BIOCHEMICAL LINKS BETWEEN SUGAR UTILIZATION, METABOLISM AND DISEASE

By

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ABSTRACT

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This study shows how the concentration and type of carbohydrate source can affect various cell properties by modifying the flux of intermediates through carbohydrate-processing pathways. Alteration of the carbohydrate sources (major and minor) available to living systems can interfere with the equilibria between sugars and change many cell properties. This work is mostly concerned with pathways involved in the metabolism of fructose and its derivatives. When fructose is the main carbon source provided to mammalian cells, distinct shifts in carbohydrate surface antigens are found relative to those found on cells grown in glucose. Changes in cellular morphology such as cell shape and granularity are also found when cells are grown on these two carbohydrate sources. These shifts are, however, cell type- and time-dependent. Fructose as carbon source also alters the cellular balance between hexoses and hexosamines. An evident increase in hexosamine biosynthesis is observed in fructose-fed cells in contrast to glucose. Another cellular characteristic affected by fructose is gene expression, with a significant induction of the expression of genes related to glycine and glycine betaine metabolism. This fact is highly relevant to one-carbon metabolism and methylation processes.

Other carbohydrate sources present in human diet can also change the flux through carbohydrate pathways by functioning as metabolic inhibitors of these routes. Glucose 6-sulfonate, also known as sulfoquinovose, is a significant dietary sugar found in plants. This study showed that this monosaccharide can affect the flux through the pentose phosphate and hexosamine

biosynthetic pathways and glycolysis. Sulfoquinovose does not participate in the pentose phosphate pathway but is a competitive inhibitor for the entry of glucose 6-phosphate into this pathway. Sulfoquinovose is also an inhibitor of glycolysis by providing only half of the energy for this route when compared to glucose or fructose. Sulfoquinovose can be converted *in vivo* in the first step of the hexosamine biosynthetic pathway to glucosamine 6-sulfonate, as is evident by the presence of this amino sugar in mammalian blood. Glucosamine 6-sulfonate possesses a broad antibacterial activity by acting as a competitive inhibitor of hexosamine biosynthesis. Both glucose 6-sulfonate and glucosamine 6-sulfonate are found to affect the growth of cancer cell lines in a concentration-dependent manner. This work brings into perspective the importance that carbohydrate metabolism and diet have on human health. The ingestion of high quantities of certain monosaccharides (e.g. fructose) may have detrimental effects on cellular health; others (such as sulfoquinovose), on the other hand, may be used as nutritional sources of inhibitors for carbohydrate pathways that are increased in many metabolic diseases.

DEDICATION

I dedicate this work to my parents, Aldino and Maria Jose, and my family, Paulo and Lily. They are the main reason I got this far by providing me love, support and unconditional care in every moment of my life.

I would also like to dedicate this work to my professor, Dr. Rawle Hollingsworth. He was an example of what a scientist and a human being should be.

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AD	Alzheimer's disease
AGE	Advanced end-glycation products
Antigen A	Antigen containing N-acetyl-galactosamine as terminal sugar residue
Antigen B	Antigen containing galactose as terminal sugar residue
Antigen O	Antigen containing fucose as terminal sugar residue
BCA	Bicinchoninic acid
bEnd.3	Brain endothelial cells
CDG	Congenital diseases of glycosylation
3,5-DNB-Cl	3,5-dinitrobenzoyl chloride
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide monohydrochloride
ER	Endoplasmic reticulum
FACS	Flow activated cell sorter
FU	Fluorescent units
GABA	γ-Aminobutyric acid
GAGs	Glycosaminoglycans
Gal	Galactose
GC-MS	Gas chromatography-Mass spectometry
GFAT	Glutamine: fructose-6-phosphate amidotransferase
GalNAc	N-acetyl-galactosamine
GlcNAc	N-acetyl-glucosamine
Glu	Glucose

G6PDH	Glucose-6-phosphate dehydrogenase
GSI4	*Griffonia simplicifolia* isolectin B4
HBP	Hexosamine biosynthetic pathway
hCG-H	Hyperglycosylated human chorionic gonadotropin
HFCS	High fructose corn syrup
HPA	*Helix pomatia* lectin
HPI	Hexose phosphoisomerase
IC_{50}	Concentration that inhibits 50% of cell growth
LB	Luria-Bertani medium
Man	Mannose
ManNAc	N-acetyl-mannosamine
MEF	Mouse embryonic fibroblasts
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PDHC	Pyruvate dehydrogenase complex
PEP	Phosphoenolpyruvate
PPP	Pentose phosphate pathway
RA	Rheumatoid arthritis
SDHB/C/D	Succinate dehydrogenase complexes B, C and D
SQDG	Sulfoquinovosyl diacylglycerol
TCA	Tricarboxylic cycle
T2D	Type 2 diabetes

THF	Tetrahydrofolate
ULE	*Ulex europeaus* lectin

CHAPTER 1

CHAPTER 1

CARBOHYDRATE METABOLISM, GLYCOSYLATION AND DISEASES.

1. Overview

Carbohydrates are the most abundant organic molecules in a cell. They are present in the structure of membranes, enzymes, structural proteins, nucleic acids, hormones, growth factors, cytoskeleton molecules, vesicles, cell wall polysaccharides, extracellular matrix molecules among others. They participate in most metabolic pathways within a cell, playing therefore innumerous roles in cellular metabolism. They are, for instance, involved in providing energy[1,2], amino acids[3], fatty acids[4], nucleotides[5] and amino sugars[6] for the cell metabolism, as well as acting as secondary messengers[7]. Besides the important role they play in the cellular catabolism and anabolism, they are also important post-translational modifications of many proteins known. Therefore, it is not surprising that they are involved in whole context of cell growth, survival and differentiation.

Because of the importance of carbohydrates in the cell metabolism, affecting the flux of molecules formed in each pathway they are involved in, it is essential to appreciate carbohydrates not as merely contributors of protein function (as post-translational modifications) but also as modulators of cellular properties, since they provide many essential molecules that will affect the physiological status of the cell/tissue. In the studies described here I will explore the relationship between the types and concentrations of carbohydrates available for the growth of cells and organisms and metabolic pathways that are utilized. I will link the pathways and the types and

concentrations of metabolites that are formed to the chemical makeup of the resulting cellular ultra-structure.

2. Carbohydrate Occurrence in Biological Systems

Carbohydrates are divided into four different groups based on their degree of polymerization[8]: monosaccharides, disaccharides, oligosaccharides and polysaccharides. Monosaccharides are the smallest, non-hydrolysable carbohydrate units. The most common ones found in living systems are: D-glucose, D-fructose, D-mannose, D-galactose, D-fucose, L-rhamnose, D-glucosamine, D-galactosamine, D-mannosamine, D-N-acetyl-neuraminic acid (sialic acid), D-xylose, L-arabinose, D-arabinose, D-ribose and D-2-deoxyribose. In vertebrates, most monosaccharides are found in the D-form, with exception of rhamnose, fucose and arabinose, which are found in both isomeric forms[9]. Figure 1.1 shows the structures of the most common hexoses and sialic acid, which are major sugars in mammalian systems (besides ribose and 2-deoxyribose) and their routes of biosynthesis. There are several pathways by which sugars can be interconverted, which are an important cellular adaptation when the relative concentrations of sugars available for growth vary.

Monosaccharides can be found in the living systems freely or combined to high-energy donor molecules, such as nucleotides. In the last case, they are frequently used in alkylation reactions with proteins and lipids, forming glycoproteins and glycolipids respectively through the process known as glycosylation[10]. The majority of proteins are glycosylated, and this post-translational modification is important for proper protein folding, function and resistance to

protease degradation. Glycosylation of lipids also confer them the properties mentioned above. The glycosylation process is described in section 3.

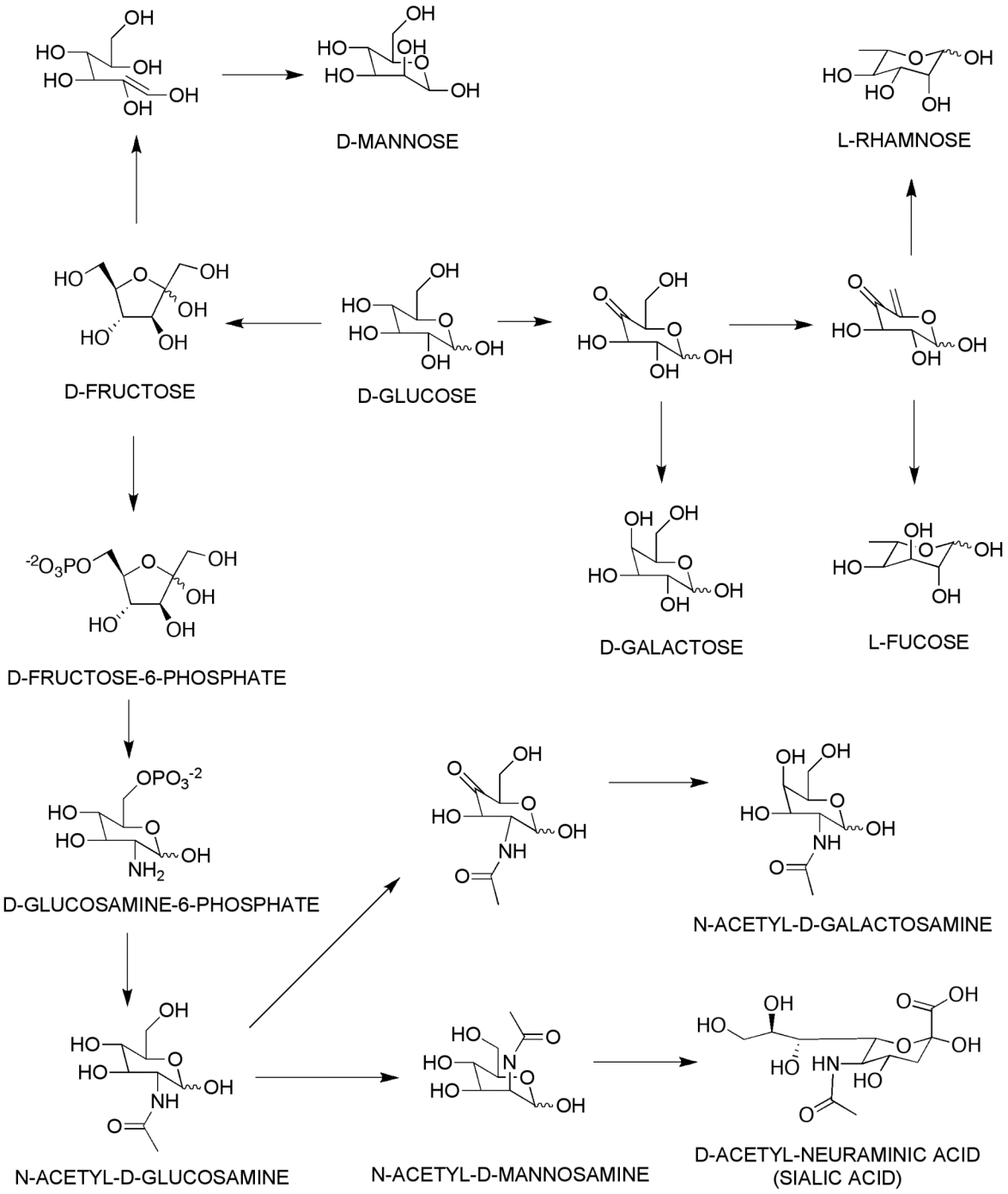

Figure 1.1. Structure and biosynthetic routes of most common monosaccharides found in living systems.

3. The Glycosylation Process: Post-Translational Modification of Proteins and Lipids

3.1. Biosynthesis of Glycoconjugates

The biosynthesis of glycoproteins in eukaryotes starts in the membrane of the endoplasmic reticulum (ER), where UDP-N-acetyl-glucosamine (UDP-GlcNAc) is transferred to the lipid precursor dolichol phosphate (Dol-P), generating Dol-P-P-GlcNAc. In general, fourteen sugars are then added to this precursor, forming the sequence $Glc_3Man_9GlcNAc_2$-P-P-Dol. This precursor is transfer *en bloc* to an asparagine residue (Asn-X-Ser/ Thr, where X is different from proline) of a protein as it is synthesized by ribosomes attached to the ER. This process is called N-glycosylation. This glycosylated protein is further modified in the ER and Golgi complex by various glycosyltransferases and glycosidases that can add or remove monosaccharide units to or from these glycans. They can generate forms that are classified as high-mannose, hybrid or complex glycans[11].

Proteins can also have monosaccharides or short oligosaccharide sequences added to the oxygen of serine, threonine or tyrosine residues in the Golgi complex through a the process of O-glycosylation. In contrast to N-glycosylation, there is no *en bloc* transfer of carbohydrates[11]. Again the glycan structure can vary tremendously in any given position.

