases and glycosidases.

2.4.1 Glycosyltransferases

Glycosyltransferases, which are the natural biosynthetic enzymes that produce the oligosaccharides present in glycoconjugates, have been used for carbohydrate synthesis (*e.g.* Jung *et al.*, 1989; Cheetam *et al.*, 1989; Soh *et al.*, 1989; Palcic *et al.*, 1989; & Nilsson, 1989). These enzymes catalyze the transfer of a monosaccharide from a donor molecule to an acceptor molecule (see Figure 5).

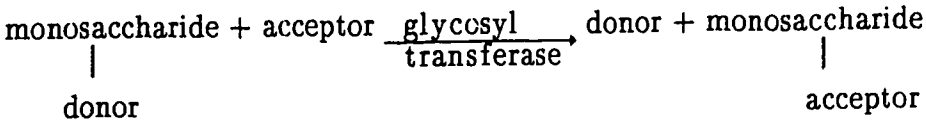


Figure 5. *A Glycosyltransferase–catalyzed Reaction.*

The reaction is very specific and has a high yield. The disadvantage is that the donor must be a complex, expensive glycosyl nucleotide (see Figure 6). Also, glycosyltransferases in animal cells are present in low concentrations and are bound to intracellular membranes, thus requiring special purification techniques (Nilsson, 1988). Only a few glycosyltransferases are available commercially.

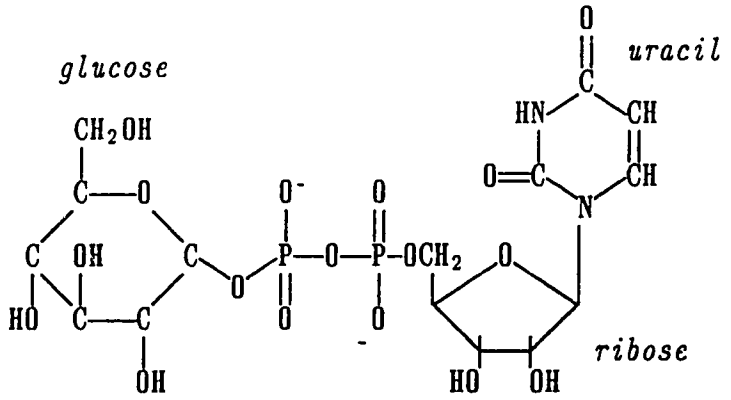


Figure 6. *Uridine Diphosphate Glucose* (UDPG), *a typical glycosyl nucleotide substrate for a glycosyltransferase.*

2.4.2 Glycosidases

Glycosidases are found in a wide range of organisms and are therefore more readily and cheaply available than glycosyltransferases. Glycosidases catalyze the reaction shown in Figure 7.

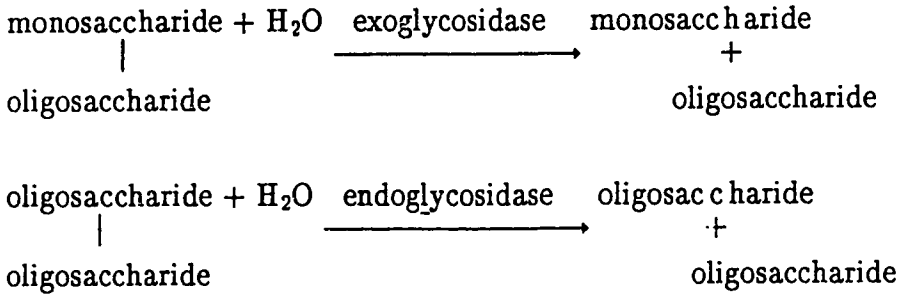


Figure 7. *Glycosidase–catalyzed Reactions.*

An exoglycosidase removes the terminal monosaccharide unit from a carbohydrate polymer, whereas an endoglycosidase cleaves a glycosidic bond within the polysaccharide.

The equilibrium lies strongly in favor of the forward hydrolysis reaction, but the reverse reaction ("reverse hydrolysis") does provide an avenue for the synthesis of carbohydrates. Alternatively, the hydrolysis reaction can be viewed as a transfer reaction in which water acts as the acceptor. If a mono– or disaccharide is used as an acceptor in place of water, a transfer reaction suitable for synthesizing carbohydrates results.

Glycosidases have the advantage of being able to use simple, cheap mono– and disaccharides as substrates. Also, glycosidases do not have the strict structural requirements for donor and acceptor stereochemical conformation characteristic of glycosyltransferases. Consequently, a single glycosidase can be used to catalyze several different synthetic reactions depending on the donors and acceptors provided. A disadvantage of this lack of substrate specificity is that a mixture of products with different linkages is often obtained.

2.4.3 β – Galactosidase

The glycosidase β–galactosidase (EC 3.2.1.23) from *Escherichia coli* was chosen as a model system for this study. β–galactosidase catalyzes the reaction shown in Figure 8, cleaving a β–linked galactose moiety from a donor substrate and transferring it to a hydroxyl group–containing acceptor (usually water, resulting in a hydrolysis reaction).

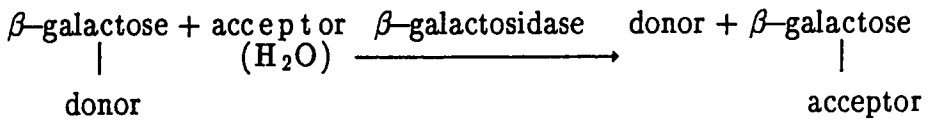


Figure 8. *The Reaction Catalyzed by β–Galactosidase.*

β–galactosidase has been the focus of many scientific investigations (see Table 2) largely because the genetic control system regulating its production was one of the first such systems to be extensively studied. β–galactosidase is also

Table 2. Representative Studies of β-Galactosidases

Reference	*Enzyme source*	*Type of study*
Sykes *et al.*, 1983	*Aspergillus niger*	physical characterization & substrate specificity
Li *et al.*, 1975	Jack bean	physical characterization & substrate specificity
Distler & Jourdian, 1973	Bovine testes	substrate specificity
Bahl & Agrawal, 1969	*Aspergillus niger*	substrate specificity
Lind *et al.*, 1989	*Thermoanaerobacter*	physical characterization & substrate specificity
Deshpande *et al.*, 1989	*Aureobasidium pullulans*	determination of optimum hydrolysis conditions
Simos *et al.*, 1989	Jack bean, barley, tobacco, spinach, corn, rye, & wheat germ	physical characterization & substrate specificity
Priyolkar *et al.*, 1989	*Corynebacterium murisepticum*	physical characterization & substrate specificity
Huber *et al.*, 1983, 1984	*Escherichia coli*	substrate specificity

known as lactase and is used in the dairy industry to break down lactose (galactose β 1→4 linked to glucose), the major sugar found in milk. Lactose is the second most abundantly occurring natural disaccharide (after sucrose), and consequently β–galactosidases are found in a wide variety of organisms.

Synthesis of di– and oligosaccharides using β–galactosidase can proceed via either reverse hydrolysis or transgalactosylation.

2.5 Reverse Hydrolysis

Goldberg and Tewari (1989) studied the equilibrium of lactose hydrolysis catalyzed by β–galactosidase. They reported a thermodynamic equilibrium constant of 34. The equilibrium constant, K, was given as

$$K = \{[glc]\cdot[gal]/(m^{o}\cdot[lac])\}\cdot\{\gamma(glc)\cdot\gamma(gal)/(a(H_2O)\cdot\gamma(lac))\}$$

where square brackets denote the molalities of the carbohydrates in solution, γ is the activity coefficient of the indicated solute, $a(H_2O)$ is the activity of the water, and m^{o} is equal to 1 mol/kg. The concentration of lactose at equilibrium was only 0.2% of the total carbohydrates in solution. Unfortunately, they did not report the source of their enzyme.

The equilibrium of the hydrolysis reaction lies far towards the monosaccharides. One strategy for shifting the equilibrium towards the disaccharides is the addition of high concentrations of monosaccharides. Johansson *et al.* (1986) synthesized mannose–mannose disaccharides by mixing jack bean α–mannosidase with a 85% w/w mannose solution. Not only were three different mannose–mannose disaccharides produced (the 1→6, 1→3, and 1→2 linkages), but 13 trisaccharides and 20 tetrasaccharides were also formed. In a later paper on the same reaction, Johansson *et al.* (1989) reported the yield of di– and oligosaccharides to be 70%. They also stated that product composition altered according to varying initial substrate concentration, temperature, and reaction time.

Huber and Hurlburt (1986) produced β–galactosyl–glucopyranoses and β–galactosyl–galactopyranoses by adding high concentrations of galactose and glucose to a solution containing β–galactosidase from *E. coli.* Although 10

β–galactosyl–glucopyranoses and 10 β–galactosyl–galactopyranoses were formed, allolactose (β–Gal(1→6)Glc) was by far the most prevalent product, representing 50% of the total disaccharides at equilibrium. Lactose (β–Gal(1→4)Glc) was the second most prevalent product. An equilibrium constant, defined as [total disaccharides]/[gal][glc], was reported as 9.5 mM^{-1}.

Another strategy for driving the equilibrium towards disaccharides is to continuously remove the product from the reaction mixture. Ajisaka *et al.* (1987) have used the apparatus shown schematically in Figure 9 to employ this strategy. The activated carbon column selectively adsorbed disaccharides and oligosaccharides in preference to monosaccharides (Abe *et al.*, 1983), shifting the equilibrium towards the products and allowing the monosaccharides to recirculate through the immobilized enzyme column. This method will henceforth be referred to as the "carbon adsorbent process". Using β–galactosidase from *E. coli* as the catalyst, and galactose and fructose as reactants, lactulose (β–Gal(1→4)Frc) and allolactulose (β–Gal(1→6)Frc) were produced. The yields were 3.3% and 8.0% respectively. When galactose and N–acetylglucosamine were employed as reactants, lactosamine (β–Gal(1→4)GlcNAc) and allolactosamine (β–Gal(1→6)GlcNAc) were produced. The yields were 0.9% and 9.1% respectively. By removing the activated carbon column from the system, eluting the disaccharides with an ethanol solution, and then placing the regenerated column back in the system, Ajisaka's group was able to increase the overall yields. After three column regenerations, the overall yields of lactosamine and allolactosamine were 3.2% and 31.6% respectively.

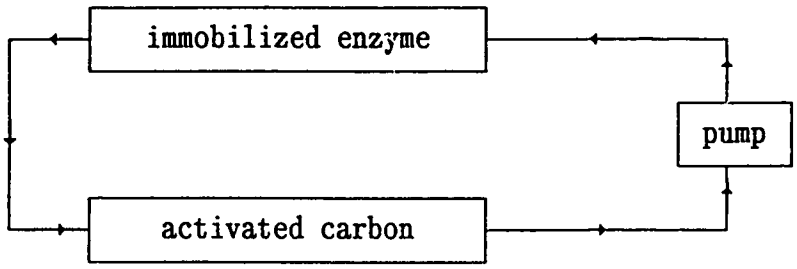


Figure 9. *Reactor System of Ajisaka* et al. (1987).
(*Carbon adsorbent process*)

Ajisaka *et al.* (1988) compared yields from their carbon adsorbent process (circulating monosaccharides through their reactor system, but without column regeneration) to yields obtained in batch reactions (reactions run in beakers using soluble enzyme with no activated carbon present). Using galactose and N–acetylglucosamine as reactants, the product proportions were 20% lactosamine and 80% allolactosamine. The yields were 7.6% for the batch reaction and 16.0% for the carbon adsorbent process reaction. Using galactose and glucose as reactants, they produced four galactopyranosyl–glucopyranoses. The product proportions and overall yield of disaccharides are given in Table 3. Galactose and fructose were also used as substrates, and the product proportions and overall yield of disaccharides are shown in Table 3.

Table 3. Product Compositions of Batch vs. Carbon Adsorbent Process Reactions (Ajisaka *et al.*, 1988)

Method	*Substrates*	*Product Composition (%) by Linkage*					*Yield*[1]
		(*1→1*)	(*1→2*)	(*1→4*)	(*1→5*)	(*1→6*)	(%)
Batch	gal + glc	0	13	6	27	54	2.9
C.A.P.[2]	gal + glc	0	9	9	28	54	6.0
Batch	gal + frc	61	0	19	12	8	55.0
C.A.P.	gal + frc	51	0	24	10	15	11.3

[1]*Yield of total disaccharides based on galactose.*

[2]*Carbon adsorbent process.*

Table 3 shows that the product distribution differed between the batch and carbon adsorbent process reactions. The authors theorized that the difference in

product compositions was the result of transgalactosylation reactions. Disaccharides can act as acceptors for the transfer of a galactose moiety, as shown schematically in Figure 10. If the step leading to the formation of trisaccharides is slow, then (in the carbon adsorbent process) the disaccharides may be adsorbed onto the activated carbon before they have the opportunity to participate in a transgalactosylation reaction.

$$E + gal \rightleftharpoons E \cdot gal + A \rightleftharpoons E \cdot gal \cdot A \rightleftharpoons E + gal\text{–}A$$

$$E \cdot gal + gal\text{–}A \rightleftharpoons E \cdot gal \cdot gal\text{–}A \rightleftharpoons E + gal\text{–}gal\text{–}A$$

Figure 10. *A transgalatosylation reaction depicting the transfer of galactose* (gal) *to either a monosaccharide acceptor* A (*e.g. glucose or fructose*), *and subsequent transfer of another galactose to the newly formed disaccharide* gal–A, *resulting in the formation of the trisaccharide* gal–gal–A. (E stands for enzyme; E·gal, E·gal·A, & E·gal·gal–A represent enzyme–substrate complexes).

This contention is supported by the fact that fructose is a poorer acceptor than glucose by an order of magnitude (Huber *et al.*, 1984), and the disparity in product compositions for the galactose + fructose case is greater than that for the galactose + glucose case. Unfortunately, the comparison of product compositions for the batch versus carbon adsorbent process runs for the galactose + fructose case is not completely justified because 100% (w/v) fructose was used in the batch run, whereas only 50% (w/v) fructose was used in the carbon adsorbent process run. The difference in overall yields for this case may also be attributable to the difference in initial substrate concentrations.

Fujimoto *et al.* (1988) used the same reactor system to study the production of glucose–glucose disaccharides (glucobioses) using several different enzymes: an α–glucosidase, a glucoamylase, and β–glucosidases from two different sources. Again they found that the product compositions differed between the batch and carbon adsorbent process reactions, and proposed that transgalactosylation reactions (as previously described) were responsible for the different product distributions. A contributing factor to the disparity in product compositions was that the batch

reactions were run with a glucose concentration of 90%. This solution was too viscous to run in the carbon adsorbent process system, so a solution of 50% glucose was employed. The theory that transgalactosylation reactions are responsible for the difference in product compositions would be supported by either a demonstration that different product compositions can be obtained under identical reaction conditions, or by a comparison of the rate of galactose transfer to the appropriate disaccharides versus glucose and fructose. The authors also noted that the yields of total disaccharides for the batch reactions were approximately 30% compared to yields of approximately 5% for the carbon adsorbent process reactions. Again, this disparity may be at least partially accounted for by the difference in initial substrate concentrations.

The carbon adsorbent process reactor system has been used in further studies (Ajisaka & Fujimoto, 1989; Ajisaka & Fujimoto 1990) to produce trisaccharides using various substrates and enzymes (see Table 4 A & B). In all cases, the product compositions of the batch and carbon adsorbent process runs differed, and total product yields were higher for batch reactions than carbon adsorbent process reactions. The initial substrate concentrations were always higher for the batch run than the carbon adsorbent process runs. Often, however, unique products, which could not be produced by batch reaction methods, were produced in the carbon adsorbent process system.

The repeated attainment of higher yields for the batch versus carbon adsorbent process reactions suggests that increasing substrate concentration is more effective than removing product (by adsorption) in shifting the equilibrium.

Table 4 A. Trisaccharides Produced by Ajisaka and Fujimoto (1990)

Enzyme	*Source*	*Substrates*	*Method: Oligosaccharides formed*	*Yield* [1]
β-Galactosidase	*Aspergillus oryzae*	galactose trehalose	C.A.P.[2]: βGal(1→3)Tre, βGal(1→4)Tre, & βGal(1→6)Tre	7 %
			Batch: βGal(1→3)Tre, βGal(1→4)Tre, βGal(1→6)Tre, βGal(1→2)Tre	18 %
β-Galactosidase	*Escherichia coli*	galactose trehalose	C.A.P.: βGal(1→4)Tre, βGal(1→6)Tre	4.5 %
			Batch: βGal(1→4)Tre, βGal(1→6)Tre, βGal(1→2)Tre	14 %
α-Glucosidase	*Saccharomyces*	glucose trehalose	C.A.P.: αGlc(1→4)Tre, αGlc(1→6)Tre	2, 7 %
β-Glucosidase	Almond		C.A.P.: βGlc(1→4)Tre, βGlc(1→6)Tre	1, 5 %
Glucoamylase	*Rhizopus niveus*		C.A.P.: αGlc(1→4)Tre, αGlc(1→6)Tre	2, 13%

Abbreviations: Gal = galactose, Glc = glucose, Tre = trehalose.
Trehalose is α-glucopyranosyl(1→1)-α-glucopyranose.
[1] Total yield of oligosaccharides or, when two numbers are given, the respective yields of each oligosaccharide.
[2] Carbon Adsorbent Process

Table 4 B. Trisaccharides Produced by Ajisaka and Fujimoto (1989)

Enzyme	*Source*	*Substrates*	*Method: Oligosaccharides formed*	*Yield*
α-Galactosidase	*M. vinacea*	galactose sucrose	C.A.P.: αGal(1→6)-αGlc(1→2)-βFrc	17.6 %
			Batch: αGlc(1→2)-βGal(1→6)-βFrc αGal(1→6)-αGlc(1→2)-βFrc	N.R.
β-Galactosidase	*E. coli*		C.A.P.: βGal(1→6)-αGlc(1→2)-βFrc	10.6 %
			Batch: βGal(1→6)-αGlc(1→2)-βFrc αGlc(1→2)-βGal(1→6)-βFrc	N.R.

Abbreviations: Gal = galactose, Glc = glucose, Frc = fructose, N.R. = not reported.
Sucrose is α-glucopyranosyl(1→2)-β-fructofuranoside

2.6 Transglycosylation

Carbohydrate synthesis via the transglycosylation activity of glycosidases offers an alternative method to synthesis by reverse hydrolysis. Researchers studying glycosidase–catalyzed hydrolysis of high concentrations of disaccharides have found transient production of oligosaccharides. At high disaccharide concentrations, the disaccharide will act as both the donor and acceptor in a transfer reaction, resulting in the production of trisaccharides (see Figure 11).

$$E + lac \rightleftharpoons E \cdot lac \rightleftharpoons E \cdot gal \cdot glc \rightleftharpoons E \cdot gal + glc$$

$$E \cdot gal + lac \rightleftharpoons E \cdot gal \cdot lac \rightleftharpoons E + gal\text{–}lac$$

Figure 11. *A transglycosylation reaction showing lactose* (lac) *as both the donor and acceptor of a galactose moiety. A glucose molecule* (glc) *is released during the reaction.*

The trisaccharides may then also be used as acceptors by the enzyme, producing higher oligosaccharides. Many researchers have studied the production of oligosaccharides via glycosidase–catalyzed transglycosylation, and a representative sampling of their reports is given in Table 5.

Huber *et al.* (1976) studied the transglycosylation activity of β–galactosidase from *E. coli.* They found that β–galactosidase produces allolactose (βGal(1→6)glc), the product of a transfer reaction, at all initial lactose concentrations studied. This reaction has great physiological significance for *E. coli* because allolactose is a regulatory molecule responsible for controlling the expression of several *E. coli* genes. The ratio of the rate of allolactose production to the rate of glucose (a hydrolysis product) production was constant at 0.88 for all lactose concentrations.. The ratio of transfer to hydrolysis declined below pH 6.0, was constant between pH 6 to pH 7.8, and increased dramatically at pH's greater than 7.8. Galactose–glucose disaccharides with 1→3, 1→2, and 1→1 linkages were also formed, but at only 3% of the amount of allolactose produced. At lactose concentrations over 0.05 M, oligosaccharides were produced via a reaction like the one shown in Figure 11 (*i.e.*

Table 5. Representative Studies of Transglycosylation by β-Galactosidases

Reference	*Enzyme source*	[Lac]i*	*Oligosaccharides formed*
Toba, *et al.*, 1985	*Aspergillus oryzae*	30 %	βGal-(1→6)-Glc (allolactose) βGal-(1→3)-βGal-(1→4)-Glc βGal-(1→6)-βGal-(1→4)-Glc βGal-(1→4)-βGal-(1→6)-Glc + two tetrasaccharides + one pentasaccharide
Greenberg & Mahoney, 1983	*Streptococcus thermophilus*	5 %	βGal-(1→6)-Glc (allolactose) βGal-(1→6)-βGal
Toba, *et al.*, 1978	*Saccharomyces fragilis* *Aspergillus niger*	30 %	βGal-(1→2)-Glc βGal-(1→3)-Glc βGal-(1→6)-Glc βGal-(1→3)-Gal βGal-(1→6)-Gal + seven trisaccharides
Pazur, *et al.*, 1958	*Saccharomyces fragilis*	20 %	βGal-(1→6)-Gal βGal-(1→3)-Glc βGal-(1→6)-Glc + two trisaccharides
Suyama, *et al.*, 1986	*Escherichia coli*	25 % + 25 % sucrose	βGal-(1→6)-Glc (allolactose) βGal-(1→6)-βGlc-(1→2)-Frc + trace trisaccharides & tetrasaccharides

** initial lactose concentration*

lactose and allolactose acted as both the donors and acceptors of galactose moieties).

To study transgalactosylase activity, Huber *et al.* (1976) added 0.5 M glucose to several lactose concentrations (exact concentrations not reported) in the presence of β–galactosidase. At high lactose concentrations (>0.1 M), no increase in allolactose production was observed, but at lower concentrations of lactose, allolactose production increased as much as 100%. At high concentrations of lactose, free glucose rapidly accumulates from the hydrolysis of lactose (see Figure 11), therefore adding a high initial concentration of glucose to the reactions produces little or no effect.

Huber *et al.* (1981) studied the effect of anomeric configuration on transgalactosylation activity, and found that the rate of allolactose production increased as the percentage of α–lactose in the substrate solution was increased. They also found that β–galactosidase retained the anomeric configuration of the reactants: α–glucose and α–allolactose were produced from α–lactose, and β–glucose and β–allolactose were produced from β–lactose. Nilsson (1987) also studied the effect of anomeric configuration on transglycosylation. In experiments with β–galactosidase from *E. coli* and ONPG (o–nitrophenyl–β–D–galactopyranoside) as donor, he found that using methyl α–D–galactopyranoside (αGal–O–Me) as acceptor produced mainly βGal(1→6)–αGal–O–Me, whereas using methyl β–D–galactopyranoside as acceptor produced mainly βGal(1→3)–βGal–O–Me. These reactions were not optimized, but yields were often greater than 20%.

Hedbys *et al.* (1984) produced βGal(1→6)GalNAc from lactose and N–acetylgalactosamine using immobilized β–galactosidase from E. coli. A 20% yield (based on GalNAc added) was obtained. The product mixture for this transfer reaction also contained βGal(1→6)Gal and allolactose (βGal(1→6)Glc). βGal(1→6)GalNAc was also formed in a reverse hydrolysis reaction using galactose and N–acetylgalactosamine as substrates, but the yield was only 2–3%. The low yield for the reverse hydrolysis results from the use of equimolar initial concentrations (0.31 M) of galactose and N–acetylgalactosamine. A simplified sequence of events for both the transfer and reverse hydrolysis reactions is shown in Figures 12 & 13.

$$E + lac \rightleftharpoons E \cdot lac \rightleftharpoons E \cdot gal \cdot glc \rightleftharpoons E \cdot gal + glc \rightleftharpoons E + gal$$

$$E \cdot gal + galNAc \rightleftharpoons E \cdot gal \cdot galNAc \rightleftharpoons E + gal\text{–}galNAc$$

$$E \cdot gal + glc \rightleftharpoons E \cdot gal \cdot glc \rightleftharpoons E + gal\text{–}glc$$

$$E \cdot gal + gal \rightleftharpoons E \cdot gal \cdot gal \rightleftharpoons E + gal\text{–}gal$$

Figure 12. *The simplified sequence of events occurring in a tranfer reaction that produces βGal(1→6)GalNAc.*

$$E + gal + galNAc \rightleftharpoons E \cdot gal + galNAc$$

$$E \cdot gal + galNAc \rightleftharpoons E \cdot gal \cdot galNAc \rightleftharpoons E + gal\text{–}galNAc$$

$$E \cdot gal + gal \rightleftharpoons E \cdot gal \cdot gal \rightleftharpoons E + gal\text{–}gal$$

Figure 13. *The simplified sequence of events occurring in a reverse hydrolysis reaction that produces βGal(1→6)GalNAc.*

In both cases, the monosaccharides competed with each other for binding to the enzyme–galactose complex. In the case of the transfer reaction, the initial concentrations of galactose and glucose were zero, and the initial concentration of N–acetylgalactosamine was 0.22 M. In the case of the reverse hydrolysis, however, the concentrations of galactose and N–acetylgalactosamine were initially equal, resulting in a low yield of βGal(1→6)GalNAc due to the formation of βGal(1→6)Gal. A high ratio of GalNAc/Gal should have been used to increase the yield of βGal(1→6)GalNAc.

Hedbys *et al.* (1989) synthesized the TF antigen (βGal(1→3)GalNAc) by an ingenious method employing the sequential use of β–galactosidases from bovine testes and *E. coli.* Using lactose as donor and N–acetylgalactosamine as acceptor, a transgalactosylation reaction catalyzed by bovine testes β–galactosidase produced a mixture of βGal(1→3)GalNAc (yield 37%), βGal(1→6)GalNAc, and small amounts

of other disaccharides and trisaccharides. This product mixture was then subjected to hydrolysis by *E. coli* β–galactosidase, which has a very low specificity for 1→3 linkages, until all contaminating di– and trisaccharides had been hydrolyzed to monosaccharides. The yield of TF antigen was 27% at this stage. The βGal(1→3)GalNAc was then separated from the monosaccharides by column chromatography, resulting in a final yield of 21%. This study highlights the enormous potential for the use of enzymes in the synthesis of biologically important carbohydrates.

2.7 Kinetics of β–Galactosidase from *Escherichia coli*

Escherichia coli has been the most extensively studied bacterium to date. The genetic system regulating the metabolism of lactose (and therefore the production of the enzyme β–galactosidase) in *E. coli* was the first such system to be widely studied by molecular geneticists. Consequently, β–galactosidase has become one of the most intensively studied enzymes. Many studies have concentrated on the hydrolysis of lactose, ignoring the transglycosylation reactions catalyzed by β–galactosidase. The following sections will discuss kinetic studies relevant to the transglycosylation reactions catalyzed by the β–galactosidase from *E. coli*. The focus of the discussion will be on the determination of the kinetic parameters pertinent to this project.

2.7.1 Overall Kinetics

A common approach used in the study of enzyme kinetics is the initial rate method. By considering only early times in the reaction, the concentrations of products may be assumed to be zero (*i.e.* reverse reactions may be ignored), and thus the kinetic equations describing the reaction are greatly simplified. These equations are often of the form shown in Equation (2.7.1)

$$-\frac{dS}{dt} = \frac{k_{cat} E_0 S}{K_m + S} \qquad (2.7.1)$$

where E_0 is the total enzyme concentration, t is time, S is the substrate concentration, and k_{cat} and K_m are kinetic constants. This equation is known by enzyme kineticists as the Michaelis–Menten equation.

a complete discussion of the derivation of the Michaelis–Menten equation.

The Michaelis–Menten parameters (k_{cat} & K_m) for the β–galactosidase–catalyzed hydrolysis of many substrates have been determined, and the results of some of these investigations are presented in Table 6, which gives overall kinetic parameters for the disappearance of substrate.

Table 6. Michaelis–Menten Parameters for the β–Galactosidase–catalyzed Hydrolysis of Various Substrates.

Substrate	k_{cat} (s^{-1})	K_m (mM)
ONPG[1]	750	0.11
ONPG[2]	753	
PNPG[1]	90	0.03
PNPG[2]	76	
PNPG[3]	163.4 ± 4.7	0.048 ± 0.004
Lactose[3]	66.4 ± 2.3	1.35 ± 0.12
Allolactose[3]	102.0 ± 1.02	0.94 ± 0.22

[1]Viratelle & Yon (1973); [2]Huber *et al.* (1983); [3]Huber & Gaunt (1983).
ONPG & PNPG are *o*– & *p*–nitrophenyl–β–D–galactopyranoside, respectively.

ONPG and PNPG are often used in studies of β–galactosidase because the release of the nitrophenyl group causes a chromogenic change that can be monitored spectrophotometrically.

Because the rate of allolactose hydrolysis is comparable to (or less than) the rates for substrate hydrolysis, the data shown in Table 6 suggest the the yield of allolactose by transgalactosylation would be low.

The following sections describe experiments used to calculate intrinsic kinetic parameters.

2.7.2 Nucleophilic Competition Studies

A key aspect of the transglycosylation reaction is the competition between the desired acceptor compound and water.

Viratelle and Yon (1973) studied the kinetics of some β–galactosidase–catalyzed reactions in the presence of nucleophiles added to compete with water. Three nitrophenyl β–D–galactosides, two aminophenyl β–D–galactosides, phenyl β–D–galactoside, and *o*–nitrophenyl α–L–arabinoside were employed as substrates. The authors proposed the scheme shown in Figure 14 for analyzing their data:

$$E + S \overset{K_S}{\rightleftharpoons} E\cdot S \xrightarrow{k_2} E\cdot gal + P_1$$

$$E\cdot gal \xrightarrow{k_3'} E + gal$$

$$E\cdot gal \xrightarrow[+\ N]{k_4} E + gal\text{–}N$$

Figure 14. *Reaction of β–galactosidase in the presence of an added nucleophile.*

where E and S represent enzyme and substrate, E·S and E·gal are the enzyme–substrate complex and enzyme–galactose complex, P_1 is the phenyl–containing group of the substrate, gal is galactose, and gal–N is the transfer product obtained from the addition of the nucleophilic compound N to galactose. For the reaction of *o*–nitrophenyl β–D–galactoside (ONPG) with methanol as an added nucleophile, the reaction would be as shown in Figure 15.