In lipids, the glycosylation process starts in the ER with the addition of β-galactose or β-glucose to a ceramide unit. This goes to the Golgi complex where it is further processed by the Golgi enzymes. It can also receive many other modifications in the Golgi, such as sulfation and acetylation. Most glycolipids are components of membranes and are responsible for structural and signaling functions within the cell. Figure 1.2 exemplifies the biosynthesis of a type of glycolipid called ganglioside. They are classified as sialilated sphingolipids found in high concentrations in

the human brain. This figure exemplifies how the variety of structures that can originate from an initial structure depending of the substrate utilized and how it is modified by glycosyltransferases and glycosidases. As seen, the concentration of monosaccharides available in the cell, the order of substrate utilization by the glycosyltransferases, in conjunction with structural factors such as size stereochemistry, number of available sites for modification and energetics of the final structure is what determines the final structure to be formed[11].

As demonstrated in Figure 1.2, it is important to remember that the glycosylation process is not template driven. This opens the chance for the generation of an endless repertoire of structure combinations. These can vary in size, sequence and branching types. Each monosaccharide can accommodate different linkages in several positions with another and each sugar and they can also be individually modified by non-carbohydrate substituents such as phosphorylation, sulfation, formylation, methylation and acetylation. All possible sites for glycosylation on all copies for a given molecule may not be modified at the same time or by the same thing, resulting in different combinations of glycosylation patterns between two otherwise equal molecules. Thus, the number of different branch types in an oligosaccharide is virtually infinite[11-15]. Over 100 glycoforms have been identified for some glycoproteins and glycolipids with many more still unidentified[16]. But the numbers for other glycoconjugates can be even greater. For instance, a glycoprotein with 23 glycosylation sites can have more than 300 glycoforms identified[14].

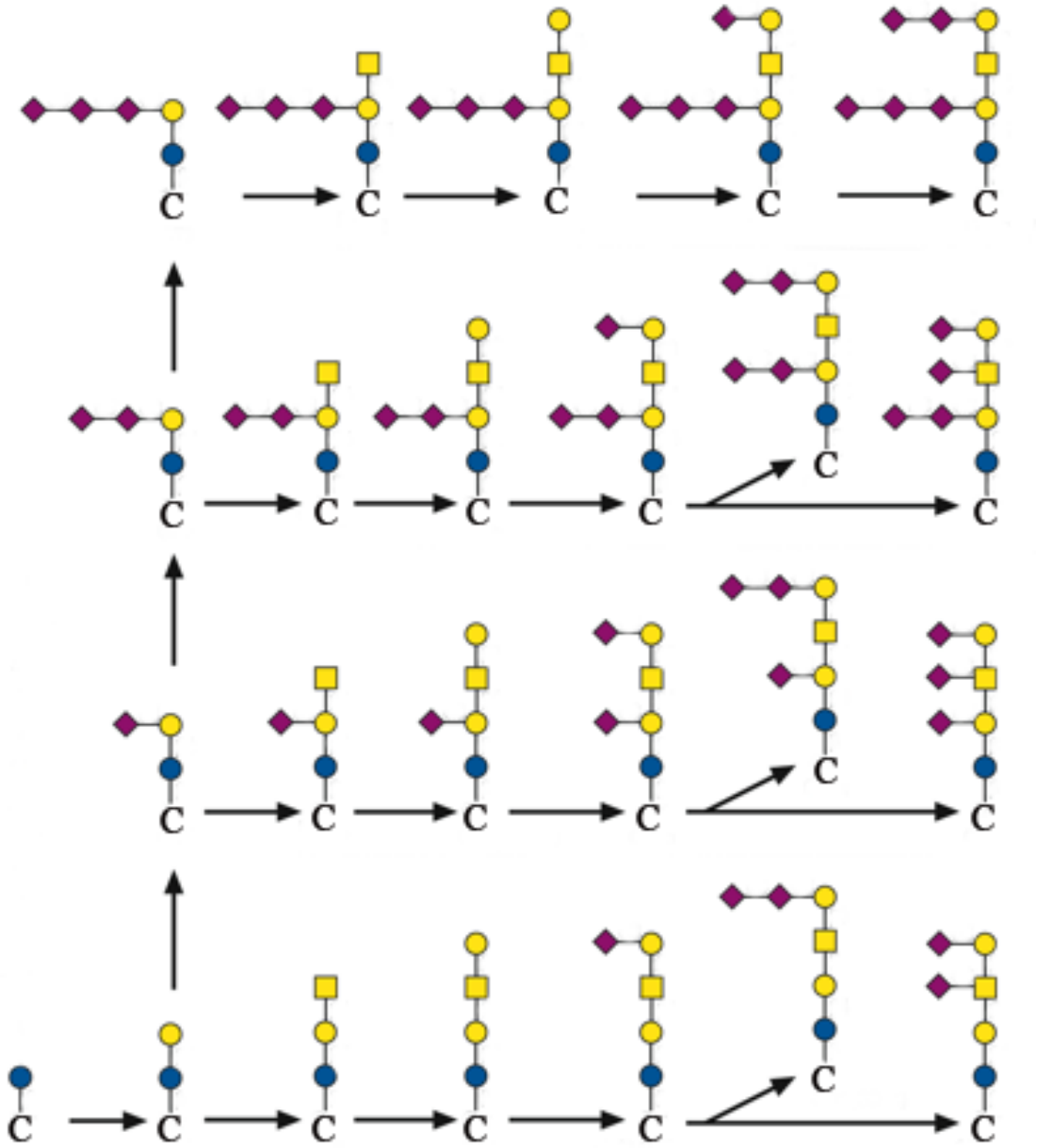

Figure 1.2. Biosynthetic pathway for brain gangliosides. The final structure generated from the same substrate is highly dependent on the order and type of enzymes that react with it. Legend: yellow circle: galactose; square: N-acetyl-galactosamine; blue circle: glucose; diamond: sialic acid; C= ceramide (figure from reference 11, 2009, The Consortium of Glycobiology Editors, La Jolla, California). (For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation).

3. 2. Control and Regulation of the Glycosylation Process

The central feature of nucleic acids and proteins biosynthesis is that these molecules are synthesized based on templates. This feature is not true of glycoconjugate synthesis. Nucleic acid and protein synthesis generates molecules with, in theory, invariant sequences of predictable sizes and properties, while carbohydrate structures are not template driven. It has been shown though that even nucleic acid biosynthesis is subject to sequence alterations (also known as mutations) depending on the concentrations of the different types of deoxynucleotides available at the time of their biosynthesis[17]. For all glycoforms, then, that do not follow any template-defined, coded pre-determined sequence, their structure is highly dynamic. These types of glycoforms can be constantly altered because they are extremely sensitive to the nutrient status of the cell. They are solely determined by the instantaneous and past concentrations of enzymes and activated monosaccharides (substrates) within the cell in conjunction with the structural features mentioned earlier. Because carbohydrates are important structural and regulatory components of cells, this relatively loose mode of regulation of synthesis can confer a great degree of adaptability from the standpoint of substrate availability. On the other hand, it can lead to a wide spectrum of undesirable consequences if control falls outside of defined limits[18]. Loss of control of the regulation of oligosaccharide synthesis through radical changes in the monosaccharide pools or by induction or suppression of some glycosidases/ glycosyltransferases can have a severe impact on the cellular structure and properties of organisms, compromising these vital processes and leading eventually to diseases.

Four forces drive the glycosylation process: enzyme activity, substrate type and concentration, the availability of acceptor sites and structural/ energetic considerations. Enzyme

activity can be disrupted completely or partially as seem in many mutations or by nonspecific activity of the enzyme in the presence of unusual amounts of substrates. In this case, a substrate for which the enzyme might have a low preference is included in the product because its concentration happens to be high. Nonspecific activity based on substrate concentration and type is actually very common[19]. All carbohydrate-processing pathways are interconnected. Substrate-dependent differences in one will ultimately be reflected in many aspects of cell glycosylation affecting cell properties such as antigenicity, morphology and cell-cell interactions. The potential impact of differences between the ratio and rate of substrates that go into each pathway at any given time is explored using the ABO blood group system as an example.

The ABO blood group system is one of the best-known example of how carbohydrates play a role in the development of self-immune reactivity and underlies the need for blood compatibility during blood transfusions[20]. The simplified picture of the biosynthesis of ABO blood groups is that they are absolutes and are determined by one highly specific gene that encodes for a specific glycosyltransferase, which is responsible for the addition of either a galactose (B type) or a N-acetyl-galactosamine (A type) to the basic chain that is present in the O type (H antigen) (Figure 1.3)[11]. The structural difference between the glycosyltransferases A and B resides in only 2 amino acids, in positions 266 and 268[21].

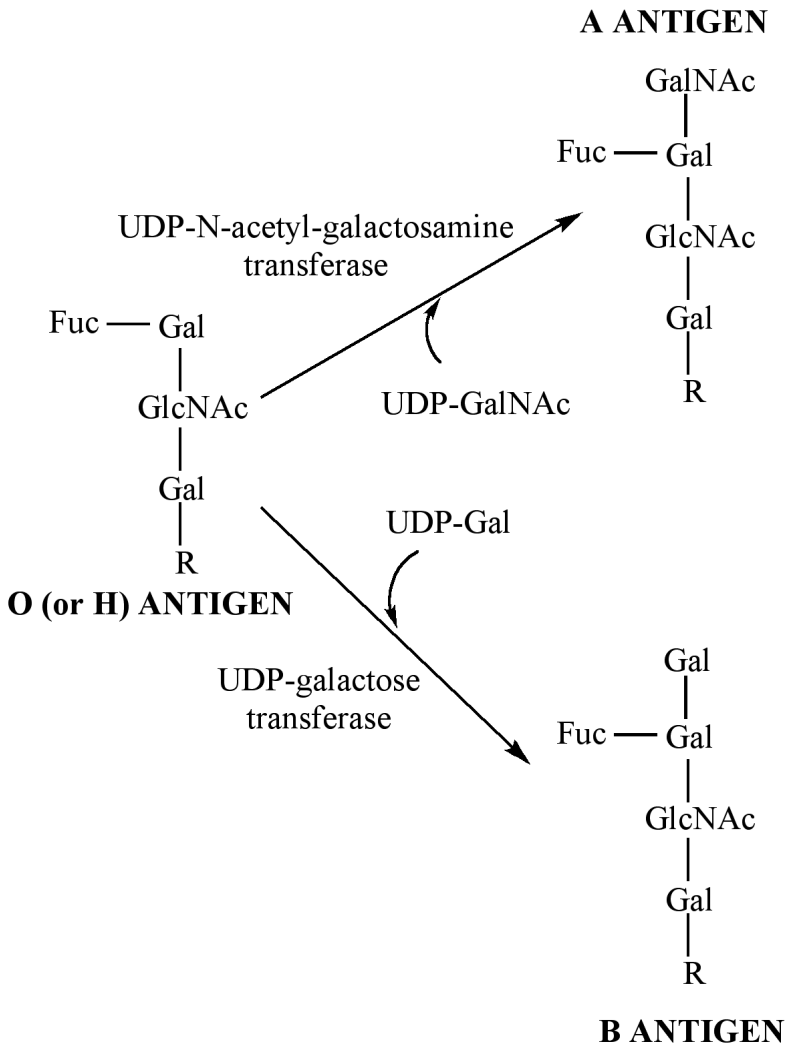

Figure 1.3. The formation of the ABO histo-blood groups. The O blood type is defined by the H antigen, which encodes for a truncated glycosyltransferase with no enzymatic activity. The A type is characterized by the addition of GalNAc to the O antigen. The B type is defined by the presence of Gal added to the H antigen.

It is now known that this high level of specificity is overstated and that there is a totally different reality with respect to the stringency with which these rules are followed. There are significant differences between expected genotypes based on heredity and phenotypes based on

actual gene expression and enzyme activity[21,22,23]. There are instances where transferases use either activated galactose or N-acetyl-galactosamine as substrate[24,25]. Another example is the B (A) phenomenon in which individuals that were genotyped as BO or BB but the serological assay indicates significant A-transferase activity due to the presence of A antigens[22]. The opposite is also seen where individuals with genotype A can synthesize B antigens[24]. The overlapping in activity between the glycosyltransferases (A and B) participating in the process of definition of these antigens is significantly frequent[26,27,28]. Therefore, changes in the concentration of the substrates provided to these cells are the main source of the generation of unexpected variability among the phenotypes. One pathway responsible for the production of carbohydrate residues that defines the differences between the ABO phenotypes is the hexosamine biosynthetic pathway (HBP). N-acetyl-galactosamine defines the group A, differentiating it from groups O and B. If the flux through the HBP is altered, we consequently expect changes in the expression of ABO antigens in the cell membrane. Galactose addition characterizes the group B epitope. The donor molecule UDP-galactose is formed by the epimerization of UDP-glucose, which in turn is formed from glucose 1-phosphate. Metabolic fluxes that lead to increases of glucose 1-phosphate (which can be formed from glucose 6-phosphate) will lead to the production of more UDP-galactose and an increase in B-epitope. This will occur if flux through the HBP or glycolysis is reduced[14,15,16].

The question of heterogeneity should be addressed here again in connection with what structurally defines the A, B or O antigen. The structures cited earlier are only mean structures. The extent of substitution varies greatly from cell to cell in the same population leading to a complex spectrum of molecules with a consensus serotype. This is well illustrated in analyses that

were performed on erythrocyte glycolipids with an O-serotype in which structures with the general formula $NeuAc_{(1)}Fuc_{(y)}Hex_{(x+2)}HexNAc_{(x)}Ceramide$ where x varied from 5 to 17 and y from 0 to 6 were identified. This leads to scores of structures based on weights of sugars and potentially hundreds based on the identity of hexose and hexosamine[15].