The release of galactose from the enzyme–galactose complex requires the addition of a water molecule to the galactose, and therefore k_3' is a lumped parameter consisting of both the second–order rate constant for that step and the concentration of water (*i.e.* $k_3' = k_3[H_2O]$).

$$E + ONPG \overset{K_S}{\rightleftharpoons} E\cdot ONPG \xrightarrow{k_2} E\cdot gal + ONP$$

$$E\cdot gal \xrightarrow{k_3'} E + gal$$

$$E\cdot gal \xrightarrow[+\ MeOH]{k_4} E + \text{Methyl } \beta\text{–D–galactopyranoside}$$

Figure 15. *The reaction catalyzed by β–galactosidase with ONPG as substrate and methanol as an added nucleophile* (MeOH *represents methanol*).

Evolution of P_1 (ONP or PNP) was followed spectrophotometrically, and the kinetic parameters (k_{cat} and K_m) for the Michaelis–Menten equation (Equation 2.7.1) were determined.

For the scheme shown in Figure 7:

$$k_{cat} = \frac{k_2.(k_3' + k_4[N])}{k_2 + k_3' + k_4[N]} \qquad (2.7.2)$$

and

$$K_m = K_S\cdot\frac{k_3' + k_4[N]}{k_2 + k_3' + k_4[N]} \qquad (2.7.3)$$

From the change in k_{cat} as a function of the initial concentration of N, k_2 and k_3' were determined. The results for ONPG and PNPG are shown in Table 7.

For PNPG, no change in k_{cat} as a function of nucleophile concentration was observed, indicating that k_2 is the rate constant for the rate–limiting step.

Table 7. Kinetic Parameters for the Hydrolysis of ONPG & PNPG. (Viratelle & Yon, 1973)

Substrate	*Parameter*	
	k_2 (s^{-1})	k_3' (s^{-1})
ONPG	2100	1200
PNPG	90	

The reaction of water with the enzyme–galactose complex (and therefore k_3') is common for all substrate that form galactose as a product, whereas k_2 is specific to a given substrate.

Huber *et al.* (1983) confirmed the values of 2100 and 1200 s^{-1} for k_2 and k_3' respectively using the nucleophilic competition method of Viratelle & Yon (1973). This method was employed to determine the values of k_2 and k_3' as a function of pH (see Table 8).

Table 8. Values of k_2 and k_3' for ONPG Hydrolysis as a Function of pH. (Huber *et al.*, 1983)

pH	k_2 (s^{-1})	k_3'(s^{-1})
7.0	2100	1200
8.6	1900	600
10.0	900	150

2.7.3 Kinetics of Inhibitors

Initial rate studies are designed to minimize the effects of products on reaction kinetics, but in following the time course of transgalactosylation, products accumulate. These compounds may competitively inhibit the reaction of the substrate, therefore the magnitude of inhibition is important.

Deschavanne *et al.* (1978) studied the β–galactosidase–catalyzed hydrolysis of ONPG and PNPG in the presence of various inhibitor compounds. They proposed the following model:

$$E + A \underset{}{\overset{K_i}{\rightleftharpoons}} E\cdot A$$

$$E + S \overset{K_s}{\rightleftharpoons} E\cdot S \xrightarrow{k_2} E\cdot gal + P_1$$

$$E\cdot gal \xrightarrow{k_3'} E + gal$$

$$E\cdot gal + A \overset{K_i''}{\rightleftharpoons} E\cdot gal\cdot A \xrightarrow{k_4} E + gal\text{–}A$$

Figure 16. *β–Galactosidase–catalyzed hydrolysis in the presence of added inhibitor* (A), (gal–A *represents the transfer product*).

The Michaelis–Menten parameters for this model are given by

$$k_{cat,i} = \frac{k_2\cdot\left[k_3' + k_4\cdot\dfrac{[A]}{K_i''}\right]}{k_2 + k_3' + (k_2 + k_4)\cdot\dfrac{[A]}{K_i''}} \qquad (2.7.4)$$

and

$$K_{m,i} = K_s\cdot\left[1 + \frac{[A]}{K_i}\right]\cdot\frac{k_3' + k_4\cdot\dfrac{[A]}{K_i''}}{k_2 + k_3' + (k_2 + k_4)\cdot\dfrac{[A]}{K_i''}} \qquad (2.7.5)$$

where $k_{cat,i}$ and $K_{m,i}$ are k_{cat} and K_m in the presence of inhibitor.

Linearization of Equation (2.7.4) gives

$$k_{cat,i} = \frac{(k_{cat,i} - k_{cat,o})}{[A]}\left[\frac{k_2 + k_3'}{k_2 + k_4}\right]K_i'' + \frac{k_2k_4}{k_2 + k_4} \quad (2.7.6)$$

where $k_{cat,o} = \dfrac{k_2k_3'}{k_2 + k_3'}$ (k_{cat} in the absence of inhibitor).

In the absence of transfer product formation (i.e. k_4 is negligible), Equation (2.7.4) simplifies to

$$k_{cat,i} = \frac{k_2k_3'}{k_2\left[1 + \dfrac{[A]}{K_i''}\right] + k_3'} \quad (2.7.7)$$

which can be rewritten as

$$\frac{1}{k_{cat,i}} = \frac{1}{k_{cat,o}} + \frac{[A]}{k_3'K_i''} \quad (2.7.8)$$

Also, the ratio of $K_{m,i}/k_{cat,i}$ gives the following relation:

$$\frac{K_{m,i}}{k_{cat,i}} = \left[\frac{K_s}{k_2}\right]\left[1 + \frac{[A]}{K_i}\right] \quad (2.7.9)$$

By measuring k_{cat} at various inhibitor concentrations and using Equations (2.7.6), (2.7.8), and (2.7.9), the authors estimated the kinetic parameters K_i, K_i'',

and k_4. The estimated parameters for lactose, galactose, and glucose are given in Table 9.

Table 9. Kinetic Parameters for the Inhibition of ONPG Hydrolysis. (Deschavanne *et al.*, 1978)

Inhibitor	*Parameter*		
	K_i (mM)	K_i'' (mM)	k_4 (s^{-1})
Lactose	1		
Galactose	40		
Glucose	630	34	330

Lactose and galactose will also act as acceptors to form transfer products, but only at sufficiently high concentrations. Deschavanne and co–workers used less than 3 mM lactose and did not report the concentrations of galactose used. The concentrations of glucose used were up to 750 mM.

Huber & Gaunt (1983) also studied the β–galactosidase–catalyzed hydrolysis of ONPG in the presence and absence of various inhibitors, using the same model and theoretical treatment (Equations 2.7.4 to 2.7.9) as Deschavanne *et al.* (1978). In addition, Huber & Gaunt used the inhibition of PNPG hydrolysis to estimate K_i for some inhibitors. Since k_2 is the rate–limiting step for PNPG hydrolysis, the expressions for the Michaelis–Menten parameters (Equations 2.7.4 & 2.7.5) simplify to

$$k_{cat,o} = k_2 \qquad (2.7.10)$$

and

$$K_{m,o} = K_s \qquad (2.7.11)$$

Substituting these two equations into Equation (2.7.9) gives

$$\frac{K_{m,i}}{k_{cat,i}} = \left[\frac{K_{m,o}}{k_{cat,o}}\right] \cdot \left[1 + \frac{[A]}{K_i}\right] \qquad (2.7.12)$$

from which K_i can be determined.

For galactose, Huber & Gaunt (1983) estimated K_i to be 20.1 mM based on inhibition of ONPG hydrolysis and 34.7 mM based on inhibition of PNPG hydrolysis. These values are in reasonable agreement with the value of 40 mM reported by Deschavanne *et al.* (1978).

2.7.4 Transgalactosylation Kinetics Using Glucose as an Acceptor

When glucose is used as the acceptor, β–galactosidase catalyzes the formation of the gal–glc disaccharide allolactose (Galβ(1→6)Glc, see Figure 3).

Huber *et al.* (1983) studied the hydrolytic and transgalactosylation reactions of β–galactosidase using glucose as an acceptor for the transfer reaction. Again, the model of Deschavanne *et al.* (1978) was employed with the substitution of individual rate constants for K_i'', as shown in Figure 17(a) & (b). The kinetic equations are therefore the same as Equations (2.7.4) to (2.7.6).

Huber *et al.* (1976) had previously shown that the rate of allolactose production is equal to the rate of galactose production, demonstrating that k_4 and k_{-a} are approximately equal. The definition of K_i'' is then $(k_4 + k_{-a})/k_a$ rather than k_{-a}/k_a.

Table 10 shows the effect of added glucose on the apparent k_{cat} for the hydrolysis of both ONPG and PNPG at various pH values.

From the data in Tables 8 & 10 and using Equation (2.7.6), the k_4 value for glucose was determined to be 444 ± 14 s^{-1} at pH 7.6 and 460 ± 24 s^{-1} at pH 10 (essentially unchanged over the pH range studied). The K_i'' value for glucose was

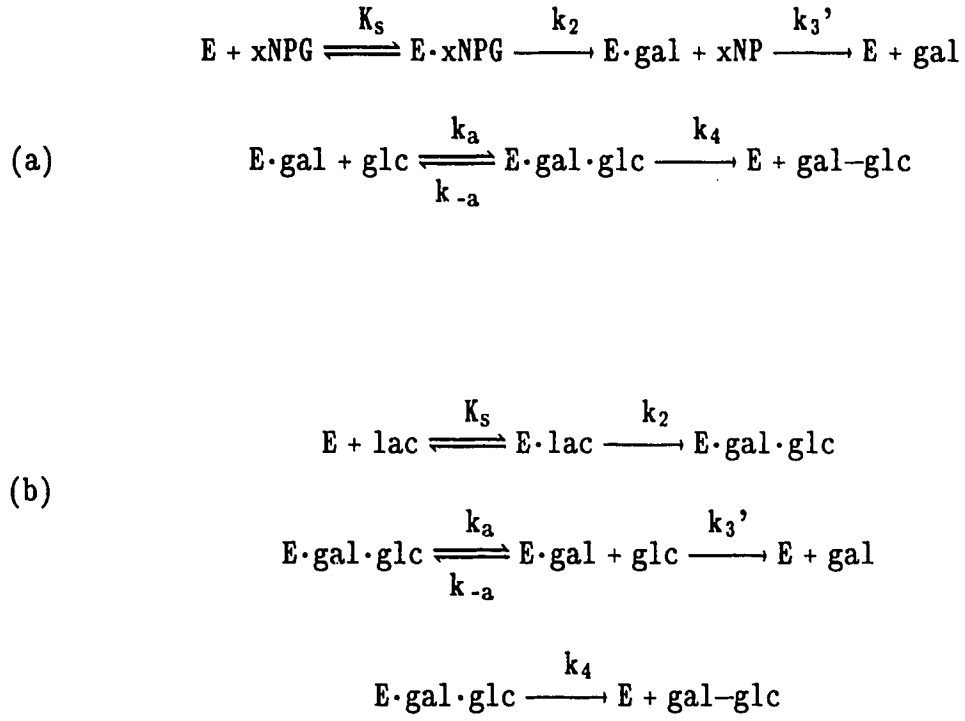


Figure 17. *The Reactions of β–Galactosidase with Glucose as Acceptor and* (a) *ONPG or PNPG* (*xNPG*) *and* (b) *lactose as substrate.*

Table 10. Effect of Glucose on the Apparent k_{cat} Values for ONPG and PNPG Hydrolysis (Huber *et al.*, 1983).

	k_{cat} (s^{-1})					
	pH 7.0		pH 8.9		pH 10.0	
[Glc] (mM)	PNPG	ONPG	PNPG	ONPG	PNPG	ONPG
0	76	753	ND	375	25	162
20	76	615	ND	381	24	210
60	72	506	ND	375	26	267
100	73	465	ND	371	27	284

also unchanged over the pH range studied, and was estimated to be equal (within experimental error) to the value of 34 mM previously reported by Deschavanne *et al.* (1978).

Huber *et al.* (1984) continued studying the transfer reaction of β–galactosidase using various sugars and sugar alcohols as acceptors. The model shown in Figure 11 and the theoretical treatment given by Equations (2.7.4) to (2.7.9) were employed to determine values for k_4 and K_i''. The values of these parameters for galactose and glucose are given in Table 11. ONPG was used as the donor for the transfer reaction.

The value of k_4 should depend only on the acceptor molecule, because the enzyme–galactose complex is independent of the substrate. The range of reported values for k_4, from 330 s^{-1} (Deschavanne *et al.*, 1978) to 440 s^{-1} (Huber *et al.*, 1976), illustrates the difficulty in obtaining reliable kinetic parameters for some key steps.

Table 11. K_i'' and k_4 Values for Galactose and Glucose. (Huber *et al.*, 1984)

Acceptor	K_i'' (mM)	k_4 (s^{-1})
Galactose	200	380
Glucose	34	380

By measuring products formed at very early times (as early as 200 ms), Huber *et al.* (1976) had shown that β–galactosidase can catalyze the direct conversion of lactose to allolactose without first releasing a glucose molecule. So by using lactose as the substrate with no acceptor present, the ratio of k_4 to k_{-a} can be determined by measuring the rate of glucose production relative to the rate of allolactose production. This ratio and the values of K_i'' and k_4 can then be used to calculate the values of k_{-a}, k_a, and K_{diss} ($= k_{-a}/k_a$). The ratio of k_4/k_{-a} was found to be 1.05. K_{diss} and k_{-a} for glucose were calculated to be 17 mM and 360 s^{-1}

respectively.

2.7.5 Literature Survey Summary

Many of the individual rate constants for β–galactosidase–catalyzed reactions have been estimated by initial rate studies. Initial rate methods use conditions designed to make the effects of reaction products negligible. The disaccharides produced by the transgalactosylase activity of β–galactosidase are not actually end–products, but transient intermediates which are eventually hydrolyzed to monosaccharides. The goal of this study was to maximize the yield of allolactose, therefore the emphasis was on the collection of time–course data at longer reaction times.

Chapter 3. MATERIALS & METHODS

3.1 Materials

β–Galactosidase (EC 3.2.1.23) from *Escherichia coli* (Grade X) was obtained from Sigma Chemical Company. Lactose, galactose, glucose, ONPG, PNPG, melibiose, and *myo*–inositol were also purchased from Sigma Chemical Company.

3.2 Experimental Methods

3.2.1 Enzyme Assays

The enzyme was assayed in 0.1 mM sodium phosphate buffer containing 1 mM $MgSO_4$, 145 mM NaCl, and 0.02% sodium azide at 23° C. The assay mixture (3 mL total volume) contained 2.0 mM ONPG and 6.9 x 10^{-3} mg/mL enzyme. The release of ONP was monitored at 410 nm with a Shimadzu UV–160 UV–visible recording spectrophotometer. One unit was defined as the amount of enzyme required to release 1 μmol of ONP per minute under the stated assay conditions.

3.2.2 Reactions with Lactose as Donor and Glucose as Acceptor

The effect of glucose on lactose hydrolysis was studied by adding various amounts of glucose to different initial concentrations of lactose. Reactions (5 mL total volume) were run in 0.1 mM sodium phosphate buffer containing 1 mM $MgSO_4$, 145 mM NaCl, and 0.02% sodium azide at 23° C with 3 x 10^{-4} mg ml^{-1} enzyme. 0.5 mL samples were taken at various time intervals and boiled for 5 minutes to destroy enzyme activity. Samples were then frozen for later analysis by high–performance liquid chromatography (HPLC) and gas chromatography (GC) (see Sections 3.3.1 & 3.3.2).

3.2.3 Reactions with ONPG as Donor & Glucose as Acceptor

The effect of glucose on ONPG hydrolysis was studied by adding various amounts of glucose to 7 mM ONPG. Reactions (100 mL total volume) were run in 0.1 mM sodium phosphate buffer containing 1 mM $MgSO_4$, 145 mM NaCl, and 0.02% sodium azide at 23° C with 1.05 U mL^{-1} enzyme. 2 mL samples were taken at

various time intervals and immediately filtered through a 30,000 Dalton molecular weight cut–off filter (Microsep Centrifugal Microconcentrator, Filtron Technology Corporation) to physically remove the enzyme. An appropriate aliquot of the sample was diluted to 3 mL and the ONP concentration determined by measuring the absorbance at 410 nm. The remaining sample was analyzed by HPLC to quantitate the amounts of galactose, glucose, and allolactose present.

3.2.4 Production of Allolactose

Allolactose was produced (for the purpose of confirming its identity) by adding 146 U of enzyme to a solution of 25 mM ONPG and 105 mM glucose in 0.1 mM sodium phosphate buffer containing 1 mM $MgSO_4$, 145 mM NaCl, and 0.02% sodium azide at 23° C. The reaction was monitored by spectroscopy and HPLC as described in the previous section. When all the ONPG had been consumed, the reaction was terminated by boiling the entire reaction mixture for 5 minutes. The reaction mixture was then evaporated to dryness in a Brinkmann Rotavapor–M at 90° C, and redissolved in 2 mL water. The redissolved mixture was separated by HPLC and fractions were collected. The fractions with a retention time corresponding to a disaccharide were pooled, evaporated to dryness, and then analyzed by NMR spectroscopy (see Section 3.3.3).

3.3 Analytical Methods

3.3.1 High–Performance Liquid Chromatography (HPLC)

Sugars were analyzed using a Waters HPLC system consisting of a model 6000A pump, a model U6K injector system, a Sugar Pak I column, an R401 refractive index detector, and a model 730 data module (integrator). The column, a fixed–ion (Ca^{++}) resin, was operated at 90° C in a DuPont Instruments Column Compartment equipped with a Shimaden temperature controller. The mobile phase was water containing 50 mg L^{-1} calcium EDTA. Galactose and glucose were quantitated by means of standard curves (using *myo*–inositol as an internal standard). Allolactose (Galβ(1→6)Glc) was quantitated using a lactose (Galβ(1→4)Glc) standard curve. Melibiose (Galα(1→6)Glc) produced the same standard curve as lactose, therfore using the lactose standard curve to quantitate allolactose was justified.

Sample preparation: Frozen samples were thawed in a 30° C water bath. Equal volumes of sample and a solution of 10mM *myo*–inositol (internal standard) were mixed and then filtered through a Millipore microcentrifuge ultrafiltration unit (nominal molecular weight limit 100,000 Daltons). 15 to 25 μL were injected into the HPLC system.

For experiments where lactose was used as substrate, the HPLC system was incapable of separating lactose and allolactose.

3.3.2 Gas Chromatography (GC)

Because of the inability of the HPLC system to separate lactose and allolactose, GC was employed to determine the presence of allolactose in some samples.

GC was performed on a Hewlett–Packard 5890A gas chromatograph equipped with a J & W DB–1 column, flame–ionization detector (FID), and a 3393A integrator. The temperature program began at 205° C, was raised 10°/min to 295° C, and then raised 5°/min to 320° C and kept at that temperature for 15 minutes.

Sample preparation: Frozen samples were thawed in a 30° C water bath. Equal volumes of sample and 1 mM *myo*–inositol were mixed and then evaporated to dryness. Samples were redissolved in 300 μL DMSO at 60° C. The samples were then converted to trimethlysilyl ethers by the addition of trimethylchorosilane (TMCS) and hexamethyldisilazane (HMDS). After the reaction mixture had formed two layers (approximately 10 minutes), 1 to 2 μL of the top phase was injected into the GC.

3.3.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy was used to confirm the identity of allolactose. ^{13}C NMR spectra were performed by the Spectrophotometry Laboratory in the Department of Chemistry. The spectra were done on a 200 MHz Bruker WH200 spectrophotometer using D_20 as the solvent.

Chapter 4. RESULTS & DISCUSSION

4.1 Determination of k_{cat} for Lactose Hydrolysis

At saturating substrate concentrations, an enzymatic reaction proceeds at the maximum velocity. A rule of thumb for achieving saturating conditions is to use an initial substrate concentration equal to 10 times the K_m value. A plot of substrate (or product if the stoichiometry is 1:1) concentration versus time will have a slope equal to the maximum velocity.

Two initial concentrations of lactose (7.5 & 37.5 mM) were hydrolyzed using β–galactosidase. The evolution of glucose and galactose were followed by HPLC. A typical concentration versus time plot is shown in Figure 18. The complete data sets for the hydrolysis of lactose are located in Appendix A.

Product concentration versus time data were fitted to a zero–order equation to determine the maximum velocity.

Huber *et al.* (1983) reported a k_{cat} of 66.4 s^{-1} and a v_{max} of 30 U mg^{-1} for lactose hydrolysis. One unit (U) is defined as the release of 1 μmol min^{-1} of glucose. The equation relating v_{max} and k_{cat} is

$$v_{max} = k_{cat}E_o \tag{4.1.1}$$

where E_o is the total enzyme concentration. For this equation to be dimensionally consistent, Huber *et al.* must have expressed E_o in μmol mg^{-1}, *i.e.* (molecular weight)$^{-1}$ x 1000. An effective molecular weight can, therefore, be back–calculated from the published values for k_{cat} and v_{max}:

$$E_o = \frac{30 \text{ U mg}^{-1}}{(66.4 \text{ s}^{-1}) \cdot (60 \text{ s min}^{-1})} = 7.53 \times 10^{-3} \ \mu\text{mol mg}^{-1} \tag{4.1.2}$$

and

$$\text{molecular weight} = (7.53 \times 10^{-3} \ \mu\text{mol mg}^{-1})^{-1} \cdot (1000 \ \mu\text{mol mg}^{-1})$$

$$= 132{,}800 \text{ mg mmol}^{-1} \tag{4.1.3}$$

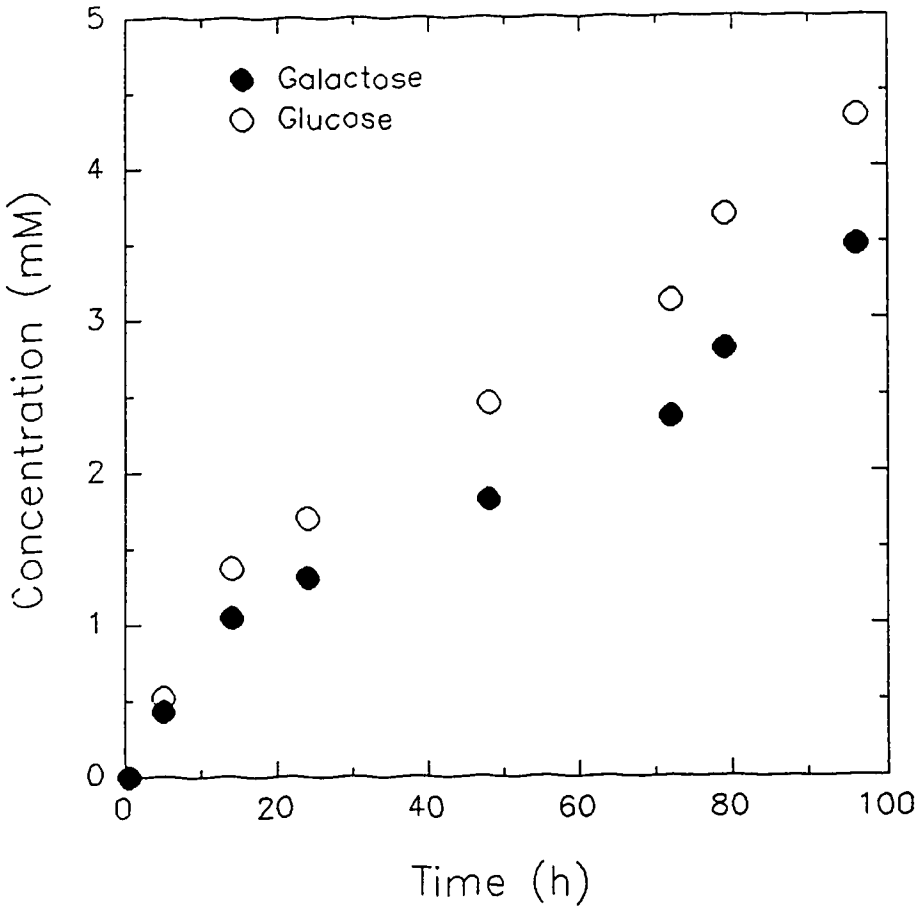


Figure 18. *Evolution of glucose and galactose during the β–galactosidase–catalyzed hydrolysis of lactose.* 0.0119 mg *of β–galactosidase* (350 U mg^{-1}) *was added to* 37.5 mM *lactose at* 22.5° C.

β–galactosidase is a tetramer composed of four identical polypeptide chains (each with an independent active site). A molecular weight of 128,000 mg $mmol^{-1}$ for a monomeric unit has been reported by Wallenfels & Weil (1972), which agrees with the value of 132,800 mg $mmol^{-1}$ calculated from the data of Huber *et al.* (1983).

Using this effective molecular weight and adjusting for the difference in specific activities between the enzyme preparation used by Huber *et al.* and the enzyme preparation used in this study, the maximum velocities for lactose hydrolysis were converted into k_{cat} values. The k_{cat} values are reported in Table 12.

Table 12. Values of k_{cat} for the Hydrolysis of Lactose (Based on Glucose Production)

$[lactose]_{initial}$ (mM)	n	Mean k_{cat} (s^{-1})	Range of k_{cat} (s^{-1})
7.5	1	69.4	
37.5	3	75.0	45 to 130

These values are the same order of magnitude as the value of 66.4 s^{-1} reported by Huber *et al.* (1983).

4.2 Determination of k_{cat} for ONPG hydrolysis

The k_{cat} value for ONPG hydrolysis was determined by following the β–galactose–catalyzed release of ONP with time. An initial concentration of 2 mM ONPG was used (*i.e.* a saturating concentration of substrate). The slope of a zero–order equation fitted to the concentration versus time data was therefore equal to v_{max}. The v_{max} value obtained was converted to a k_{cat} value as described in the previous section. The complete data sets for these experiments are located in Appendix B.

The value of k_{cat} for ONPG hydrolysis was calculated to be 1115 s^{-1} ± 6 (95% confidence interval, n = 4). A k_{cat} equal to 1115 s^{-1} is in reasonable agreement with

the values of 753 s^{-1} (Huber *et al.*, 1983) and 670 s^{-1} (Deschavanne *et al.*, 1978) previously reported.

The amount of error found in determining k_{cat} for lactose hydrolysis (75 s^{-1} with a range of 45 to 130 s^{-1}) and k_{cat} for ONPG hydrolysis (1115 s^{-1} ± 6, 95% confidence interval) indicates the error involved in determining the k_{cat} values in the following sections.

4.3 Effect of Added Glucose on β–Galactosidase–Catalyzed Hydrolyses

The transgalactosylation activity of β–galactosidase was examined by studying the effect of added glucose on the k_{cat} values for the hydrolysis of lactose and ONPG. The following two sections discuss the results of these experiments.

4.3.1 Effect of Added Glucose on the Hydrolysis of ONPG

Varying amounts of glucose were added to reactions using ONPG as substrate. The k_{cat} values for these reactions are shown in Table 13. The ONPG hydrolysis in the absence of glucose was performed 4 times, the other experiments were performed once. Typically 5 or 6 data points for concentration as a function of time were used to determine k_{cat}. The K_m for ONPG is 0.11 mM (Viratelle & Yon, 1973), therefore the initial concentration of 0.5 mM ONPG was not saturating, resulting in slightly lower estimates for k_{cat}.

Glucose can act as a competitive inhibitor (binding unproductively to free enzyme), but a correction for this effect can be made. The Michaelis–Menten equation for an enzymatic reaction in the presence of a competitive inhibitor is

$$-\frac{dS}{dt} = \frac{k_{cat}E_oS}{K_m^{app} + S} \qquad (4.3.1)$$

where $K_m^{app} = K_m\left[1 + \frac{[A]}{K_i}\right]$

Table 13. Effect of Glucose on Apparent k_{cat} (based on ONP production) for the Hydrolysis of ONPG

Initial concentrations (mM)		*Rate constants* (s^{-1})			
[ONPG]	[Glc]	k_{cat}^{app}	k_{cat}^{corr}[1]	k_{cat}^{rel}[2]	k_{pred}^{rel}[3]
2.0	0	1115	1115	1.00	1.00
2.0	7.5	1026	1084	0.97	0.95
2.0	450	626	712	0.64	
0.5	7.5	847	1038	0.93	
0.5	450	290	450	0.40	
	0	753[4]		1.00	1.00
	20	615[4]		0.82	0.87
	60	506[4]		0.67	0.69
	100	465[4]		0.62	0.58

[1]k_{cat} corrected for the competitive inhibition effect of glucose.
[2]Ratio of apparent k_{cat} to k_{cat} in the absence of glucose (1115 s^{-1}).
[3]k_{cat}^{rel} predicted from a fit to Equation (4.3.4).
[4]Data of Huber *et al.* (1983).
Complete data sets are located in Appendix C.

Bailey & Ollis (1986) provide a full discussion of the derivation of the Michaelis–Menten equation.

The effect of an inhibitor (A) can be compensated for by multiplying the apparent k_{cat} by a correction factor as shown by

$$k_{cat}^{corr} = \left[\frac{S}{K_m^{app} + S}\right] \cdot k_{cat}^{app} \qquad (4.3.2)$$

The corrected k_{cat} values (k_{cat}^{corr}) are given in Table 13.

The binding constant of glucose to free enzyme (K_i) is very high (Huber & Gaunt (1983) report $K_i > 300$ mM and Deschavanne *et al.* (1978) report $K_i = 630$ mM). To allow for the maximum possible effect, a value of $K_i = 300$ mM was used to calculate the k_{cat}^{corr} values shown in Table 13.

Table 13 also presents relative k_{cat} values (k_{cat}^{rel}) which were calculated as

$$k_{cat}^{rel} = \frac{k_{cat}^{corr}}{k_{cat}^{act}} \qquad (4.3.3)$$

where k_{cat}^{act} is the actual k_{cat}, as measured in the absence of glucose.

The k_{cat}^{rel} values for initial concentrations of 0.5 and 2.0 mM ONPG, and the k_{cat}^{rel} values calculated form the data of Huber *et al.* (1983), show a definite inhibition of ONPG hydrolysis by glucose. All the data sets presented in Table 13 show the same trend of lower k_{cat}^{rel} values at higher glucose concentrations, and that the inhibitory effect of glucose was larger than expected (from k_{cat}^{corr}).

The inhibition of ONPG hydrolysis by high concentrations of glucose can be explained by examining a schematic diagram of the reaction, as pictured in Figure 19.

$$E + ONPG \overset{K_S}{\rightleftharpoons} E\cdot ONPG \xrightarrow{k_2} E\cdot gal + ONP \xrightarrow{k_3'} E + gal$$

$$E\cdot gal + glc \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} E\cdot gal\cdot glc \xrightarrow{k_4} E + allo$$

Figure 19. *Hydrolysis of* ONPG *by β–galactosidase in the presence of glucose.*

As the glucose concentration is increased, the steady–state concentration of the E·gal·glc complex increases. Because the total amount of enzyme is fixed, a

concomitant decrease in the steady–state concentration of the E·gal complex will occur, thus causing the observed decline in the k_{cat}^{app}. Thus the inhibitory effect of glucose is two–fold: at low glucose concentrations the inhibition is caused mainly by the alteration of the steady–state concentrations of enzyme–intermediate complexes (i.e. by glucose participating as a reactant), whereas at high glucose concentrations the effect is due to competitive inhibition. Because the competitive inhibition decreases the k_{cat}^{app} of all the reaction pathways, the k_{cat}^{app} approaches a minimum as the glucose concentration becomes saturating.