4. Carbohydrate Metabolism

Carbohydrates participate in many pathways within a cell. As stated in section 3, two of the main forces driving chemical reactions are (1) the concentration of substrates and (2) the activity of enzymes. Therefore, changes in the flux through the various carbohydrate pathways create changes in others that can have global implications in cellular chemistry and physiology. Here some of the major pathways that carbohydrates are involved in will be described.

4.1. Glycolysis and the Tricarboxylic Acid Cycle (TCA)

Glycolysis is the primary way of obtaining energy in all organisms. It occurs in the cytoplasm and it starts with the phosphorylation of glucose to glucose 6-phosphate. After a series of enzymatic reactions, two molecules of pyruvate are formed which can be redirected to many pathways. The two paths used for energy generation are the aerobic and anaerobic respiration. If pyruvate is used by lactate dehydrogenase, it forms lactate and two molecules of ATP are generated per glucose. This pathway is called “anaerobic respiration”. If, however, pyruvate is used through the process of “aerobic respiration”, it is then decarboxylated to acetyl-CoA by the pyruvate dehydrogenase complex (PDHC), which can enter the tricarboxylic acid cycle (TCA)[1,2]. This is followed by the reactions of the respiratory complex, resulting in a net of thirty-eight ATPs. Both TCA and respiratory chain happen in the mitochondria, more precisely in the

mitochondrial matrix and the inner membrane respectively. The aerobic pathway clearly produces more energy, but it takes a longer time and is dependent on the presence of oxygen when compared to the anaerobic respiration.

The TCA cycle is also closely related to the metabolism of amino acids, since some of them are synthesized from some intermediates of this cycle. Glutamate, for example, is a derivative of α-ketoglutarate and aspartate, a derivative of oxaloacetate. Therefore, it is not surprising that amino acid metabolism is dependent on energy-related pathways within the cell and they can actually be used as a source for energy generation or they can be synthesized from it when there is an amino acid starvation condition[29].

Many molecules involved in glycolysis and the TCA cycle are actually shared by several other pathways. Such examples are glucose 6-phosphate and glyceraldehyde 3-phosphate, which are also used by the pentose phosphate pathway (PPP); glucose that is used in glycogen and sorbitol metabolism; acetyl-CoA that is also used for fatty acid biosynthesis; phosphoenolpyruvate for sialic acid biosynthesis; fructose 6-phosphate that can be directed for the HBP, among others. Another important example of the interconnections between pathways is the participation of intermediates of the TCA cycle with general amino acid synthesis and hexosamine synthesis. Hence α-ketoglutarate from the TCA cycle is converted to glutamic acid from which glutamine is derived (amino acid biosynthesis). Glutamine is a donor of ammonia for the conversion of fructose 6-phosphate to glucosamine 6-phosphate (hexosamine biosynthesis). In another example, the glycolytic product 3-phosphoglycerate is converted to serine and then glycine, sarcosine and betaine, which can all be used as methyl donors. These methyl groups are transferred to methionine that is converted to S-adenosylmethionine, and the last one is the actual methylation agent for many processes. These are all strong illustrations of how the activities of pathways in

living systems are not isolated but actually very dependent on each other and explain why an imbalance in one pathway will affect the function of another.

4.2. Pentose Phosphate Pathway (PPP)

The pentose phosphate pathway (PPP) has two distinct parts with specific roles: the oxidative branch that generates NADPH for reductive reactions non-oxidative path, and the non-oxidative reactions, which are responsible for the production of pentoses. In animals, the pentose phosphate pathway happens in the cytosol[30]. By producing ribose 5-phosphate in the non-oxidative phase, this pathway is responsible for the synthesis of ribonucleotides and deoxynucleotides and, consequently, RNA and DNA. The production of NADPH in the oxidative phase is very important as a reducing molecule in fatty acid biosynthesis as well as for reducing glutathione. This last reaction is well known to protect the cell against oxidative stress damage[31].

PPP is closely connected to glycolysis since it produces and also uses intermediates of the last pathway. Glucose 6-phosphate, fructose 6-phosphate and glyceraldehyde 3-phosphate are intermediates that are shared by both pathways, and the enzymes reacting with them define their utilization. Therefore, the rates of glycolysis and PPP are intimately connected.

4.3. Hexosamine Biosynthetic Pathway (HBP)

The HBP is responsible for the synthesis of activated amino sugars, such as UDP-N-acetyl-glucosamine (UDP-GlcNAc), UDP-N-acetyl-galactosamine (UDP-GalNAc) and UDP-N-acetyl-mannosamine (UDP-ManNAc). In general, 2-5% of all fructose 6-phosphate in a cell go to this pathway and these molecules are used for the N- and O-glycosylation of lipids and proteins, hence conferring their functionality. The N-glycosylation is essentially dependent on the pool of

GlcNAc, since this amino sugar is always present as the two first residues of any N-glycan structure known[11,32]. Although the residues can vary in composition of O-glycans, there is a high frequency of GlcNAc and GalNAc on them[33,34]. Amino sugars are also an important component of extracellular matrices and cell walls[34].

The hexosamine biosynthetic pathway starts with the conversion of fructose 6-phosphate to glucosamine 6-phosphate through the action of an enzyme called glutamine: fructose-6-phosphate amidotransferase (GFAT, EC 2.6.1.16)[33]. This enzyme has two structural domains, one for binding glutamine (glutaminase domain) and another for the binding of fructose 6-phosphate (isomerase domain)[35,36]. This enzyme catalyzes the transfer of the amino group from glutamine to fructose 6-phosphate producing glutamic acid and glucosamine 6-phosphate. This enzyme is a thiol protease and the mechanism of action is through the formation of acyl intermediate between the cysteine residue (Cys1) and the glutamine in the glutaminase domain, releasing ammonia; the intermediate is hydrolyzed forming glutamic acid and regenerating the free enzyme. Meanwhile, in the isomerase domain, the fructose 6-phosphate is attached by its C2 to a lysine residue (Lys603) through a Schiff base reaction and it is then attacked by the ammonia produced in the glutaminase domain, recovering the free lysine and forming a 2-imine derivative. By the action of a glutamic acid residue (Glu488), the derivative is then isomerized to glucosamine 6-phosphate, the product of the overall reaction[36]. After that, glucosamine 6-phosphate is acetylated, isomerized and combined to a UDP to form UDP-GlcNAc and other hexosamines. Figure 1.4 illustrates the overall pathway.

As seen in Figure 1.4, the HBP also has major points of overlap with glycolysis, the TCA cycle and the PPP. It stands to reason that perturbations due to elevated or much reduced amounts of certain substrates in these pathways can affect the rate and ratio of sugars utilized for amino sugars biosynthesis. Since the HBP is a major provider of intermediates (amino sugars) for the glycosylation process, imbalances in other pathways can indirectly affect the biosynthesis of glycoforms.

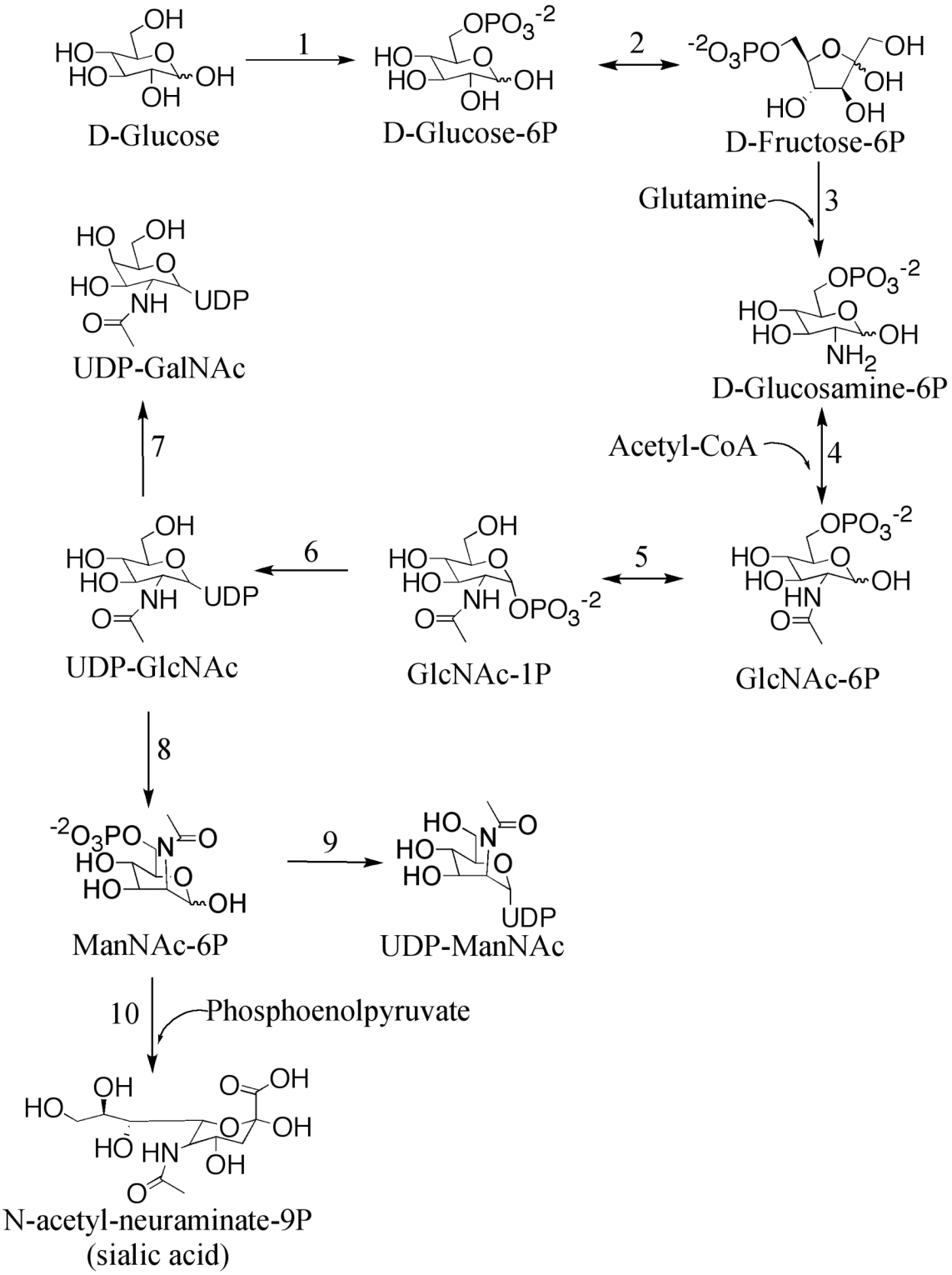

Figure 1.4. Hexosamine biosynthetic pathway. Legend for the enzymes: 1. Hexokinase; 2. Phosphoglucose isomerase; 3. Glutamine: Fructose-6-phosphate amidotransferase; 4.

Glucosamine-6-phosphate acetyltransferase; 5. Phosphoacetylglucosamine mutase; 6. N-acetylglucosamine pyrophosphorylase; 7. UDP-galactose-4-epimerase; 8 and 9. UDP-*N*-acetylglucosamine 2-epimerase; 10. Sialic acid synthase. GalNAc: N-acetyl-galactosamine; GlcNAc: N-acetyl-glucosamine; ManNAc: N-acetylmannosamine 6-phosphate

Furthermore, the HBP also provides amino sugars that are heavily incorporated in glycosaminoglycans (GAGs), such as heparin and chrondroitin sulfate, and various mucins[6]. These GAGs are important components of the extracellular matrix, and complications in their biosynthesis or maintenance are related to the development of rheumatoid arthritis and Alzheimer's disease. A growing amount of evidence also indicates the participation of the HBP in development of Type II Diabetes too[37,38]. As underlined earlier, the substrate concentrations and types on the various pathways can affect the other routes of carbohydrate metabolism, including this one (HBP). Since this route is closely related to glycolipids/ glycoproteins biosynthesis, disequilibrium in the HBP would strongly affect the biosynthesis of glycoforms and, therefore, proper cell function.

5. Disorders of Glycosylation

5.1. Genetic Based Disorders

5.1. 1. N-glycosylation related diseases: Congenital Disorders of Glycosylation (CDG)

Defects in glycosidases and glycosyltransferases functions are described in many diseases. Mutations in specific glycosidases can lead to accumulation of large amounts of sugars resulting

in disease (e.g. Gierke, Pompe, Forbes, Tarui's Diseases)[39,40]. Other mutations in either glycosidases or glycosyltransferases can result in hypoglycosylation or hyperglycosylation of N-glycans, resulting in the so-called congenital disorders of glycosylation (CDG). The CDG comprise 18 diseases, each caused by a mutation in a gene encoding for an enzyme related to N-glycan assembly, processing, activation or transport. Table 1.1[40] summarizes the type of CDG and the defective enzyme in each case.

Table 1.1. Summary of the Congenital Diseases of Glycosylation.