An apparent K_i (K_i^{app}) for glucose was estimated by fitting the k_{cat}^{rel} values given in Table 13 (including those calculated from the data of Huber *et al.*, 1983) to Equation (4.3.2) rearranged into the form

$$k_{cat}^{rel} = \frac{S}{K_m\left[1 + \frac{[A]}{K_i^{app}}\right] + S} \qquad (4.3.4)$$

The k_{cat}^{rel} values predicted from this fit (k_{pred}^{rel}) are given in Table 13.

The data point at 450 mM glucose was not used in the fit because the form of the equation does not account for the fact that as the reaction pathway involving glucose and the competitive inhibition effect of glucose both become saturating the k_{cat}^{app} approaches a limiting value.

K_i^{app} was estimated to be 6.6 mM. This value differs greatly from previous estimates of K_i (>300 mM by Huber *et al.*, 1983 and 630 mM by Deschavanne *et al.*, 1978) because of the different experimental methods used in determining K_i. Previous estimates have used the inhibition of initial velocities extrapolated back to zero glucose concentration to determine K_i, thus accounting for only the competitive inhibition effect of glucose. Fitting Equation (4.3.4) to the data from Table 13 (without the 450 mM glucose data point) accounts mainly for the

inhibition caused by glucose acting as a reactant. Obviously, the latter type of inhibition is much more significant in the design of transglycosylation experiments.

4.3.2 Effect of Added Glucose on the Hydrolysis of Lactose

Varying amounts of glucose were added to reaction mixtures using lactose as substrate. The k_{cat}^{app} values for these reactions are shown in Table 14.

Monitoring the change in lactose concentration is the only way to fully assess the activity of β–galactosidase. Although lactose concentration versus time data were collected, these data were highly unreliable and therefore could not be used.

Table 14. Effect of Glucose on Apparent k_{cat} (based on galactose production) for the Hydrolysis of Lactose

Initial concentrations (mM)		*Rate constants* (s^{-1})			
[Lac]	[Glc]	k_{cat}^{gal}[1]	k_{cat}^{glc}[2]	k_{cat}^{rel}[3]	*Number of Runs*[4]
7.5	0	34.8	69.4		3
7.5	7.5	40.3			3
7.5	75	21.7			3
37.5	0	46.4	75.0	1.00	3
37.5	7.5	40.6		0.86	3
37.5	75	27.5		0.59	3

[1]apparent k_{cat} based on galactose production.

[2]apparent k_{cat} based on glucose production.

[3]Ratio of apparent k_{cat} to k_{cat} for saturating substrate in the absence of glucose.

[4]Typically 4 to 6 data points from each run were used in determining the apparent k_{cat}. Complete data sets are located in Appendix D.

Neither the k_{cat} based on galactose production nor the k_{cat} based on glucose production represents the full activity of β–galactosidase. Huber *et al.* found that β–galactosidase catalyzed the direct intramolecular conversion of lactose to allolactose. In other words, glucose need not be released from the enzyme–galactose–glucose complex prior to the formation of allolactose. The sequence of reactions occurring with lactose as substrate is illustrated in Reactions (4.3.1) to (4.3.6).

$$E + lac \rightleftharpoons E \cdot lac \rightleftharpoons E \cdot gal \cdot glc \qquad (4.3.1)$$

$$E \cdot gal \cdot glc \rightleftharpoons E \cdot allo \rightleftharpoons E + allo \qquad (4.3.2)$$

$$E \cdot gal \cdot glc \rightleftharpoons E \cdot gal + glc \qquad (4.3.3)$$

$$E \cdot gal + lac \rightleftharpoons E \cdot gal \cdot lac \rightleftharpoons E + gal\text{–}lac \qquad (4.3.4)$$

$$E \cdot gal + allo \rightleftharpoons E \cdot gal \cdot allo \rightleftharpoons E + gal\text{–}allo \qquad (4.3.5)$$

$$E \cdot gal \rightleftharpoons E + gal \qquad (4.3.6)$$

The scheme depicted by Reactions (4.3.1) to (4.3.6) shows that the rate of glucose formation will be equal to the rate of lactose consumption minus the rate of allolactose production. Likewise, the rate of galactose production will be equal to the rate of lactose consumption minus the sum of the rate of all reactions consuming galactose. Therefore the k_{cat} based on galactose production is not the actual k_{cat} for lactose consumption, but is instead a "net" k_{cat}. The effects of these side reactions will be discussed more fully in Section 4.5.

The K_m for lactose is 1.35 mM (Huber & Gaunt, 1983), therefore the initial concentration of 7.5 mM lactose was not saturating, resulting in slightly low estimates for k_{cat}.

For the experiments using an initial lactose concentration of 37.5 mM, a relative k_{cat} equal to

$$k_{cat}^{rel} = \frac{k_{cat}^{app}}{k_{cat}^{act}}$$

was calculated, where k_{cat}^{act} was taken to be the k_{cat}^{gal} in the absence of added glucose. Figure 20 plots these k_{cat}^{rel} value[illegible] k_{cat}^{rel} values calculated from the data of Huber *et al.* (1983). A K_i^{app} wa[illegible] fitting the data shown in Figure 20 to Equation (4.3.4). The estima[illegible] 2.4 mM. This value is similar to the one obtained for ONPG hydrolysis because K_i^{app} reflects mainly the inhibitory effect due to the participation of glucose as a reactant. This inhibition occurs because of a change in the steady–state concentration of the E·gal complex, which is common to both the ONPG and lactose hydrolytic pathways. However, the maximum level of inhibition by glucose (set by the competitive inhibition effect of glucose) was greater for lactose than for ONPG because lactose (K_m = 1.35 mM) binds free enzyme less effectively than ONPG (K_m = 0.11 mM).

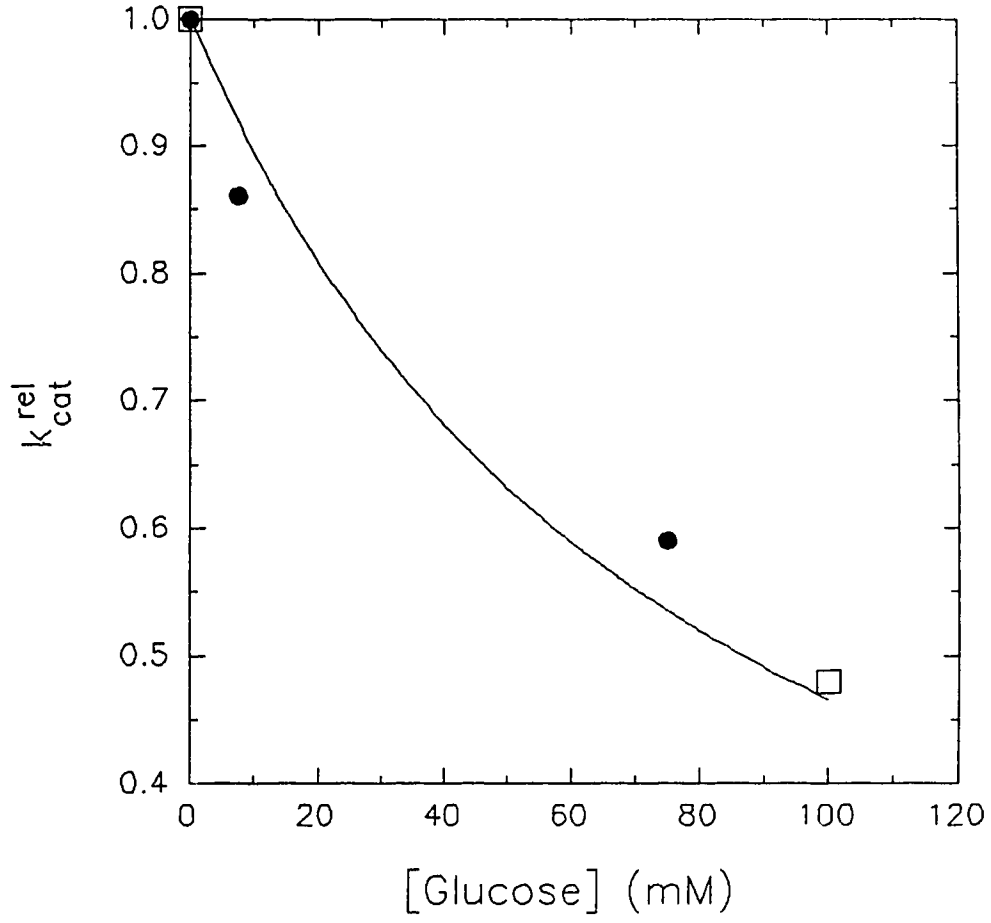


Figure 20. *The effect of glucose on the hydrolysis of lactose by β–galactosidase. Lactose* (37.5 mM) *was hydrolyzed by β–galactosidase* (0.0119 mg, 350 U mg–1) *both in the absence of glucose and in the presence of* 7.5 & 75 mM *glucose* (•). *Also plotted are points calculated from the data of Huber* et al., 1983 (□).

4.4 Strategy for Maximizing Allolactose Yield

Developing a strategy for maximizing the production of allolactose by β–galactosidase requires careful consideration of the reaction mechanism. The β–galactosidase–catalyzed formation of allolactose from a β–galactoside can be illustrated schematically by the following series of reactions:

Hydrolysis:

$$E + gal\text{–}x \rightleftharpoons E\cdot gal\text{–}x \longrightarrow E\cdot gal + x \rightleftharpoons E + gal \qquad (4.4.1)$$

Transfer to glucose:

$$E\cdot gal + glc \rightleftharpoons E\cdot gal\cdot glc \rightleftharpoons E + allo \qquad (4.4.2)$$

Transfer to galactose:

$$E\cdot gal + gal \rightleftharpoons E\cdot gal\cdot gal \rightleftharpoons E + gal\text{–}gal \qquad (4.4.3)$$

Transfer to a disaccharide (gal–gal, allo, or gal–x *when the donor is a disaccharide):*

$$E\cdot gal + DS \rightleftharpoons E\cdot gal\cdot DS \rightleftharpoons E + gal\text{–}DS \qquad (4.4.4)$$

If the dissociation of allolactose from the E·gal·glc complex (Reaction 4.4.2) is in equilibrium, then the reaction governing the production of allolactose is

$$E\cdot gal + glc \xrightarrow{k_a} E\cdot gal\cdot glc \qquad (4.4.5)$$

and the rate of this reaction is given by

$$v = k_a[E\cdot gal]\,[glc] \qquad (4.4.6)$$

The rate constant k_a, intrinsic to the enzyme, and the nature of the reacting

species cannot be altered. Raising the glucose concentration will increase the rate of reaction. Also, because several reactions (Reactions 4.4.2 to 4.4.4) compete for the E·gal complex, the selectivity of the reaction will be increased by using a high glucose concentration. Once this has been done, the rate of transfer to an acceptor now depends on the concentration of the E·gal complex. For the case where selectivity is maximal (*i.e.* no significant reactions of the E·gal complex with galactose or disaccharides), the total amount of enzyme (E_0) present can be represented by the following balance equation:

$$E_0 = E + E\cdot gal{-}x + E\cdot gal + E\cdot gal\cdot glc \qquad (4.4.7)$$

In order to maximize the concentration of the E·gal complex, the concentrations of the free enzyme (E) and the E·gal–x complex must be minimized.

The amount of free enzyme is determined by the reaction

$$E + gal{-}x \rightleftharpoons E\cdot gal{-}x \qquad (4.4.8)$$

and can be minimized by using a very high substrate (gal–x) concentration.

The concentration of enzyme–substrate complex (E·gal–x) is regulated by the reaction

$$E\cdot gal{-}x \longrightarrow E\cdot gal + x \qquad (4.4.9)$$

and can be minimized by selecting a substrate that is hydrolyzed at a high rate.

Based on these considerations, ONPG should be a superior donor to either lactose or PNPG (because of its higher rate of hydrolysis), and the maximum possible yield of allolactose should be obtained using high concentrations of ONPG as donor and glucose as acceptor.

4.5 Production of Allolactose

An allolactose production run was performed employing the concepts outlined in the previous section. The time course of the reaction is shown in Figure 21.

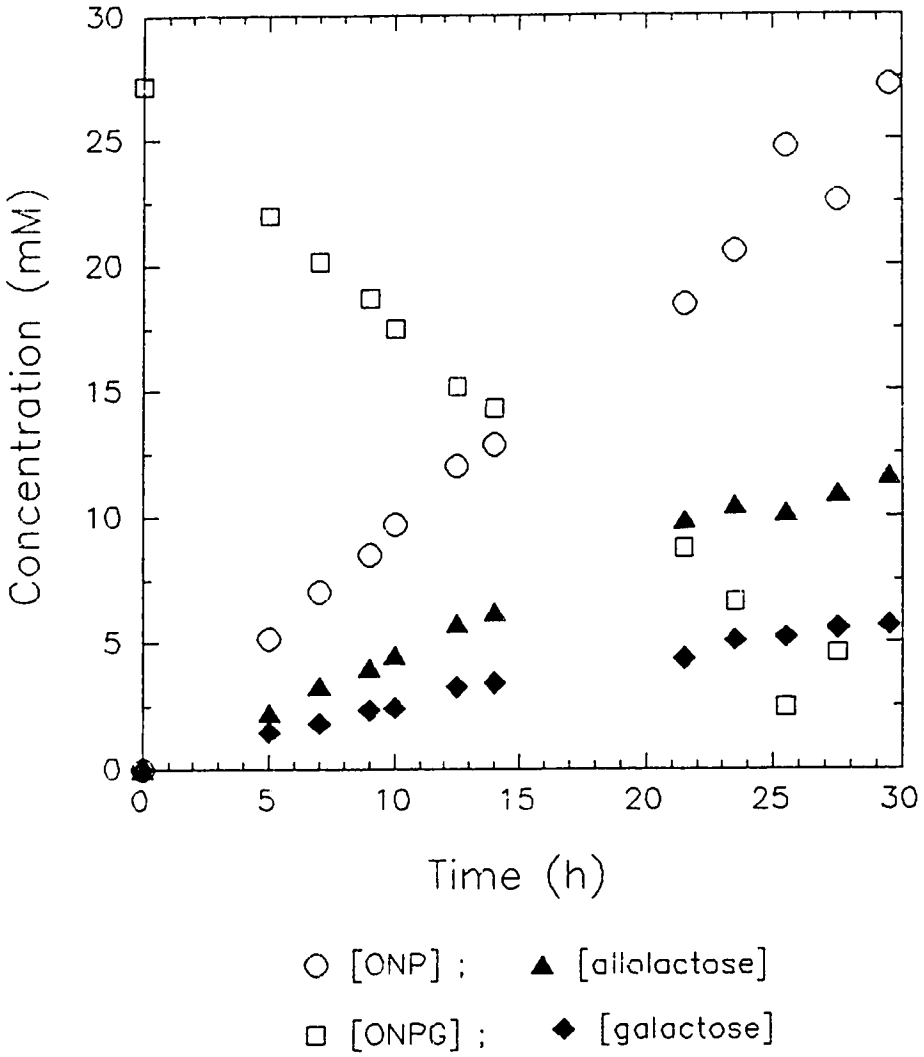


Figure 21. *Time–course data for the production of allolactose.* 0.0119 mg *of β–galactosidase* (169 U mg^{-1} *specific activity) was added to a solution of* 25 mM ONPG *and* 105 mM *glucose at* 22.5° C.

Four experiments were also performed to systematically study the effect of added glucose on allolactose production. The kinetic data for these experiments and the allolactose production run are presented in Table 15.

Table 15. The Effect of Added Glucose on Allolactose Production

Initial concentrations (mM)		*Rate constants* (s^{-1})			
[ONPG]	[Glc]	k_{cat}^{onp} [1]	k_{cat}^{allo} [2]	k_{cat}^{gal} [3]	Yield [4]
7	0	814	0	492	0
7	7.5	803	33	578	4
7	75	773	351	226	31
7	450	683	352	0	50
25	105	581	270	135	53

[1]apparent k_{cat} for ONP production.
[2]apparent k_{cat} for allolactose production.
[3]apparent k_{cat} for galactose production.
[4]yield of allolactose based on ONPG.
Complete data sets located in Appendix E.

The yield of allolactose for the allolactose production run (25 mM ONPG + 105 mM glucose) was 53% based on ONPG. This yield is superior to the 22% allolactose yield obtained by Huber et al. (1976) using lactose as substrate. Huber et al. used an initial concentration of 500 mM lactose with no glucose added as acceptor. No glucose was added because at initial lactose concentrations greater than 100 mM, added glucose had no effect on allolactose production.

The k_{cat}^{onp} for the hydrolysis of 7 mM ONPG in the absence of added glucose was 814 s^{-1} considerable less than the value of 1115 s^{-1} determined for the hydrolysis of 2 mM ONPG. The lower rate at the very high substrate concentration

of 7 mM (approximately $70 \cdot K_m$) could be due to substrate inhibition.

Using Equation (4.3.2) and the previously determined estimate for K_i^{app} (6.6 mM), the level of glucose inhibition for the hydrolysis of 7 mM ONPG was predicted to be less than that for 2 mM ONPG (see Figure 22), but still showing the same downward trend with increasing glucose concentration.

The observed inhibition by glucose was even less than expected (see Figure 22). At this extremely high substrate concentration, the ONPG may be better able to compete with glucose for free enzyme, thus lessening the decrease in k_{cat}^{app} caused by increasing glucose concentration.

If galactose were not involved in any side reactions (i.e. if allolactose were the only product formed from ONPG and glucose), the amount of ONPG consumed would be equal to the sum of the amounts of allolactose and galactose produced. At saturating conditions, this relation can be expressed as

$$k_{cat}^{onp} = k_{cat}^{allo} + k_{cat}^{gal} \qquad (4.5.1)$$

Equation (4.5.1) is not true for any of the experiments listed in Table 15, indicating that galactose is indeed involved in side reactions.

A set of possible reactions occurring during the hydrolysis of ONPG in the presence of glucose is shown in Reactions (4.5.1) to (4.5.5).

$$E + ONPG \rightleftharpoons E.ONPG \rightleftharpoons E.gal + ONP \qquad (4.5.1)$$

$$E.gal + glc \rightleftharpoons E.gal.glc \qquad (4.5.2)$$

$$E.gal.glc \rightleftharpoons E.allo \rightleftharpoons E + allo \qquad (4.5.3)$$

$$E.gal + allo \rightleftharpoons E.gal.allo \rightleftharpoons E + gal\text{–}allo \qquad (4.5.4)$$

$$E.gal \rightleftharpoons E + gal \qquad (4.5.5)$$

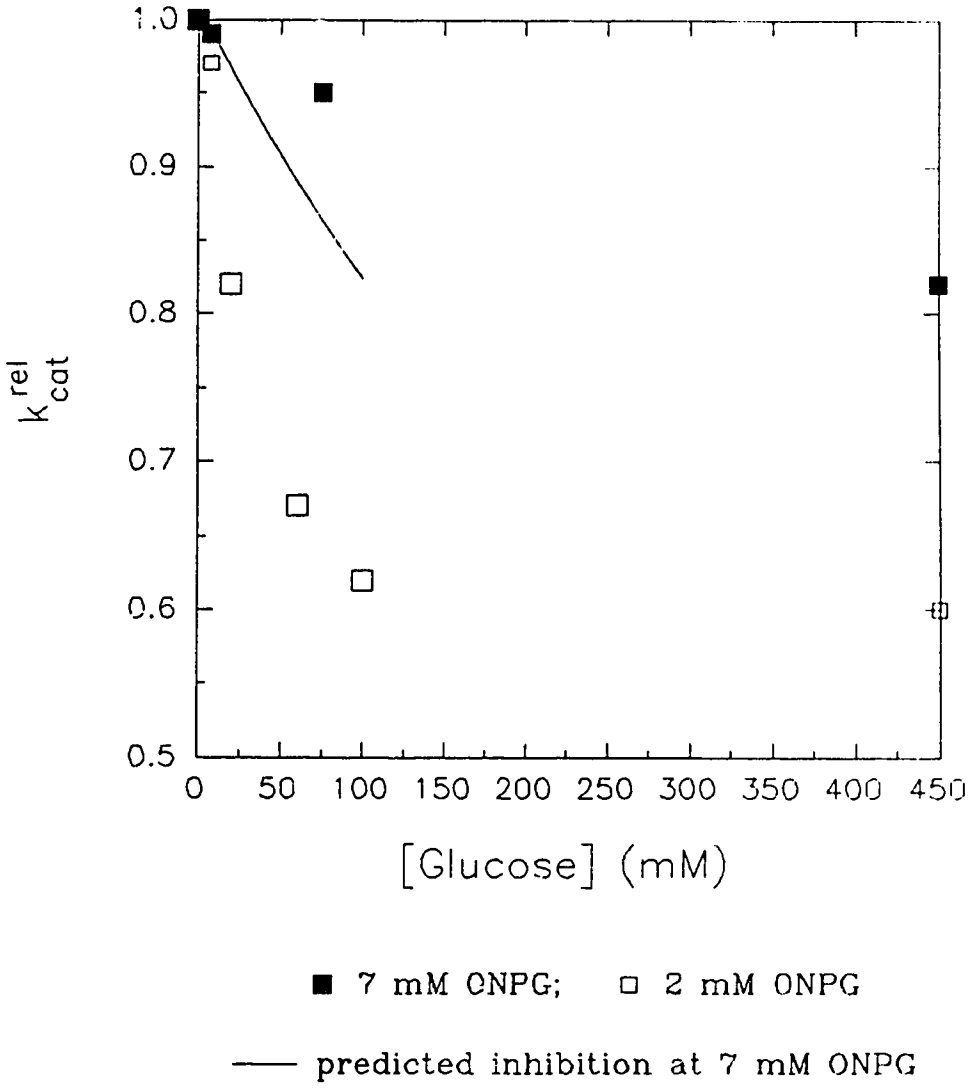


Figure 22. *Observed and predicted effects of glucose on the hydrolysis of* ONPG *by β–galactosidase.*

Here both galactose and allolactose are shown as being involved in a side reaction (Reaction (4.5.4)) that produces a trisaccharide (gal–allo).

If galactose were involved in side reactions, the time course concentration profiles of ONP and galactose would appear as shown in Figure 23.

At very early reaction times, the rate of galactose production would equal the rate of ONP production. At "intermediate" reaction times, the build–up of galactose would exhibit first–order behavior. Finally, as the side reactions became saturated, the rate of galactose production would be zero–order.

β–galactosidase and the enzyme–galactose complex both have high affinities for many saccharides, particularly β–galactosides. The K_m values for lactose and allolactose have been reported as 1.35 and 0.94 mM respectively (Huber & Gaunt, 1983). If a side reaction such as Reaction (4.5.4) (the formation of a gal–allo trisaccharide) were occurring at an appreciable rate when the allolactose concentration is still less than 1 mM, the HPLC methods used to track the reaction would not reveal the initial reaction period during which $k_{cat}^{onp} = k_{cat}^{gal}$. Mono– and disaccharides could not be accurately quantitated with the HPLC until their concentrations exceeded 1 mM. Also, the first–order period of the reaction would be difficult to observe unless a large enough number of data points were taken during that period. With only a few data points, the expected non–linearity might not be detected. The emphasis in these experiments was the collection of data at longer reaction times as opposed to the collection of initial rate data as in conventional studies of enzyme kinetics.

If a trisaccharide had been formed in any of the experiments listed in Table 15, it would not have been detected by either the HPLC or GC methods used.

An interesting finding is that $k_{cat}^{onp} \neq k_{cat}^{gal}$ even for the experiment in which no glucose was added (therefore no allolactose was produced). This implies that galactose is involved in a side reaction other than the formation of a gal–allo trisaccharide. The formation of a gal–gal disaccharide is the most likely possibility.

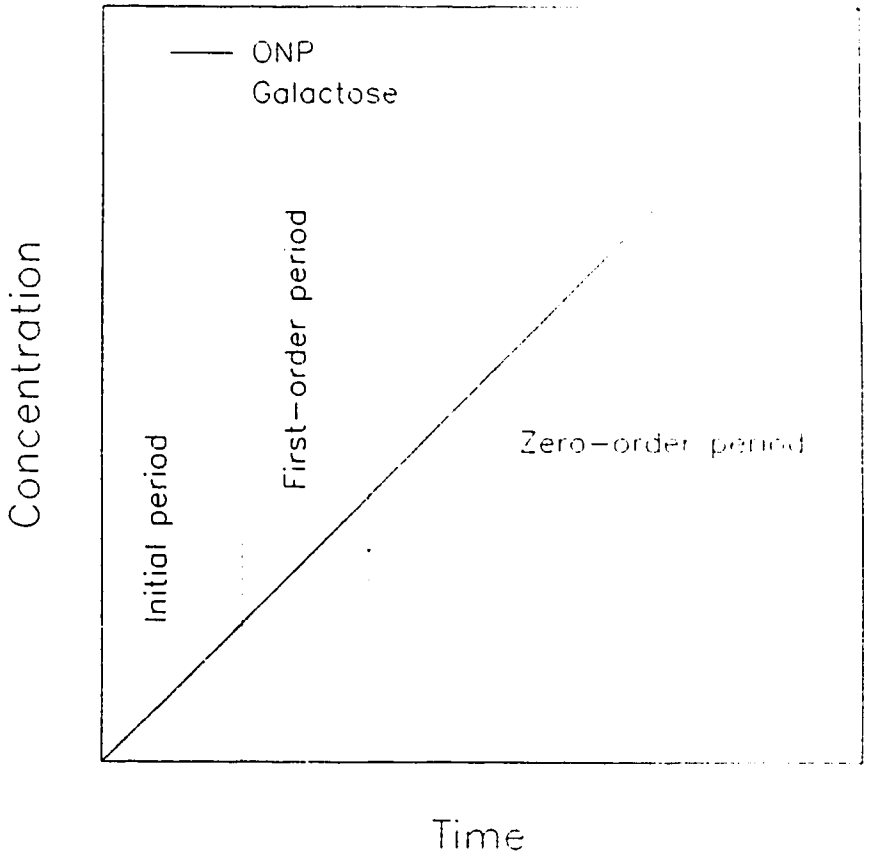


Figure 23. *The theoretical time–course of the evolution of* ONP *and galactose during the β–galactosidase–catalyzed hydrolysis of* ONPG *if galactose were being consumed by a side reaction.*

The identity of the allolactose formed in the production run was confirmed by ^{13}C NMR.

4.6 ^{13}C NMR Spectroscopy of Allolactose

The compounds in the product mixture from the allolactose production run were separated by HPLC, and the compound with a retention time corresponding to a disaccharide was collected. A ^{13}C NMR spectrum for this compound was obtained from the Spectrophotometry Laboratory in the Department of Chemistry.

In ^{13}C NMR, the position of a signal generated by a particular carbon is a consequence of its immediate electronic environment and effects diminish rapidly with distance. The appearance of the ^{13}C NMR spectrum for allolactose can be predicted from the ^{13}C NMR spectrum of lactose by examining the structural differences between the two molecules.

The ^{13}C NMR spectrum of lactose is shown in Figure 25 and the band assignments (Johnson, 1972) are given in Table 16.

Table 16. Band Assignments for ^{13}C NMR of Lactose

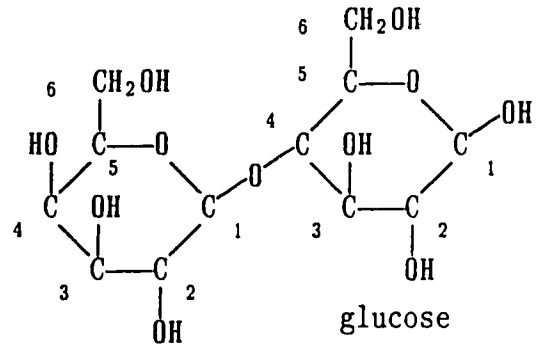
Carbon[1]	Signal (ppm)	Carbon	Signal (ppm)	Carbon	Signal (ppm)
Gal 1	103.7	α Glc 1	92.7	β Glc 1	96.6
Gal 2	71.9	α Glc 2	72.3	β Glc 2	74.8
Gal 3	73.5	α Glc 3	75.6	β Glc 3	72.1
Gal 4	69.5	α Glc 4	79.3	β Glc 4	79.4
Gal 5	76.2	α Glc 5	75.3	β Glc 5	70.9
Gal 6	61.9	α Glc 6	61.1	β Glc 6	61.0

[1]See Figure 24 for numbering system.

The structures of lactose and allolactose are depicted in Figure 24.

When the galactose–glucose linkage is shifted from $\beta 1 \rightarrow 4$ to $\beta 1 \rightarrow 6$, the carbons experiencing the greatest change in local environment are the 4 and 6 carbons of the glucose moiety. In the ^{13}C NMR spectrum of allolactose, the peak corresponding to the 4–carbon of glucose should be shifted upfield and the peak corresponding to the 6–carbon of glucose should be shifted downfield.

The ^{13}C NMR spectrum of the disaccharide isolated from the allolactose production run is shown in Figure 25. The peaks that appear at 79 ppm (the C–4 of glucose) and 61 ppm (the C–6 of glucose) have both been shifted into the 68 to 76 ppm range (an upfield shift for the C–4 of glucose and a downfield shift for the C–6 of glucose). This spectrum matches the predicted appearance of the spectrum for allolactose exactly, therefore the disaccharide produced in the allolactose production run was allolactose.