Type	Defective Enzyme
Ia	Phosphomannomutase
Ib	Phosphomannose isomerase
Ic	Dol-P-Glc: $Man_9GlcNAc_2$-PP-Dol glucosyltransferase
Id	Dol-P-Glc: $Man_5GlcNAc_2$-PP-Dol mannosyltransferase
Ie	Dol-P-man synthase I GDP-Man: Dol-P-mannosyltransferase
If	Mannose-P-dolichol utilization defect 1
Ig	Dol-P-Glc: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase
Ih	Dol-P-Glc: $Glc1Man_9GlcNAc_2$-PP-Dol glucosyltransferase
Ii	GDP-Man: $Man1GlcNAc_2$-PP-Dol mannosyltransferase
Ij	UDP-GlcNAc: dolichol phosphate N-acetylglucosamine-1-phosphate transferase
Ik	GDP-Man: $GlcNAc_2$-PP-Dol mannosyltransferase
Il	Dol-P-Man: Man_6 and $_8$ $GlcNAc_2$-PP-Dol mannosyltransferase
IIa	GlcNAcT-II
IIb	Glucosidase I
IIc	GDP-fucose transporter
IId	B14- galactosyltransferase
IIe	COG complex, subunit 7
IIf	CMP-sialic acid transporter

The CDGs type I result in hypo-glycosylated proteins and lipids due to incomplete or insufficient lipid-linked oligosaccharide precursors that are not efficiently transferred to the target molecules[41]. In all of them, the recurring phenotype presents neurological problems, hypotonia and frequent infections. Neurological problems are expected since the glycosylation process in the

brain is essential for many neuronal properties, such as neuronal growth, morphology and for the learning and memory formation processes.

The propensity of individuals to succumb to infections has also been tied to genetic disorders in carbohydrate metabolism. In CDG IIf, for instance, the impairment of sialic acid transport between Golgi and plasma membrane impairs the sialylation of many proteins. Individuals then lack sialylated antigens, such as sialyl-Le^X and sialyl-Le^A, which are important for cell-cell interactions. This leads to infections due to elevation of circulating lymphocytes and megakaryocyte immaturity[42].

5.1.2. O-Glycosylation related diseases: Congenital Muscular Dystrophies

Congenital muscular dystrophies are another class of glycosylation defects related to incorrect production or assembly of O-glycans. Most of them consist of the incorrect addition of O-mannose and O-xylose to proteins, of which the best example is the incorrect addition of O-mannose to α-dystroglycan. This protein is one of the subunits of the dystrophin glycoprotein that connects the extracellular matrix to the cell cytoskeleton. The phenotype is mainly characterized by defects in the muscular tissue connections. Many other examples are related to the incorrect addition of O-xylose to GAGs, leading to a disruption of their proper biosynthesis. That causes several phenotypes related to cartilage and bone abnormalities[41].

5.2. Diseases connected to disequilibrium in the cell metabolic status

Many diseases are correlated to the metabolic status of the cell, rather than to only a specific mutation in a gene of an enzyme from the glycosylation pathway. In these diseases, there is actually an imbalance in the levels of substrates that flow through the various carbohydrate

pathways, leading to an improper excess of certain sugars resulting in an impact in cell metabolism, physiology and antigenicity. Type II diabetes, Alzheimer's disease, rheumatoid arthritis and cancer are among these type of disorders and they have been correlated to the high concentrations of particular carbohydrates, such as glucose and fructose[43,44,45]. I will present a brief introduction of the correlations between these diseases and impairment in sugar pathways.

5.2.1. Diabetes

Type 2 Diabetes (T2D) is a metabolic syndrome that can be developed by the exposure to high concentrations of certain sugars (glucose and fructose) for a long period[46]. Dietary sugars are not metabolized properly due to improper function of the pancreatic β-cells[47]. T2D has been correlated to a breakdown in several energy-related pathways in these cells, including the flow of glycolysis through the TCA cycle when the presence of certain sugars is unusually high. In healthy β-cells, the TCA cycle contains high activities of pyruvate dehydrogenase (PDHC) and carboxylase complexes and malate-aspartate shuttle, both to ensure high levels of oxidative phosphorylation. ATP citrate lyase, which converts citrate to acetyl-CoA, is also very active to promote a regular level of fatty acid synthesis. These and other events promote adequate secretion of certain factors, such as insulin, by the β-cells[48]. However, if the TCA cycle is inhibited, diabetic features start to develop[49]. Among the many TCA-related imbalances seen in diabetes are: lower mitochondrial isocitrate concentrations, mutations in the succinate dehydrogenase B gene, higher 2-oxoglutarate dehydrogenase and UDP-glucose pyrophosphorylase levels when compared to treated-diabetic patients[50]. In addition, low hexokinase, pyruvate kinase and malate

dehydrogenase levels are also reported, which are all necessary in high concentrations for proper β-cell function[51]. These observations demonstrate that diabetes is characterized by a persistent state of non-oxidative respiration in the β-cells.

Another disorder usually linked to T2D is obesity. In obese patients, a low TCA cycle flux is also observed, which leads to an incomplete fatty acid oxidation, causing the fatty acids to be accumulated. In healthy β-cells, high pyruvate carboxylase and dehydrogenase (PDHC) activities are required for function. When high and chronic exposure to high levels of fatty acid is present in β-cells (as in obesity), the level of PDHC activity is decreased, which might also explain why obesity can lead to diabetes[52]. This high influx of carbohydrate into glycolysis that is not consumed by the TCA cycle is then redirected to other pathways, such as the PPP and HBP. Many studies show that there is an increased flux through the HBP in diabetes[53] and its association with insulin resistance[54]. Sustained increase of O-GlcNAc is a major feature of T2D, which affects the function of many cytoplasmic proteins[55,56]. Type 2 diabetes is, therefore, a metabolic syndrome that consists of alterations in the many carbohydrate pathways, with the TCA cycle being impaired and PPP and HBP being exacerbated.

5.2.2. Alzheimer's Disease (AD)

Another disease of carbohydrate metabolism is Alzheimer's. As most neurodegenerative disorders, it affects mainly older people and causes dementia. Most studies characterize this disorder by the presence of neurofibrilary tangles, accumulation of the β-amyloid protein and hyperphosphorylation (and consequent hypoglycosylation) of the tau protein, all in the brain[57-62].

Many of these features seem to be correlated to imbalances on the flux through the different carbohydrate pathways.

In AD and other neurodegenerative disorders, a reduced TCA cycle activity is also observed. This is probably the reason for the decreased metabolism that accompanies clinical AD. The activity of many enzymes involved in the TCA cycle such as PDHC, isocitrate dehydrogenase and the α-ketoglutarate dehydrogenase complex are significantly reduced[63,64,65]. The enzymatic activity of the second part of the TCA cycle, on the other hand, is increased, probably as compensatory response to the reduction of activity on the first half of this cycle. PDHC phosphorylation is controlled by tau protein kinase, which is hyper-activated in this disease as we can see by the hyper-phosphorylation of tau protein. Phosphorylation causes the PDHC to be inactivated, which may also explain the reduced flow through the TCA[66]. These imbalances in mitochondrial energy production also elevate the free radical production, which is very prominent in AD[66].

One of the most studied features in AD is the formation of the β-amyloid. The accumulation of this protein shifts cell metabolism from aerobic to anaerobic, a fact that apparently coincides with low TCA cycle activity in the neurons. The β-amyloid accumulation reduces oxygen supply, promotes insulin resistance in the brain, leads to accumulation of pyruvate and lactate, decreases the α-ketoglutarate dehydrogenase complex activity and increases succinate dehydrogenase complex II activity[66,67]. All these complications induce an increase in the flux to the PPP in an effort to obtain energy through NADPH synthesis in the neurons[67,68]. In summary, like T2D, neurons affected by AD also contain a non-functional TCA cycle and a higher PPP activity.

5.2.3. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease associated with joints in which the concentration of glycosaminoglycans (GAGs) is elevated[69]. The elevation of these polysaccharides consisting of aminosugars and iduronic acid causes a chronic inflammation of these tissues.

Evidence also shows that mitochondrial activity (TCA cycle) is decreased while other pathways are increased in arthritis. Joint areas in RA are usually hypoxic, which is again compatible with decreased TCA cycle activity[70]. On the other hand, activity of the PPP is highly elevated. This observation is noted by the activity of glucose-6-phosphate dehydrogenase (an enzyme of the PPP), which is four times higher in rheumatoid synovial cells than of healthy cells[71]. Increased PPP activity is also observed in other autoimmune diseases from connective tissues[72].

Due to redirection of intermediates from the inefficient TCA cycle to other pathways, it is reasonable to imagine that other routes than the PPP would be affected. There are indications that the flux through HBP is also deregulated in autoimmune disorders. The HBP can modulate the branching of N-glycans of T lymphocytes by regulating the supply of GlcNAc. N-glycan branching in T cells is directly related to the suppression of autoimmunity by suppressing TCR sensitivity and Th1 differentiation[73]. This whole cascade of events could contribute to the development of an autoimmune disease like the one in question. Modifications in hexosamine production could also result in disruption of this matrix, causing cells to be weakly supported and, therefore, subject to changes in cell morphology and adhesion[11,74,75].

5.2.4. Cancer

One of the most remarkable characteristics of cancer is accelerated cell growth, which is closely related to the cells' ability of producing more energy to overgrow healthy cells. The most important energy generating pathways are glycolysis and the electron transport chain, which derives most of its reducing power from the TCA cycle. Glycolysis is one of, if not the most, up-regulated pathway in cancer. Evidence supporting this fact is the up-regulation of many genes encoding for glycolytic enzymes. In pancreatic cancer, some of the most highly expressed genes are pyruvate kinase, hexokinase II, and fructose-1,6-bisphosphatase. The over expression of glycolytic enzymes (HPI, PFK1, GAPDH, PGK, ENO), especially hexose phosphoisomerase, is associated with enhanced cell motility, which is linked to the metastatic potential of the tumor in question[76,77]. Some of the frequently altered genes, such as Ras and p53, can alter the rate of glycolysis[78]. In the next generation of anticancer drugs, one important class that is gaining more focus is the anti-glycolytics[79]. There is epidemiological evidence showing a possible connection between an increase in sugar consumption and the susceptibility to breast[80,81], stomach[82], pancreatic[83,84], ovarian[85,86], upper aero digestive tract[87], endometrial[88], and colon or colorectal[89,90,91] cancers.

The up-regulation of glycolytic enzymes is directly connected to the phenomenon called the "Warburg effect", which describes the metabolic state in which cancer cells have high rates of glycolysis and low flux through the TCA cycle even in the presence of oxygen. In 1930, Otto Warburg[92] suggested that some defect in the mitochondrial machinery was the reason why cancer cells do not use glucose for oxidative respiration when oxygen is available. This has been

challenged but not disproved. Many hypotheses have been postulated to explain the Warburg effect. One is that the cancer cells need a high rate of energy production in a shorter period of time to meet the requirements for building more blocks for proliferation and survival. A second one is that high rates of glycolysis and low rates through the mitochondrial machinery would reduce the oxidative stress for the cancerous cells. A third one is that the high amounts of lactate (formed by anaerobic metabolism) would provide an acidic environment to cancer cells and protect them from the immune system. A fourth one is that high glycolytic rate protects cells from death even when survival factors withdraw[93].

The pyruvate formed in cancer cells is converted to acetyl-CoA and sent to the cytosol for fatty acid biosynthesis[94]. Very little activity is observed in the TCA cycle. Many mutations in genes encoding enzymes of this cycle are observed in cancer. Mutations in the succinate dehydrogenase complexes (SDHB, SDHC, SDHD), for instance, can cause diverse types of paraganglioma, which is a tumor in the carotid body, a chemoreceptive organ that senses oxygen levels in the blood[95,96,97]. Mutations in the fumarate hydratase enzyme are found in fibroids, renal carcinoma and uterine leiomyomas[98,99]. These facts are also compatible with the fact that many regions of mitochondrial DNA harbor mutations related to different cancers[100]. The respiratory chain is also affected by TCA mutations since heme biosynthesis is directly dependent on succinyl-CoA concentration, which is the precursor of the porphyrin ring on the heme molecule[101].

Since the TCA cycle is profoundly affected in cancer and has a very important role in amino acid biosynthesis, the cellular amino acid profile is expected to be altered in this disease.

Indeed amino acid profiles are altered in cancer cells. In normal cells, alanine and glutamine are responsible for 40 to 70% of all amino acids converted to glucose[102]. Tumor cells, however, can use a lot more glutamine as an energy source than a normal cell through a process called glutaminolysis[103]. High concentrations of certain amino acids made by tumors, such as glutamine, alanine and glycine are able to contribute to the suppression of the immune function[104].

Another pathway related to the physiology of cancer is the PPP[32,105]. Since glycolysis and PPP share many intermediates, a higher flux through glycolysis also causes a higher influx of intermediates to the PPP, generating more ribose and, consequently, a higher level of nucleic acid biosynthesis. The observation that cancer cells contain a higher DNA content than normal cells corroborates this fact. Other effects of perturbations of enzyme activities on the glycolytic pathway are also connected to a higher flux to PPP[32,106]. For instance, higher activities of hexokinase, as well as lower activities of pyruvate kinase and enolase, cause an accumulation of glucose 6-phosphate and fructose 6-phosphate in the cell, leading to higher concentrations of these metabolites that are redirected to PPP. Another enzyme with abnormal function is the thiamine-dependent transketolase, which is also an important component of the PPP[107].

The commonality of defects in the TCA cycle with the occurrence of cancer is to be expected. If pyruvic acid, the product of various carbohydrate processing pathways, is converted to lactic acid or fatty acids rather than catabolized to give carbon dioxide, there will be a buildup of intermediates and some pathways will run in reverse. More sugars will be available for modification and for conversion to nucleotides and amino acids. More cell mass will be produced. More aberrant glycosylation will result. More modifications of glycoproteins, lipids and other

carbohydrates by the large amount of un-catabolized products will be observed. More DNA will be made leading to abnormalities in chromosome structure and number. New antigens will occur on cell surfaces. New glycosyl sequences (cancer markers) will appear in serum and cell surfaces. This is the very essence of cancer.

The HBP is also altered in cancer. Abnormal high serum hexosamine levels have been reported in patients with neoplastic diseases, with that being even higher in patients with metastases[108]. Since the HBP provides amino sugars for the glycosylation processes, it makes sense that the increased flux through it is associated with higher O-glycosylation in some cancers, such as in chronic lymphocytic leukemia[109]. One of the most important changes that influence the severity of pancreatic cancer is the increased extension of the glycosylation patterns in mucins, especially in the terminal GlcNAc or GalNAc[110,111].

An enormous body of evidence is available demonstrating that altered glycosylation patterns are an important feature in cancer cells, which is a reflection of the higher flux through the carbohydrate pathways, especially HBP. Most cancer-associated markers, for instance, are carbohydrate antigens. Examples are CA 15.3, CA 19.9, CA 50, CA 125, CA 242, MCA, sialyl-Lewis^X, sialyl-Lewis^A and CEA. They usually contain aberrant patterns of syalilation, fucosylation and addition of galactose, GalNAc and GlcNAc[112]. These markers are found in the vast majority of cancers[113,114], and it is clear the contribution of amino sugars to the composition of these aberrant antigens. The composition of many other antigens that are also dependent on GlcNAc and other amino sugars are also altered in cancer. One such example is the ABO antigens[112,115]. In some carcinomas the loss of ABO antigens is correlated with a higher chance of metastasis for

bladder[116] and oral[117] cancers. In others, the presence of particular blood groups actually has a positive correlation. For instance, type A is associated with a higher risk of pancreatic cancer[118,119], breast cancer[120], gastric cancer[121], craniopharyngioma[122], colon carcinoma[123] while the group O lower risk. In another study, the opposite is actually true, with central nervous system lymphoma[124] and leukemia[125] being the lowest in blood group A.

N-acetyl-neuraminic acid (sialic acid) is another carbohydrate structure highly altered in cancer and dependent on the HBP flux. In general, tumor cells contain high amounts of this amino sugar. Sialic acid biosynthesis results from the condensation of N-acetyl-mannosamine 6-phophate (ManNAc 6-P) originated from HBP and phosphoenolpyruvate (PEP) derived from glycolysis. The amount of ManNAc 6-P and PEP available for the synthesis of these antigens are, therefore, vital for proper function of the cell. Sialic acid content is directly linked to cellular adhesion and migration[126]. Some studies positively correlated the degree of invasiveness of cancer cells with the presence of sialic acid containing-antigens[127,128,129]. The most studied antigens are the sialyl-Lewis^{A} and sialyl-Lewis^{X}. They play vital functions in the adhesion process of cancer cells to vascular endothelium[130,131], which makes them very important for the establishment of the metastatic potential in many types of cancer[132,133,134].

5.3. Interconnections between Pathways and Disease

It is important to highlight that the rate and ratio of activity of one pathway can directly or indirectly affect many other pathways. It was commented, for example, that many diseases contain an impaired TCA cycle. Since this cycle is directly connected to glycolysis, amino acid and amino

sugar biosynthesis, as well as pentose phosphate formation, it is expected that all these other routes will be affected by this lack of function. When the flux through the TCA cycle is suppressed, cells may redirect its substrates to other energy-related pathways, such as PPP, to supply ATP for anabolic processes. This increased flux through PPP, for instance, also produces more ribose 5-phosphate, a pentose involved in nucleotide biosynthesis. Higher concentrations of nucleotides available in the cell can increase the rate of DNA and RNA biosynthesis, which can affect the cell growth. The upregulation of PPP can also upregulate glycolysis since they are also connected to glycolysis by the two of the end products of PPP, fructose 6-phosphate and glyceraldehyde 3-phosphate. Higher concentrations of fructose 6-phosphate produced in the non-oxidative phase of the PPP can also enter glycolysis.

A build up in fructose 6-phosphate can also affect amino sugar biosynthesis. This build up influences all glycosylation patterns within the cells, since more hexosamines are being produced and attached to glycoforms such as membrane antigens. High amounts of pyruvate produced by glycolysis can then be condensed with ManNAc 6-P to form sialic acid, when it is not consumed by the TCA cycle. Sialic acid-containing antigens will also be affected. High concentrations of glycolytic intermediates also affect fatty acid biosynthesis by the formation of high amounts of acetyl-CoA. Another pathway directly affected by TCA cycle suppression is amino acid biosynthesis. The consequences might be the build up of some amino acids and lack of others.

It is observed that many of the imbalances related here are found in the metabolic disorders mentioned. This observation reinforces that higher flux through certain carbohydrate pathways due to blockage or efficient consumption by one route can be extremely harmful for the cell metabolism.

6. Hyperglycosylation: Advanced Glycation End (AGE) Products

Hyperglycosylation is a very common modification of diseases of carbohydrate metabolism that can lead to the formation of Advanced Glycation End products (or AGE products). The availability of higher amounts of sugars, resulting from an excess influx of their substrates through particular carbohydrate pathways, leads to hyperglycosylation. AGE products are formed by a non-enzymatic reaction between reducing sugars and proteins, lipids or nucleic acids. A reducing sugar, such as glucose, galactose, fructose or ribose forms an Schiff base with amino groups of nucleic acids, proteins or lipids resulting in "Amadori products". These products undergo a rearrangement, creating a permanent linkage between the sugar and the attacked molecule[160]. The rate of formation of these products varies according to the sugar. For instance, fructose is ten times more reactive in inducing the formation of AGE products than glucose[135].

AGE products can especially affect long-lived proteins, such as the ones participating in the extracellular matrix structure and function, producing non-functional molecules that are very resistant to degradation. This can affect tissue turnover, which leads to changes the overall structure and function of the tissue, and consequently cell structure and metabolism[135,136,137].

AGE accumulation is observed in T2D and in plaque formation in AD[138]. A person with T2D has two to five times more chance to develop AD, probably due to the natural increase in AGE products in diabetes[135]. AGE products are also present in human cancers, such as breast, colon and larynx[139] cancers.

Hyperglycosylation is, therefore, an important change of glycosylation patterns that is mainly affected by an excess of activated sugars available for this process. Since most metabolic

diseases related to carbohydrates are linked to hyper-activation of certain pathways (PPP and HBP) and hypo-activation of others (TCA cycle), it is reasonable to imagine that AGE products should feature in metabolic diseases such as T2D, AD and cancer.

7. Fructose: a Pivotal Carbohydrate in Metabolic Diseases

There are many arguments underlining the importance of carbohydrate metabolism on the development of metabolic diseases. Many studies correlate their appearance with the consumption of certain sugars, and one of the most studied is fructose due to the intake of high fructose corn syrup (HFCS). HFCS has a major presence in the modern diet and it is estimated to provide, on average, 10.2% of our daily energy intake[140]. HFCS is present in sweetened beverages, juices and in a variety of pre-packed foods. In 1970, the individual consumption of fructose was 0.5 lb/ year. In 1997, it rose to 62.4 lb/ year[141].

Epidemiological studies show strong evidence linking the consumption of sweetened beverages with increased body weight (adiposity) and occurrence of metabolic and cardiovascular disorders[142,143]. Many studies also support this data by showing that high-fructose-fed hamsters developed higher plasma triglycerides, low density lipoprotein and uric acid concentrations and decreased high density lipoprotein cholesterol[144,145], which are all risk factors for cardiovascular disorders. Apparently fructose induces lipogenesis more than other sugars, which explains why higher levels of cholesterol are found in these studies. The American Heart Association also considers fructose intake an important risk factor on the development of chronic neurodegenerative disorders, such as dementia, by directly causing it or by inducing the panel of metabolic disorders that accompanies it[146].

The dramatic increase in the incidence of T2D also coincides with the start of commercialization of HFCS[147]. Epidemiological studies show a strong correlation between the increased risk of T2D and the ingestion of HFCS, which is very important evidence to show that fructose can be directly involved in the development of T2D[148,149].

HFCS consumption and risk of pancreatic cancer are also positively associated[115]. Since HFCS is related to obesity and cancer, it is plausible that people who have a high consumption of HFCS and are obese are at increased risk of developing certain cancer types, such as adenocarcinoma of the esophagus, colon, breast, endometrial, kidney, liver, gallbladder and pancreatic cancers[149,150]. In cancer cells, fructose is mostly used by the PPP in order to generate energy and pentoses[5,151,152]. The PPP is increased by 250% in tumor cells at the expense of glycolysis when fructose is the carbon source[153]. An elevation of the activity of the PPP is congruent with the higher rate of nucleic acid biosynthesis observed in pancreatic cancer cells[152,153] and fibroblasts grown in fructose[150].

Another pathway affected by high-fructose consumption is the HBP. Higher fructose concentrations create a higher flux through the HBP, since the HBP starts with the amination of fructose 6-phosphate to glucosamine 6-phosphate. The higher flux through the HBP also increases the concentration of amino sugars, causing aberrant patterns of glycosylation, including in the cell surface antigens[154]. Cells in fructose have antigens richer in sialic acid, which causes major changes in adherence and invasiveness properties of these, with cells to be more adherent to the endothelium, as well as more invasive compared to cells grown in glucose[155].

Besides the considerable changes that high concentrations of fructose have on the different metabolic pathways, fructose and fructose 6-phosphate are also observed to cause DNA damage. When cells are exposed to different forms of glucose and fructose, fructose 6-phosphate causes the highest frequency of mutation in the thymidine kinase gene and the highest amount of single stranded breaks, followed by fructose. Another study also showed that fructose 6-phosphate triggers total DNA degradation after 3 days of exposure[156].

All these observations reinforce the great impact that high concentrations of fructose from diet can have on shifting cell metabolism, structure and physiology. Higher concentrations of fructose will lead to an increase in fructose 6-phosphate, which will augment the flux through many pathways, such as PPP and HBP. It is important to highlight that increase in the flux through the mentioned pathways are described in many metabolic diseases, which shows that the high amounts of fructose intake by in diet is an important risk factor in the development and establishment of these disorders.

The scope of this work

My overall hypothesis was that the type of sugar used as carbon source by cells can modify the relative flux through glycolysis, pentose phosphate and hexosamine biosynthetic pathways, resulting in shifts in a variety of cellular properties. This was tested by perturbing the relative flux through these three pathways with different carbon sources (fructose and glucose) or by the addition of competitive inhibitors (glucose 6-sulfonate and glucosamine 6-sulfonate) of these carbohydrate pathways. In order to show that different carbohydrate sources can modify a variety of cellular characteristics, my specific hypotheses were:

1. Treating cells with fructose instead of glucose would increase the flux through the hexosamine biosynthetic pathway, leading to an increase of hexosamine-containing antigens. The ABO blood group antigens were tracked as a sample of antigens that are dependent (A antigen) or independent (B and O antigens) of hexosamine addition for their characterization. Fluorescent-labeled lectins were used to probe these antigens by flow cytometry. Carbohydrate pool analysis (relative proportion of hexoses/hexosamines) was also used to show a relative increase of hexosamine production in fructose-fed cells in comparison to glucose-fed cells.

2. Treating cells with fructose would increase the flux through to biosynthetic processes in comparison to glucose, such as the metabolism of glycine and its derivatives. Microarray analysis was performed to show that genes correlated with glycine metabolism were increased significantly in fructose-fed cells in relation to glucose.

3. Treating cells with glucose 6-sulfonate would decrease the flux through glycolysis and the pentose phosphate pathway by competitive inhibition with glucose 6-phosphate. ^{13}C-NMR analysis was used to show which pathways glucose 6-sulfonate can participate in and enzymatic assays to demonstrate that glucose 6-sulfonate is a competitive inhibitor of glucose 6-phosphate dehydrogenase, an enzyme of the PPP. The ability of this inhibitor to decrease cell growth by inhibiting these pathways was also tested in cancer cells.

4. Treating cells with glucosamine 6-sulfonate would decrease the flux through the hexosamine biosynthetic pathway by competitive inhibition with glucosamine 6-phosphate. Chemically synthesized glucosamine 6-sulfonate was tested by its ability to inhibit glucosamine formation in

bacteria (by analysis of its cells walls), in enzymatic assays and in cancer cells mainly by competitive competition to glucosamine 6-phosphate.