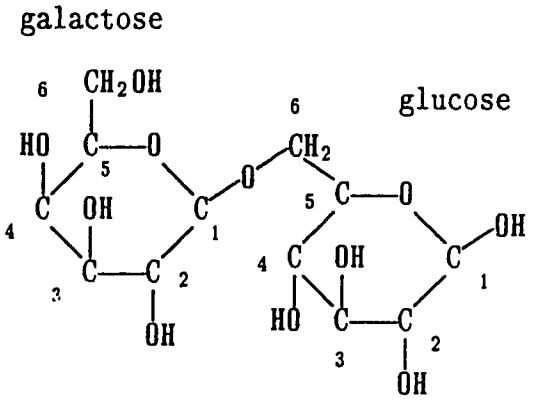


Figure 24. *Carbon numbering–schemes for lactose and allolactose. Glucose moieties are depicted in the β form but are free to mutarotate between the α & β forms. Hydrogens on ring carbons have been omitted for clarity.*

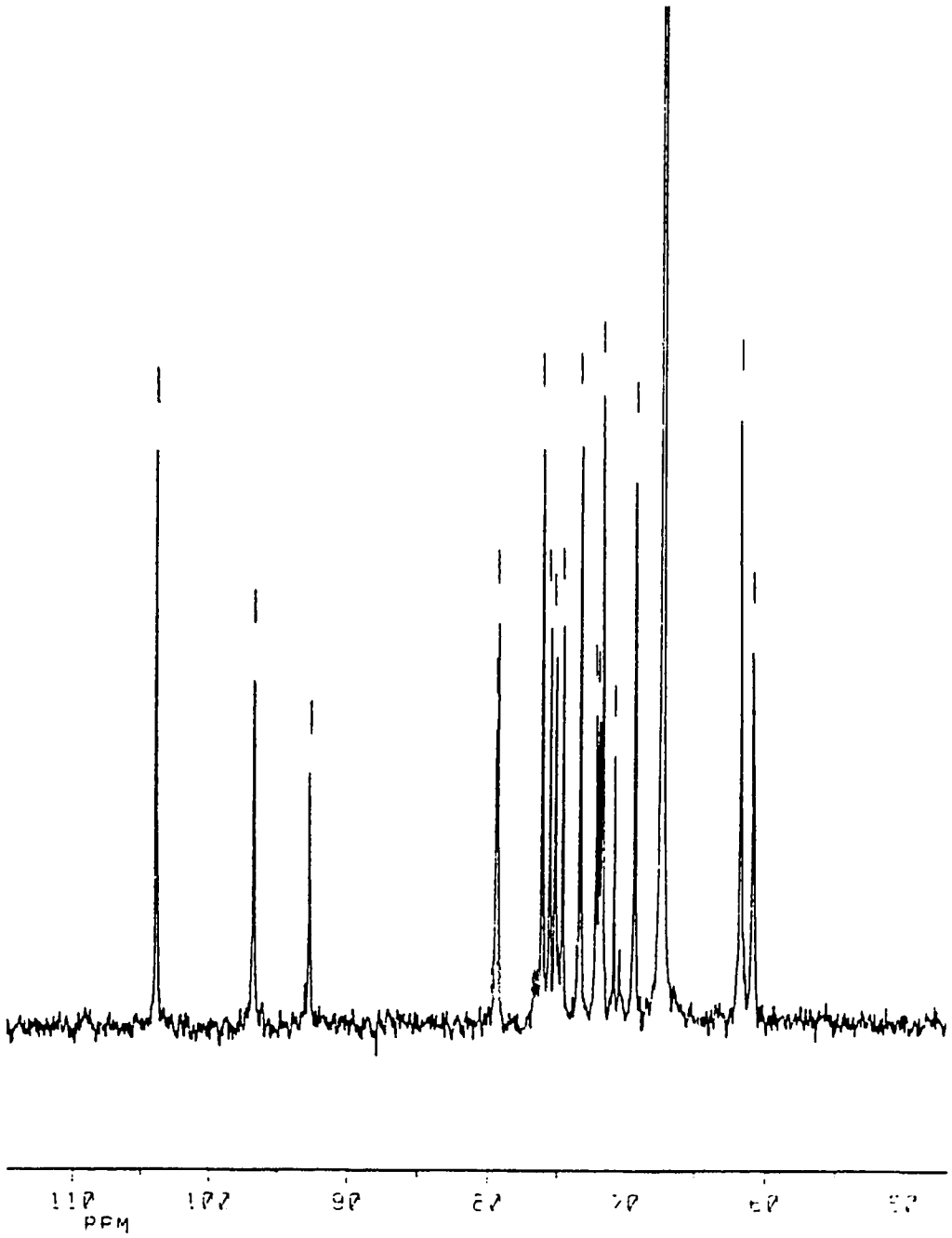


Figure 25. *The* ^{13}C *spectrum of lactose.*

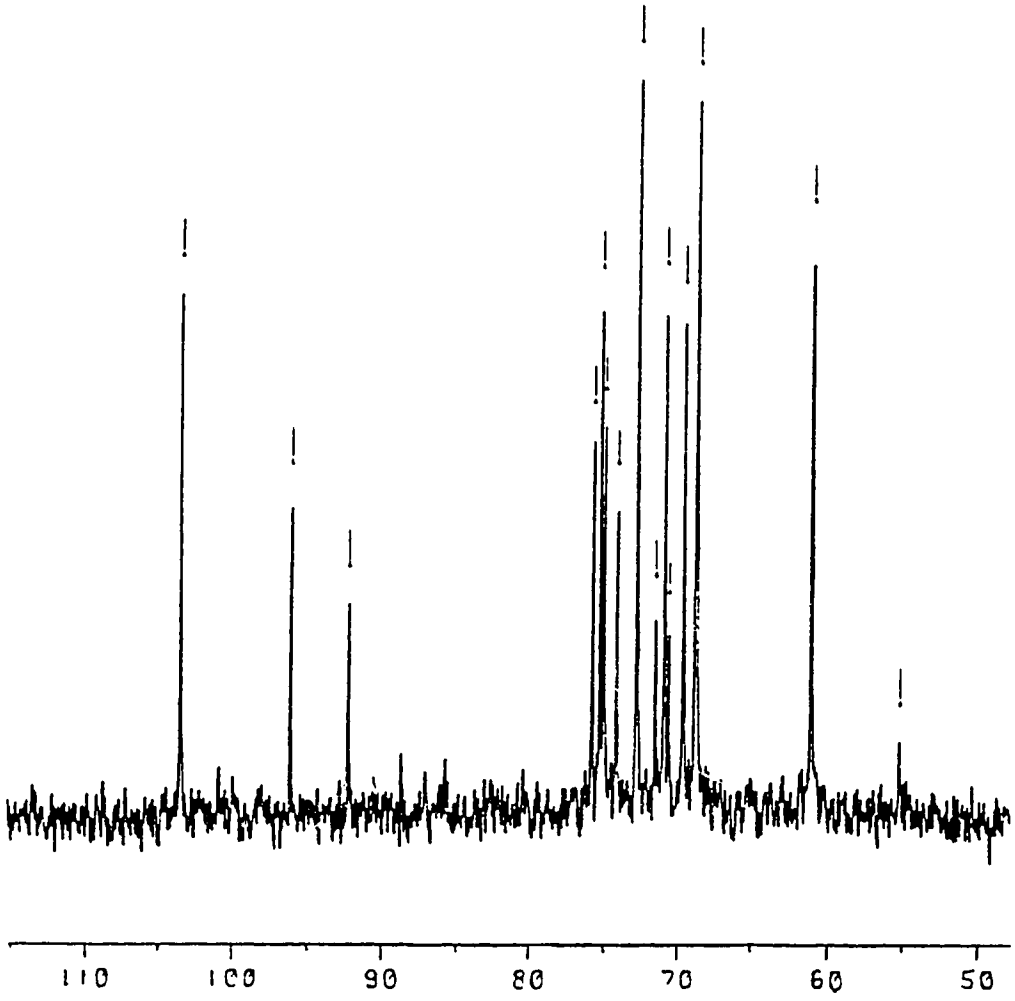


Figure 26. *The* ^{13}C *spectrum of the disaccharide produced in the allolactose production run.*

Chapter 5. CONCLUSIONS

1. The β–galactosidase–catalyzed hydrolyses of lactose and ONPG were performed in the presence and absence of added glucose. In the absence of added glucose, the catalytic rate constant (k_{cat}) for lactose hydrolysis was estimated to be 75.0 s^{-1} (based on glucose production). The k_{cat} for ONPG hydrolysis (based on ONP production) was determined to be 1115 ± 6 s^{-1}.

2. The addition of glucose to lactose and ONPG hydrolyses caused a decrease in the apparent k_{cat}. The observed decrease was greater than could be accounted for by competitive inhibition (by glucose) alone. The additional decrease in k_{cat}^{app} was attributed to an increase in the steady–state concentration of the enzyme intermediate complex involving galactose and glucose, thereby decreasing the steady–state concentration of free enzyme available for substrate hydrolysis. Apparent inhibition constants (K_i^{app}) were estimated to be 6.6 mM for ONPG hydrolysis and 2.4 mM for lactose hydrolysis. The difference in these two values likely reflects the different abilities of ONPG and lactose to compete with glucose for binding to free enzyme.

3. Examination of the effects of added glucose in terms of the reaction pathway led to the development of a three–fold strategy for maximizing allolactose yield. First, a high glucose (acceptor) to donor ratio was used to maximize the selectivity of the reaction to form disaccharides. Second, a high substrate (donor) concentration was used to minimize the amount of the free enzyme in the reaction. Third, a substrate with a high rate of hydrolysis (ONPG) was used in order to minimize the steady–state concentration of the enzyme–substrate complex and maximize the steady–state concentration of the enzyme–galactose complex (the branch point between the hydrolytic and transgalactosylic pathways).

4. The strategy for maximizing allolactose yield was tested by hydrolyzing 25 mM ONPG in the presence of 105 mM glucose. An allolactose yield of 53% (based on ONPG) was obtained.

5. The effect of added glucose on the production of allolactose was studied by hydrolyzing 7 mM ONPG in the presence of 0, 7.5, 75, and 450 mM glucose. The k_{cat}^{app}'s for ONP production were not equal to the sum of the k_{cat}^{app}'s for galactose and allolactose production for any of the cases studied, indicating that both galactose and allolactose participated in side reactions. The maximum attainable k_{cat}^{app} for allolactose production was 352 s^{-1}.

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APPENDIX A

Hydrolysis of lactose in the absence of glucose.

7.5 mM lactose

Time (h)	[Glucose] (mM)	[Galactose] (mM)
0.5	0.93	0.00
2	1.02	0.00
5	1.71	0.25
14	1.83	0.61
24	1.22	0.81
30	2.58	0.81
48	3.23	1.40
72	3.90	2.46

Enzyme: 0.0119 mg, 350 U/mg

37.5 mM lactose

Time (h)	[Glucose] (mM)	[Galactose] (mM)
0.5	0.00	0.00
5	0.51	0.43
14	1.37	1.04
24	1.69	1.31
48	2.45	1.82
72	3.11	2.36
79	3.68	2.80
96	4.34	3.48

Enzyme: 0.0119 mg, 350 U/mg

Reaction volume 5 mL

APPENDIX B

Hydrolysis of 2 mM ONPG in the absence of glucose

Time (s)	Absorbance (410 nm)	Enzyme (mg)	Activity (U/mg)
0	0.036	0.0059	9.1
10	0.046		
20	0.057		
30	0.067		
40	0.077		
50	0.088		
60	0.099		
0	0.278	0.0059	16.9
10	0.297		
20	0.317		
30	0.336		
40	0.356		
50	0.375		
60	0.395		
0	0.042	0.00069	68.3
10	0.051		
20	0.061		
30	0.071		
40	0.078		
50	0.088		
60	0.097		
0	0.006	0.00069	49.5
20	0.021		
40	0.033		
60	0.046		
80	0.061		
100	0.074		
120	0.088		
140	0.102		
160	0.115		
180	0.129		

The standard curve was: Absorbance = 2.435[ONP] (mM)

The enzyme had an initial specific activity of 350 U/mg, however, specific activity declined over time. All calculations were adjusted by a factor of 350/(observed specific activity).

APPENDIX C

ONPG hydrolysis in the presence of glucose.

	ONPG 0.5 mM Glucose 7.5 mM [ONP] mM			ONPG 0.5 mM Glucose 450 mM [ONP] mM		
Time (min)	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
3	0.037	0.039	0.037	0.024	0.025	0.022
6	0.072	0.073	0.072	0.043	0.044	0.041
9	0.106	0.108	0.107	0.062	0.064	0.061
12	0.140	0.142	0.141	0.082	0.083	0.080
	0.174	0.176	0.175	0.101	0.103	0.100
	0.251	0.254	0.253	0.144	0.147	0.145
	0.315	0.318	0.318	0.179	0.184	0.182
35	0.387	0.391	0.391	0.221	0.226	0.224
60						
65	0.625	0.635	0.636	0.377	0.391	0.389
90		0.706	0.698	0.464	0.522	0.497
120		0.719	0.710	0.513	0.583	0.559
150		0.727	0.723	0.554	0.628	0.608

	ONPG 0.5 mM Glucose 7.5 mM [ONP] mM			ONPG 0.5 mM Glucose 450 mM [ONP] mM		
Time (min)	Run 1	Run2	Run 3	Run 1	Run2	Run 3
3	0.034	0.033	0.039	0.051	0.050	0.051
6	0.058	0.057	0.063	0.092	0.088	0.091
9	0.083	0.082	0.087	0.133	0.127	0.131
12	0.109	0.107	0.112	0.174	0.166	0.172
15	0.133	0.131	0.137	0.216	0.205	0.212
22				0.312	0.298	0.308
25		0.213	0.218			
28						
30	0.260	0.254	0.258	0.421	0.400	0.414
35						
60	0.506	0.488	0.493	0.817	0.778	0.805

Enzyme 0.00035 mg. 87 U/mg Reaction volume 3 mL

APPENDIX D

Lactose hydrolysis in the presence of glucose

	Lactose 7.5 mM Glucose 7.5 mM [Galactose] mM			Lactose 7.5 mM Glucose 75 mM [Galactose] mM		
ime (h)	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
0.5	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00
14	0.00	0.00	0.79	0.00	0.34	0.14
24	0.00	0.52	0.88	0.61	0.17	0.32
30	0.51	1.73	1.83	0.78	0.61	1.30
48	1.14	1.83	2.65	0.58	0.80	1.30
72	1.58	2.95	3.07	1.03	0.97	1.91

	Lactose 37.5 mM Glucose 7.5 mM [Galactose] mM			Lactose 37.5 mM Glucose 75 mM [Galactose] mM		
ime (h)	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
0.5	0.00	0.00	0.00	0.00	0.00	0.00
2	0.21	0.00	0.00	0.00	0.00	0.00
5	0.64	1.01	1.06	0.00	0.42	0.00
14	1.07	0.95	1.46	0.00	1.62	0.00
24	1.89	2.51	1.58	0.75	2.29	1.04
30	2.31	3.36	3.10	0.84	2.80	1.31
48	2.68	0.00	3.46	1.31	2.90	2.14
72	2.55	7.21	5.11	1.01	4.99	3.45

Enzyme 0.0119 mg, 350 U/mg Reaction volume 5 mL

APPENDIX E

Effect of glucose on allolactose production

Experiments with 7 mM ONPG, 1.19 mg enzyme (9.1 U/mg)

100 mL reaction volume

Glucose 0 mM Concentrations (mM)

Time (min)	[Allo]	[Glc]	[Gal]	[ONP]	[ONPG]
0.00	0.00	0.00	0.00	0.00	6.64
15.00	0.00	0.35	2.69	1.81	4.83
30.00	0.00	1.66	2.54	3.50	3.14
45.00	0.00	0.26	3.48	5.04	1.60
60.00	0.00	0.10	3.35	6.64	0.00
75.00	0.00	0.97	2.57	6.64	0.00

Glucose 7.5 mM Concentrations (mM)

Time (min)	[Allo]	[Glc]	[Gal]	[ONP]	[ONPG]
0.00	0.00	7.50	0.00	0.00	7.00
5.00	0.00	6.19	3.89	0.68	6.28
10.00	0.00	6.70	2.83	1.45	5.52
15.00	0.00	7.73	1.75	2.00	4.97
30.00	0.16	7.60	2.13	3.87	3.09
45.00	0.22	7.45	2.81	5.59	1.38
70.00	0.00	7.03	3.16	6.97	0.00