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CHAPTER 2

CHAPTER 2

ALTERATIONS IN CELL SURFACE GLYCOCHEMISTRY, CARBOHYDRATE POOLS AND MORPHOLOGY IN MAMMALIAN CELLS CULTURED IN HIGH CONCENTRATIONS OF FRUCTOSE.

Abstract

Fructose is a major monosaccharide source in our diet and its high intake has been correlated with the development of many metabolic disorders. This has increased the concern about the effects that this sugar has on eukaryotic cellular properties. In this study, mammalian cell lines with very distinct metabolic properties (fibroblasts and endothelial cells) were grown with either glucose or fructose as carbon sources and their cell surface glycosylation (ABO blood group antigens), carbohydrate pools (hexoses and hexosamines) and morphological characteristics were analyzed. It was found that cell surface glycosylation (antigenicity), carbohydrate metabolism and cell morphology are significantly influenced by the primary carbohydrate source in the growth media and also correlated with the cell metabolic level. Metabolic changes were also reflected in qualitative and quantitative changes in the composition of free and bound carbohydrate pools. Cells have an increased production of mannose 6-phosphate and hexosamines when grown on fructose rather than glucose. This increase in hexosamine production is reflected in a higher level of expression of blood group A antigens (containing N-acetyl-galactosamine). Cells grown in fructose also present distinct morphological features when compared to cells grown in glucose, such as increase in size and granularity properties. This study adds to our knowledge of the fundamental biochemical connections

between the amount and type of carbohydrate intake and its metabolic consequences on eukaryotic cells.

Introduction

There are several biochemical links correlating the concentration and type of hexose to which cells or organisms are exposed and the metabolism of carbohydrate-related pathways they utilize[1,2]. For instance, many aspects of metabolism are affected when cells are exposed to high concentrations of fructose. These observations have been mainly fueled by the significant increase in human consumption of fructose over the past 30 years[3]. The consequence of this increase in fructose intake has been correlated with a higher incidence of metabolic diseases, such as T2D[4,5], AD and other neurological disorders[6,7], cardiovascular and lipidic disorders, obesity[8-12] and cancer[13,14]. Some of these studies correlate the presence of the disease with alterations in the flux of fructose through various metabolic pathways. For example, in pancreatic cancer cell lines, fructose induces a higher flux through the PPP supporting energy generation and nucleotide biosynthesis[15]. In other cell lines, growth on fructose causes a faster rate of cell division[16,17,18]. Tumorigenic properties such as rapid cell growth, higher degree of migration and invasiveness are also more prevalent when fibroblasts are grown in fructose in contrast to glucose[19,20]. Growth of cells in high concentrations of fructose has also been shown to result in DNA damage, especially in genes involved in nucleotide biosynthesis[21]. These observations highlight the importance of understanding the biochemical and biological outcomes that result when fructose is an abundant sugar source.

Some of the observations of the effects of fructose on metabolic pathways can be ascribed to the reactivity of this molecule in certain enzymatic reactions. When low amounts of fructose are ingested, this sugar goes to the liver and is phosphorylated to both fructose 1-phosphate and fructose 6-phosphate. However, when a high load of fructose is ingested, this sugar is also absorbed and processed by extra-hepatic tissues, in which fructose enters the cell and is rapidly phosphorylated to fructose 6-phosphate[22,23]. In both situations, the phosphorylation of fructose is extremely fast (hexokinase can act 11 times faster on fructose than on glucose[22,24]), which increases the instantaneous concentrations of fructose 6-phosphate to be utilized by the various carbohydrate pathways within the cell. This is especially crucial in tissues that absorb fructose in considerable amounts, such as liver, adipocytes, brain, sperma, muscle and pancreas[22-27]. Fructose phosphorylation in the liver is so fast that an ingestion of high amounts of fructose causes a severe, acute depletion of ATP levels. This depletion stimulates the *de novo* biosynthesis of purines in order to replenish ATP levels, which is accompanied by an increase in uric acid production that is well described in the presence of high amounts of this monosaccharide[15,22,23,24]. Glucose has a slower rate of phosphorylation in comparison to fructose, causing the available concentration of glucose 6-phosphate to be lower in comparison to fructose 6-phosphate at any given moment. This is an important concept to understand why fructose and glucose can differentially increase the flux through certain carbohydrate processing pathways.

Although significant evidence exists to indicate that cells fed fructose instead of glucose have changes in metabolic pathways, there is a gap in our knowledge of how fructose affects glycosylation of lipids and proteins that play roles as cell surface antigens. In this study the

effects that high concentrations of fructose have on general carbohydrate metabolism, especially on the biosynthesis of hexosamines and other hexoses, were evaluated. The primary focus was to identify and characterize the major metabolic connections that determine how the complex carbohydrate antigens that define cell surface chemistry are formed when fructose is the primary carbon source. An important consideration in this study was the choice of a group of carbohydrate antigens whose changes we could easily track. I chose to monitor the ABO blood group antigens, which are the best-characterized surface membrane antigens for evaluating the bridge between carbohydrate metabolism and surface glycochemistry. The A-antigen varies from the its O-counterpart by having an additional GalNAc residue. The B antigen has an additional galactose residue compared to the O structure[28]. The working hypothesis was that increased hexosamine production should lead to higher expression of A antigen. The B antigen, on the other hand, is likely to be expressed if conditions are conducive to the formation of galactose from the isomerization of UDP-glucose. Fluorescent-labeled lectins specific for each blood antigen were used in this study to facilitate their identification by flow cytometry.

A picture of the overall carbohydrate metabolism during utilization of the different carbon sources was obtained to identify and distinguish the free or simple sugars (such as simple glycosides in metabolic pools) from those tied up in complex glycoconjugates (glycoproteins and glycolipids). Mouse embryonic fibroblasts (MEF) and mouse brain endothelial (bEnd.3) cells were used for these studies. These are cell lines with markedly different properties. The first one is metabolically very active, producing and secreting a large amount of extracellular material[29]. The bEnd-3 cell line is very quiescent with low turnover and metabolism[30]. Together they allow

an examination of the impact that the carbohydrate source has on cell glycochemistry, carbohydrate pools and morphology from two extreme ends of the metabolic spectrum.

Materials and Methods

1. Cell Lines, Culture Conditions and Cell Growth

MEF, MDCK, BNL CL.2 and bEnd.3 cell lines were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids solution and 1% penicillin/ streptomycin in a 37ºC incubator with 5% CO_2 enrichment. Cells were cultured in either 4.5 g/L of fructose or glucose for 1, 5, 30, 60 and 120 days and collected for analyses. All reagents were obtained from Gibco (Invitrogen) except for fructose, which was obtained from Sigma-Aldrich. Cell growth and doubling time were monitored by cell counting in an inverted microscope. For the purpose of exemplifying the ends of the data spectrum, only the results obtained after 120 days of treatment from MEF and bEnd.3 cells are shown in this study.

2. Evaluation of Alterations of Carbohydrate Cell Surface Antigens

Flow cytometry analysis was performed on a BD FACS Vantage SE (BD Biosciences) instrument equipped with an argon laser (488 nm). The lectins used were selective for the A, B and O antigens (*Griffonia simplificifolia* isolectin B_4 – Antigen Group B; *Helix pomatia* – Antigen Group A; *Ulex europeaus* – Antigen Group O). Lectins for groups A and B were labeled with FITC (excitation 488 nm; maximum emission 521 nm) and the lectin for group O with PE-Cy5.5 (excitation 488 nm; maximum emission 667 nm) fluorochromes. All lectins were obtained from Vector Laboratories. Each assay was performed on 10,000 cells. The fluorescence profiles

were obtained using the FACS Diva software and the results were displayed using histograms of population distribution of counts (number of cells with a particular fluorescent intensity) *versus* fluorescence intensity, which was represented on a scale of 0 to 10^5 arbitrary fluorescent units. These population distribution curves were deconvoluted such that the curve derived from the sum of the individual synthetic curves was equal to the original experimental curve. This allowed the relative proportions of the individual contributing populations to be determined by direct calculation. The Weasel software package (version 2.6) from the Walter and Eliza Hall Institute (WEHI) Melbourne Australia was used for curve fitting. Alterations in surface antigenicity in response to carbohydrate source were manifested as shifts in the relative proportions of the fluorescence populations, their mean fluorescence intensity, the width of the populations, the skewness of the distributions and by the introduction of new populations.

3. Identification and Quantification of Sugars in Simple (Free) and Complex Carbohydrate (Glycoproteins/ Glycolipids) Pools

Cells were scraped from 6-well plates in deionized water and sonicated for 1 minute. Three volumes of 95% ethanol were added to precipitate the complex sugar conjugates and arrest enzymatic activity. After centrifugation at 5000 rpm for 10 min, the pellet (with glycoproteins/ glycolipids) was separated from the supernatant (free sugars) and both were collected for analysis. The supernatant with simple sugars was saved and processed later. The solvent in the pellet containing the glycoconjugate pool was removed under a stream of nitrogen at 40^{o}C and 50 µL of 2 M trifluoroacetic acid was added. The suspension was heated for 2 hr at 120ºC after which 50 µL of water and 200 µL of chloroform were added. The organic phase was separated and discarded and the aqueous phase was evaporated. Water (50 µL) and sodium borohydride

(10 mg) were added and the mixture left stirring for 12 hr. Hydrochloric acid (20 μL) was added followed by methanol (3 mL) and the solvent removed under a stream of nitrogen at 40°C. This volume of methanol was added and removed 5 times by evaporation. The mixture was dried and 200 μL of pyridine and 100 μL of acetic anhydride were added. The mixture was heated for 1 hr at 100ºC[31], dried and re-suspended in 20 μL dichloromethane. The mixture containing alditol acetates (1 μL) was injected into a gas chromatograph DB-5 (Agilent Technologies) for detection of hexosamines (glucosamine, galactosamine and mannosamine). The DB-5 column (Restek; L 30 m x 0.25 mm; d_f = 0.1 μm) conditions were as follows: 190ºC hold for 5 min followed by a temperature ramp of 5ºC/ min to 300ºC followed by a 15 min hold. Quantification and identification of neutral hexoses (glucose, galactose and mannose) was also performed by gas chromatography in a SP2330 column (Supelco; L 30 m x 0.25 mm; d_f = 0.2 μm) column, in the conditions as follows: 190ºC hold for 5 min followed by a temperature ramp of 2ºC/ min to 220ºC followed by a 20 min hold. Both columns used helium as carrier gas. The gas chromatograph was coupled to a mass spectrometer with an electron impact ionizer. Spectra were recorded in positive mode. Total ion chromatograms were recorded and selected ions characteristic of the various sugars were profiled from single samples. Components were identified based on spectra and retention times compared to authentic standards.

To characterize the simple sugars in the supernatant, the supernatant that was separated from the pellet was resuspended in water (50 μL) and sodium borohydride (10 mg) added and left at room temperate for 12 hr. The rest of the derivatization process was similar to the one described for the glycoconjugate pool. The samples were injected on the same columns under the identical conditions described earlier.

4. Evaluation of Alterations in Cell Morphology and Growth

Alterations in morphological characteristics were evaluated by several methods. The first one was by deconvolution of the forward and side scattering data obtained by flow cytometry analysis of cells labeled for the ABO antigens. Forward scatter data provided information about cell size while side scatter gave information on cell granularity. The deconvoluted data enabled the identification of the number of subpopulations or different morphological clusters. Morphological analysis was also conducted by using CMEIAS software (developed by Dr. Frank Dazzo and collaborators, MSU) to detect statistical differences in shape, size and luminosity (granularity) of cells grown in fructose or glucose based on light microscopy micrographs. The software analyzed a minimum of 400 cells of each treatment. The Mann-Whitney test was used for cellular characteristics that presented a non-parametric distribution pattern and the t-Test for characteristics that presented a parametric distribution.

Results and Discussion

Antigenic and Carbohydrate Pool Analyses of Cells Grown in Fructose or Glucose

The antigen analysis of cell lines (MEF and bEnd.3) grown in fructose or glucose for 120 days is summarized in Figures 2.1 and 2.2. The identification and quantification of the different monosaccharide pools from cells are presented in Table 2.1.

From Figure 2.1, it is clear that MEF cells cultured in fructose expressed a higher degree of blood groups A (Fig 2.1A) and B (Fig 2.1B) labeling than when cells were grown in glucose (Figs 2.1D and 2.1E respectively). On the other hand, bEnd.3 cells growth in fructose exhibited weaker O labeling (Fig 2.2C) as compared to the same line cultured in glucose (Fig 2.2F). No

significant changes in the other labels were found between the treatments within the same cell line.

There are no simple direct correlations that can be drawn concerning the composition of the complex glycoconjugate pools from that of the free pools. There are, however, several correlations between the lectin binding results and the metabolic profile obtained from the two cell lines. According to the results compiled in Table 2.1, both cell lines present higher levels of mannose in their metabolic pool (MEF: 69%; bEnd.3: 64%) when grown in fructose compared to the respective ones cultured in glucose (MEF: 6%; bEnd.3: <1%). This is consistent with the fact that once fructose enters the cell, it is rapidly phosphorylated to fructose 6-phosphate, which is readily isomerized to mannose 6-phosphate.

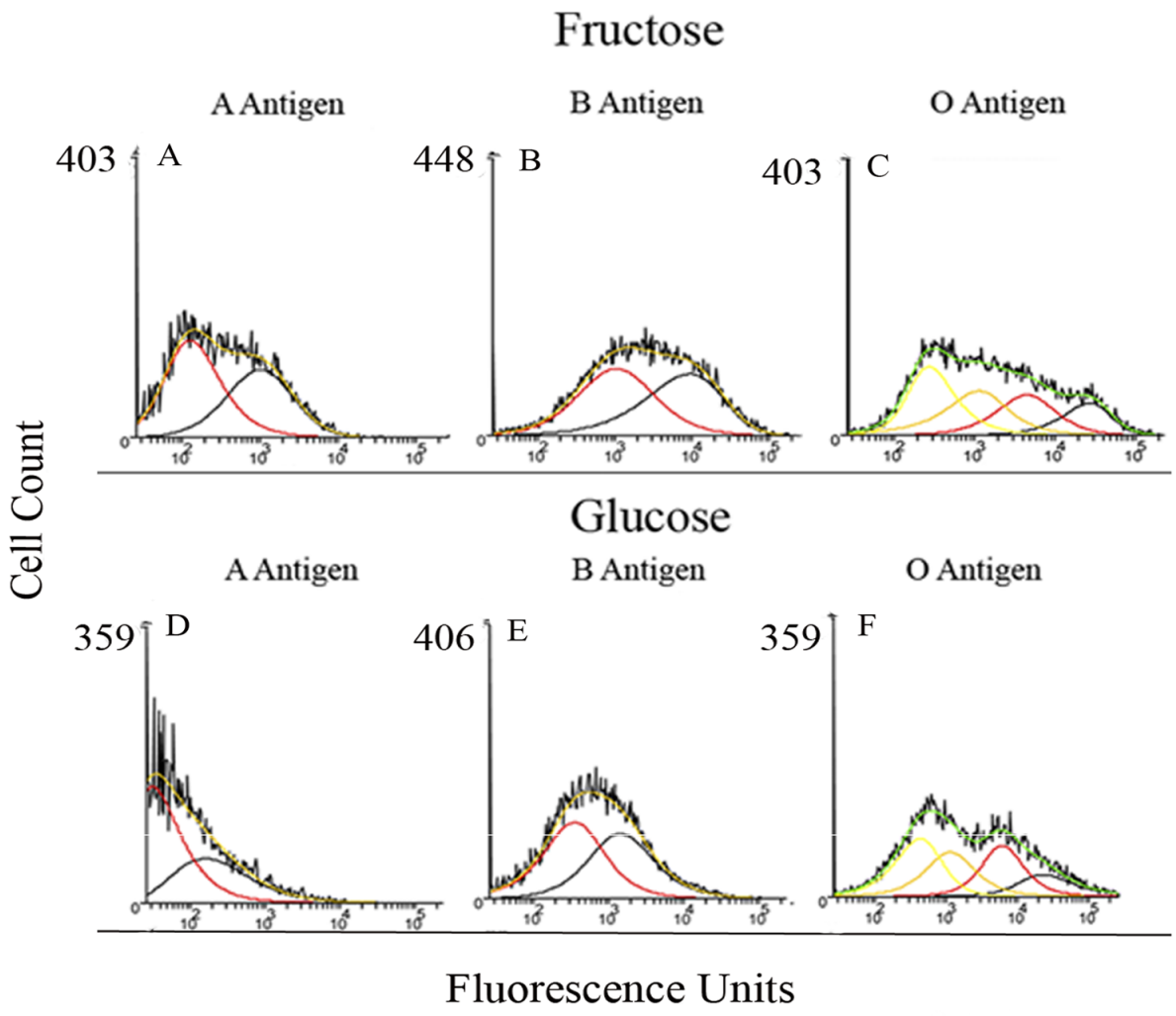

Figure 2.1. FACS analysis of ABO antigen populations of MEF cells after growth in fructose (A, B, C) or glucose (D, E, F) for 120 days showing labeling for A, B and O antigens. Each antigen was detected with specific fluorescent-labeled lectins for each blood group. 10,000 cells were analyzed in each graph. The irregular black line represents the total cell population curve obtained from the flow cytometer. The smooth curves with normal distributions (in red, yellow, black and orange) represent the individual populations mathematically obtained by the deconvolution of the total population curve.

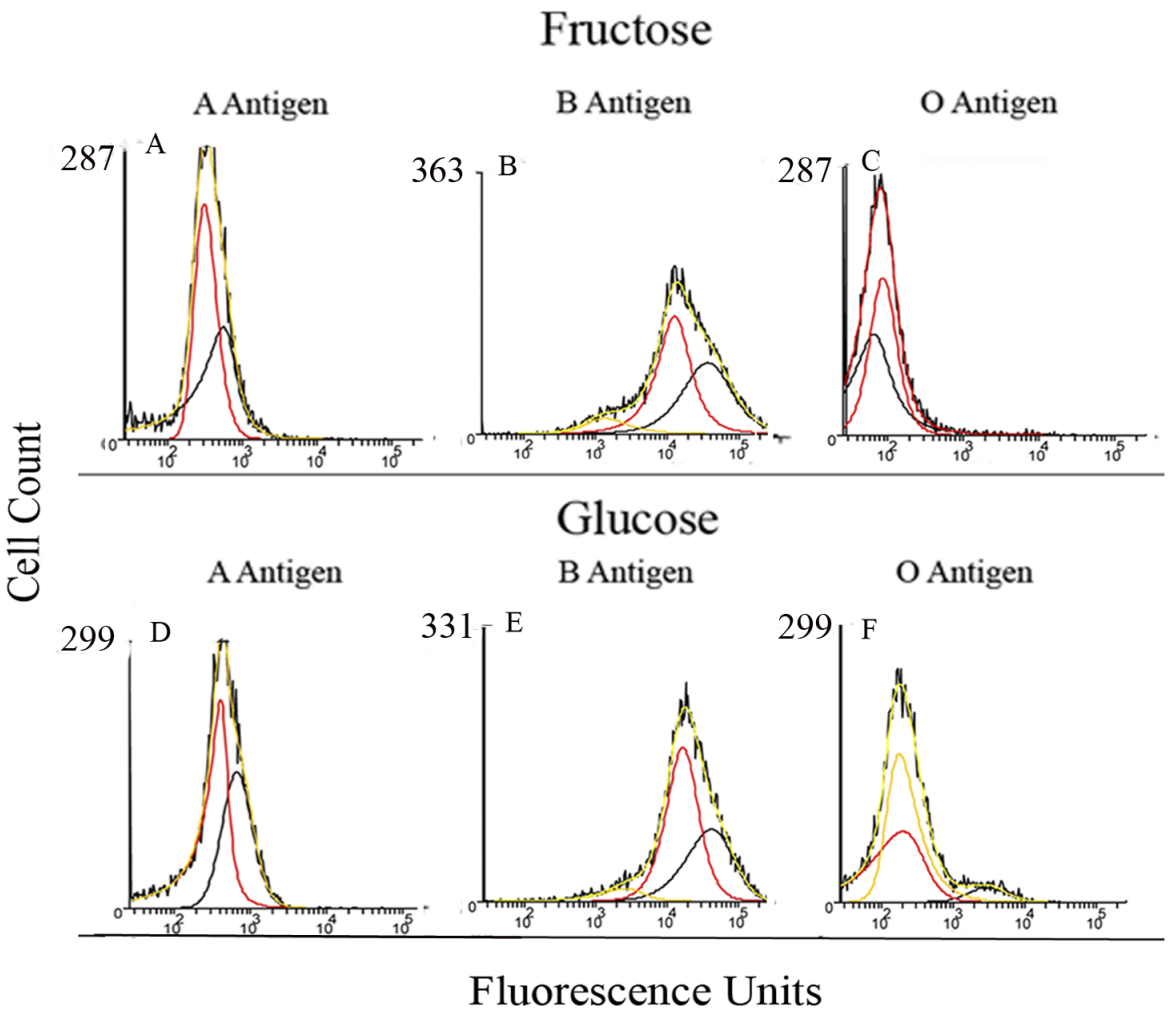

Figure 2.2. FACS analysis of ABO antigen populations of bEnd.3 cells after growth in fructose (A, B, C) or glucose (D, E, F) for 120 days showing labeling for A, B and O antigens. The irregular black line represents the total cell population curve obtained from the flow cytometry. Each antigen was detected with specific fluorescent-labeled lectins for each blood group. 10,000 cells were analyzed in each graph. The smooth curves with normal distributions (in red, yellow and black) represent the individual populations mathematically obtained by the deconvolution of the total population curve.

There are many observations of correlations between cell surface antigen expression and carbohydrate pools and the characteristics of certain cell types. It is important to keep in mind that cell function and physiology are very connected to the compositions of carbohydrate pools. MEF cells, for instance, are fibroblasts characterized by fast growth rate, high metabolic cell activity and the production of copious amounts of extracellular matrix[29]. This high-energy demand can be correlated with a high flux through glycolysis and other energy generating pathways. As a result, the glycolytic pathway has a high requirement for metabolic intermediates and the most readily available carbohydrate sources are allocated to this pathway. For this reason, it might be difficult to detect steady state concentrations of isomers of fructose and glucose (e.g. galactose) in the carbohydrate pools. This may explain why there are negligible amounts of free galactose in MEF cells in both culture conditions although this cell line contains significant amounts of B antigen (defined by the addition of galactose).

Table 2.1. Carbohydrate compositions of the metabolic (free) and glycoconjugate (glyco) pools in MEF and bEnd.3 cells cultured in either fructose or glucose.

Cell line	**MEF**				**bEnd.3**			
Sugar Source	**Fructose**		**Glucose**		**Fructose**		**Glucose**	
	Free	**Glyco**	**Free**	**Glyco**	**Free**	**Glyco**	**Free**	**Glyco**
Mannose	69	33	6	75	64	43	<1%	36
Galactose	<1%	<1%	<1%	<1%	<1%	15	<1%	36
Glucose	31	33	94	25	36	29	<1%	28
Mannosamine	<1%	6	<1%	<1%	<1%	2	<1%	<1%
Galactosamine	<1%	13	<1%	<1%	<1%	8	<1%	<1%
Glucosamine	<1%	15	<1%	<1%	<1%	3	<1%	<1%

Another observation that can be made is the higher production of total hexosamines (33% hexosamines: 64% hexoses) in MEF cells when fructose is used instead of glucose (<1%

produced). This cell line requires an intensive production of hexosamines for extracellular matrix biosynthesis, and the high hexosamine levels can be explained by the quick conversion of fructose to fructose 6-phosphate[22,24,29], which directly enters into the hexosamine biosynthetic pathway. This explains why MEF cells cultured in fructose have a much higher level of expression of A antigens (indicating the presence of GalNAc) compared to glucose-fed cells.

bEnd.3 is an endothelial line and grows more slowly and requires less energy-yielding metabolism at any given time than MEF cells[30]. This can be confirmed by the doubling time of this cell line (in glucose: 38 hr) in comparison to MEF cells (in glucose: 9 hr). In bEnd.3 cells, the percentage of total hexosamines between fructose- and glucose-grown cells changed significantly (13% versus <1% respectively) but not as much as in MEF (33% versus <1% respectively). This can be rationalized by considering that bEnd.3 cells grow more slowly and do not produce as much extracellular matrix as MEF, so the requirement of N-acetyl-glucosamine for matrix synthesis in this line is not as intensive as it is in MEF. This slower growth pattern in bEnd.3 also explains why steady state concentrations of sugars in the free metabolic pools (e.g. galactose) were higher than those observed in MEF. As a result, bEnd.3 cells are much more intensely bound by the fluorescent lectin specific for B antigen than are cells of the MEF line. A summary of the pathways that can be found increased when cells are exposed to fructose or glucose as carbon sources is found in Figures 2.3 (fructose) and 2.4 (glucose).

At first glance changes in antigenicity when cells are grown with different carbohydrate sources might appear to be inconsistent with the prevailing notion that cell surface glycosylation is highly conserved and strictly determined at a genetic level. This is to a great extent true but there is a more fundamental principle at work here. If the same complement of enzymes is present, the spectra and concentration of different available substrates can lead to different levels

of glycosylation results. It is also true that different types and levels of substrates lead to the induction of different types and levels of enzymes, again leading to different glycosylation patterns. Different cells have distinct patterns of cell growth, physiology, differentiation, morphology and gene expression[1,32,33]. This entails fundamental differences in the utilization of carbohydrates.

There are other well-documented reports of antigenic shifts in cultured cells[34,35]. In these studies, changes of antigenic response to carbohydrate source have been observed in human hybridoma lines cultured in the presence of glucose, mannose, galactose and fructose. These different carbon sources resulted in quantitative and qualitative changes in the production of anti-lung cancer human monoclonal antibody. Again, this demonstrates that the glycosylation pattern of this antibody is directly related to the carbon source that is fed to the cells[36,37].