Glucose 75 mM Concentrations (mM)

Time (min)	[Allo]	[Glc]	[Gal]	[ONP]	[ONPG]
0.00	0.00	75.00	0.00	0.00	6.77
5.00	0.27	74.73	0.00	0.73	6.04
10.00	5.64	69.36	0.51	1.35	5.42
15.00	5.72	69.28	0.00	2.01	4.76
30.00	1.91	73.09	1.15	3.68	3.09
45.00	1.92	73.08	1.09	5.24	1.53
70.00				6.77	0.00

Glucose 450 mM Concentrations (mM)

Time (min)	[Allo]	[Glc]	[Gal]	[ONP]	[ONPG]
0.00	0.00	450.00	0.00	0.00	5.97
5.00	1.51	448.49	0.00	0.72	5.25
10.00	1.21	448.79	0.00	1.31	4.66
15.00	1.29	448.71	0.00	2.22	3.75
30.00	1.68	448.32	0.00	3.07	2.90
45.00	2.14	447.86	0.00	4.23	1.74
70.00	3.09	446.91	0.00	5.97	0.00

Allolactose production run

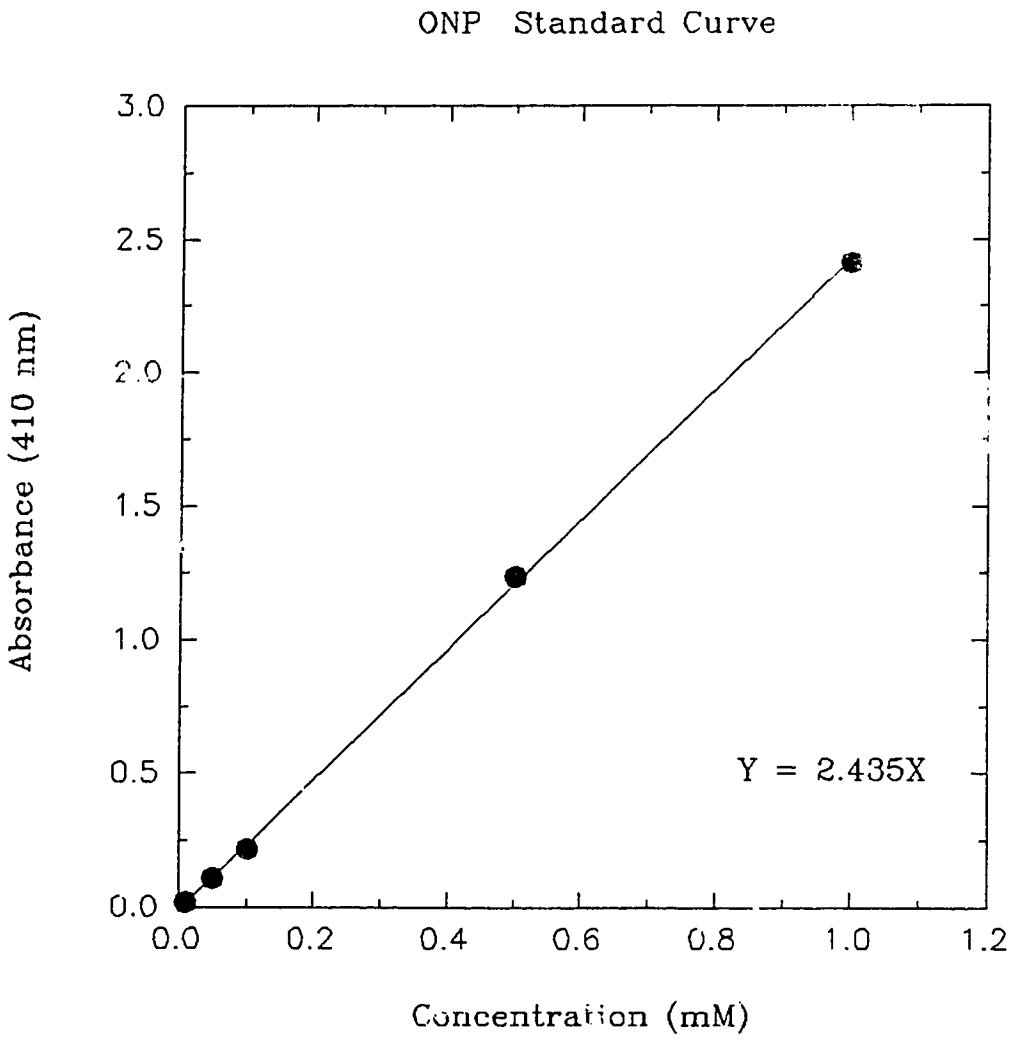
ONPG 25 mM, Glucose 105 mM

Time (h)	[Allo]	[Glc]	[Gal]	[ONP]	[ONPG]
0.00	0.00	105.50	0.00	0.00	27.16
5.00	2.24	105.45	1.47	5.18	21.98
7.00	3.30	105.17	1.82	7.02	20.14
9.00	4.02	107.16	2.33	8.49	18.67
10.00	4.50	105.19	2.41	9.68	17.48
12.50	5.77	103.95	3.24	11.98	15.18
14.00	6.22	103.30	3.41	12.84	14.32
21.50	9.85	100.38	4.36	18.43	8.73
23.50	10.45	104.41	5.09	20.53	6.63
25.50	10.14	98.17	5.20	24.69	2.47
27.50	10.88	97.08	5.57	22.57	4.59
29.50	11.58	98.19	5.68	27.15	0.01

Enzyme 0.0119 mg, 169 U/mg Reaction volume 100 mL

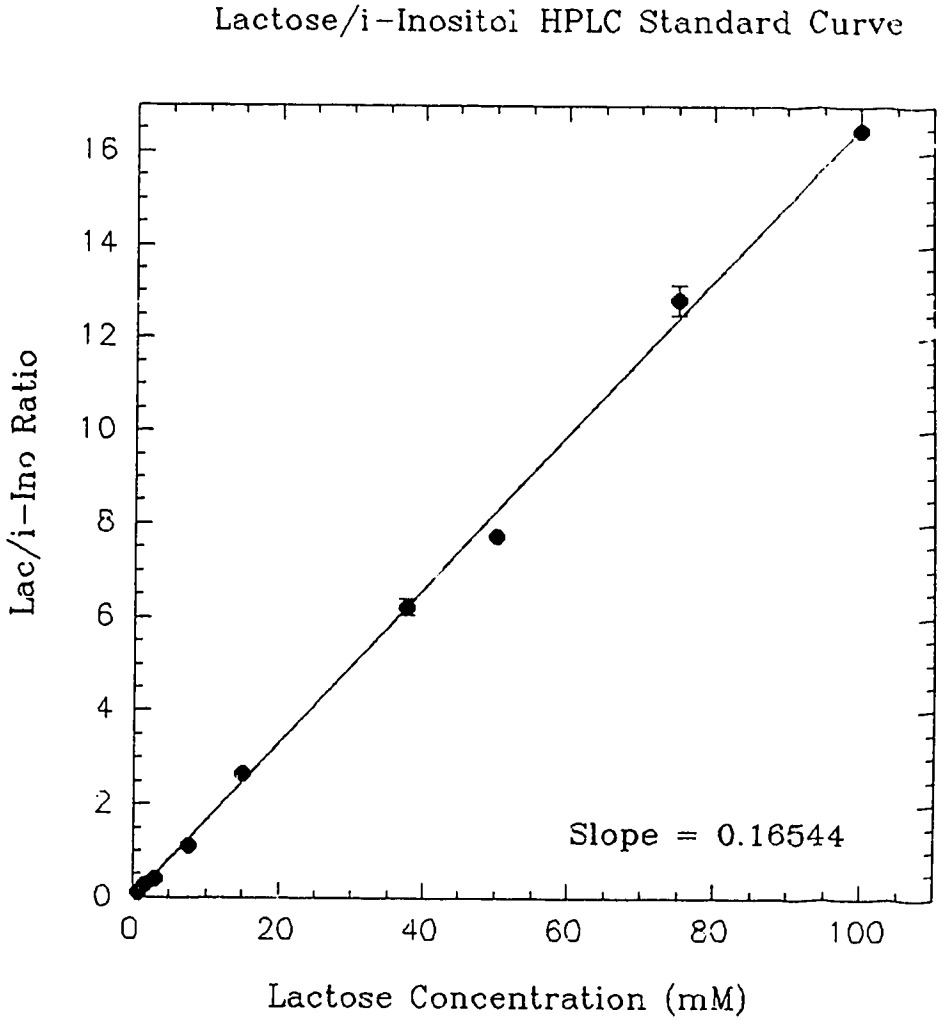
APPENDIX F

Spectrophotmeter Standard Curve

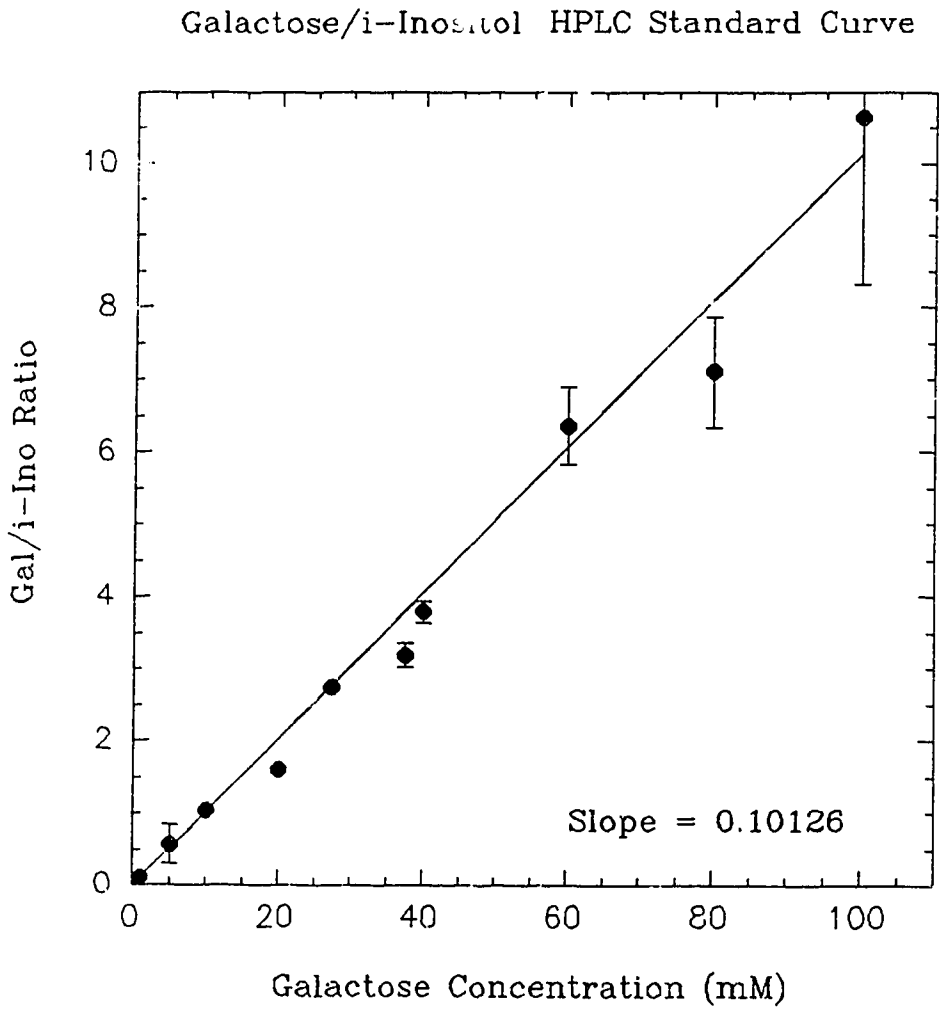


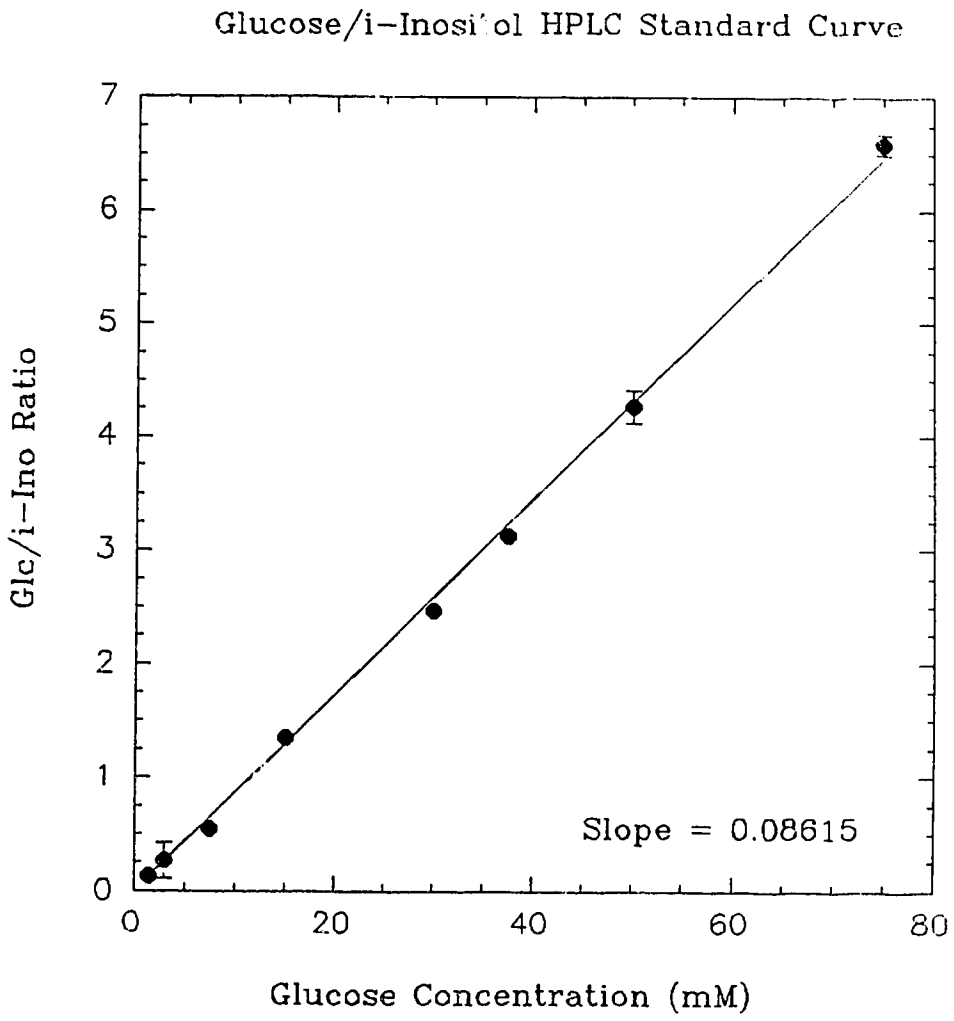
APPENDIX G

HPLC Standard Curves [1]



[1] i-Inositol was used as an internal standard. The ratio of the area of the sugar peak to the area of the internal standard peak is plotted against the sugar concentration.


Galactose/i-Inositol HPLC Standard Curve
Gal/i-Ino Ratio
Galactose Concentration (mM)
Slope = 0.10126
0
2
4
6
8
10
0
20
40
60
80
100


Glucose/i-Inositol HPLC Standard Curve
Glc/i-Ino Ratio
Glucose Concentration (mM)
Slope = 0.08615
0
1
2
3
4
5
6
7
0
20
40
60
80