Changes in other cell properties and physiology depending on the carbohydrate source provided have also been documented[1,2]. This was observed in various cell lines drawn from various lines of mouse and human fibroblasts, monkey kidney cells and HeLa cells exposed to different monosaccharides, including amino sugars, deoxy sugars and simple hexoses. Changes were observed in morphology and growth patterns of these cell lines. They were not universal but actually restricted to some combinations of cell types and sugars[2], reinforcing the notion observed in this study that changes in cell surface antigenicity and metabolic pools do not necessarily happen to the same extent in different cell lines cultured in the same sugar source.

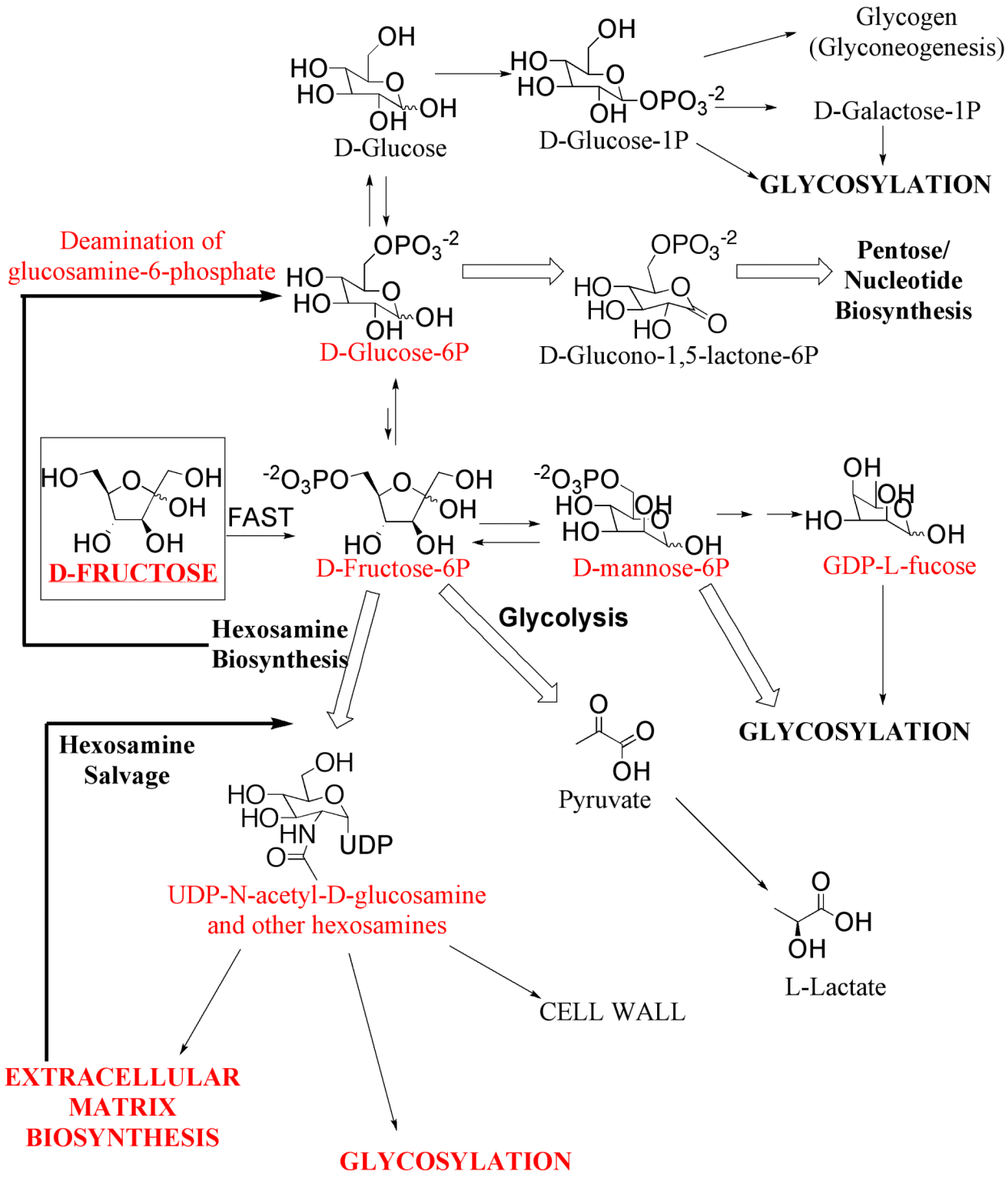

Figure 2.3. Metabolic responses when fructose is the major carbon source. Molecules/pathways highlighted in red are increased when cells are grown in this sugar.

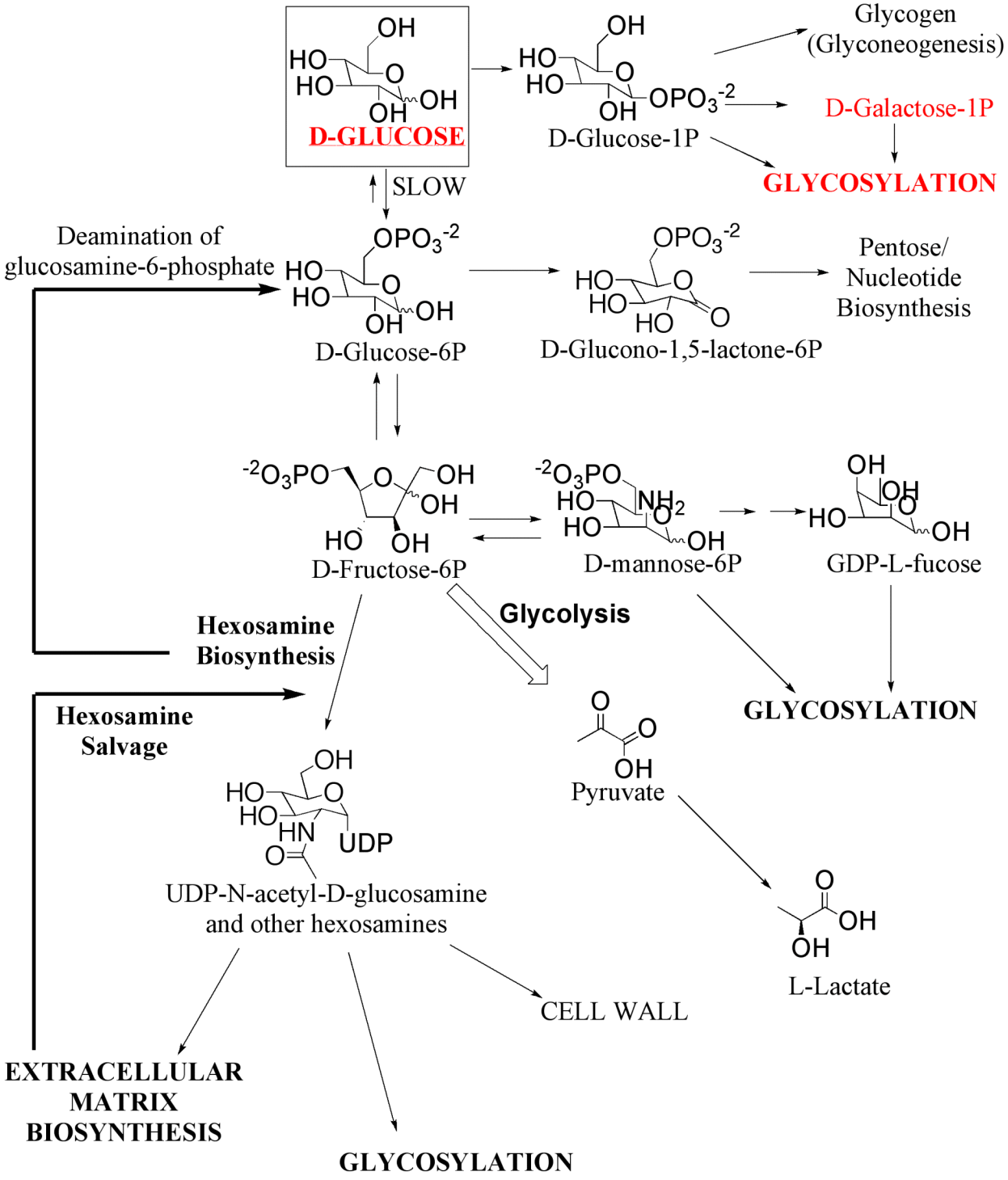

Figure 2.4. Metabolic responses when glucose is the major carbon source. Molecules/pathways highlighted in red are increased when cells are grown in this sugar.

In terms of morphology, the forward scatter data obtained from flow cytometry analysis did not show any significant changes in cell lines cultured in either fructose or glucose (data not shown). The use of methods that allow a visual evaluation of cells, however, permitted the identification of several parameters related to size and shape that are statistically different between the treatments. The most significant differences are described in Table 2.2 and are related to roundness, length/width and area of the cells. In terms of granularity, MEF cells grown on fructose displayed two subpopulations by flow cytometry analysis, with the major being less granular with a much narrower size distribution (Fig 2.5A). Cells cultured on glucose also displayed two populations (Fig 2.5B). These had the same mean granularity but one was much smaller and had a very broad distribution. These changes in granularity between the sugar sources were also confirmed by statistical analysis, which showed that cells grown in fructose or glucose presented significant differences in cell density and clumpiness. Both parameters are related to cell luminosity, which is closely associated with cell granularity. The size, shape and granularity of bEnd.3 cells changed very little under all conditions and methods used (data not shown).

A consistent change that was discernible by FACS in MEF cells was the appearance of a new population with higher granularity when cells were cultured in fructose (Fig 2.5A). This new population may be related to cells that present micronuclei vesicles (Fig 2.6A), since these vesicles increase the granular property of cells. As noticed in Figures 2.6A and 2.6.B, cells in fructose present more cytoplasmic inclusions, especially in the case of MEF. There was also evidence for cell damage in fructose as indicated by the appearance of a peak close to the axis corresponding to small granular material (Fig 2.5A, indicating cell debri). The observation of

more significant morphological changes in MEF but not in bEnd.3 reinforces, again, that the extent of changes in cell characteristics are correlated to the level of cell metabolic activity.

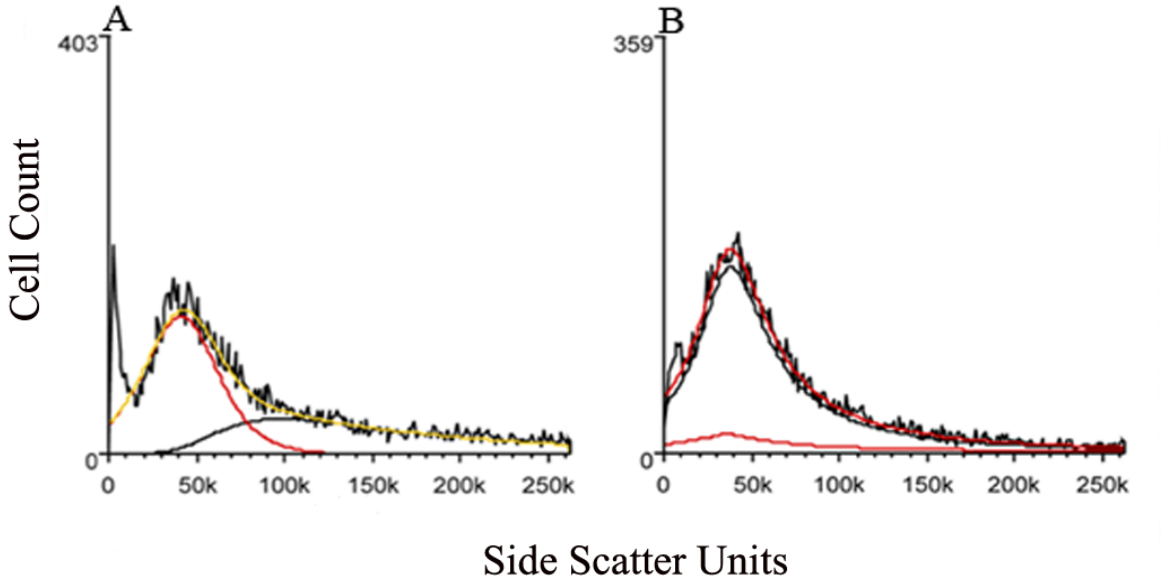

Figure 2.5. Side Scatter Profile of MEF cells grown in fructose (A) or glucose (B). The irregular black line represents the total cell population curve obtained from the flow cytometry. The regular, smooth curves with normal distributions (represented by the colors: red, yellow and black) represent the individual populations mathematically obtained by the deconvolution of the total population curve.

Table 2.2. Morphological Differences between MEF Cells treated with glucose and fructose for 120 days.

Type of Characteristic Evaluated	Specific Parameter Evaluated	Probability	Statistic Test Used
Shape	Roundness	$1.7x10^{-10}$	Mann-Whitney
Shape	Length/Width	$1.9x10^{-9}$	t-Test
Shape	Radiability	$2.3x10^{-9}$	t-Test
Size	Area	$2.9x10^{-5}$	t-Test
Size	Width	$2.1x10^{-11}$	t-Test
Luminosity	Mean Gray Level	$5x10^{-3}$	Mann-Whitney
Luminosity	Integrated Density	0.001	t-Test
Luminosity	Clumpiness	0.013	t-Test

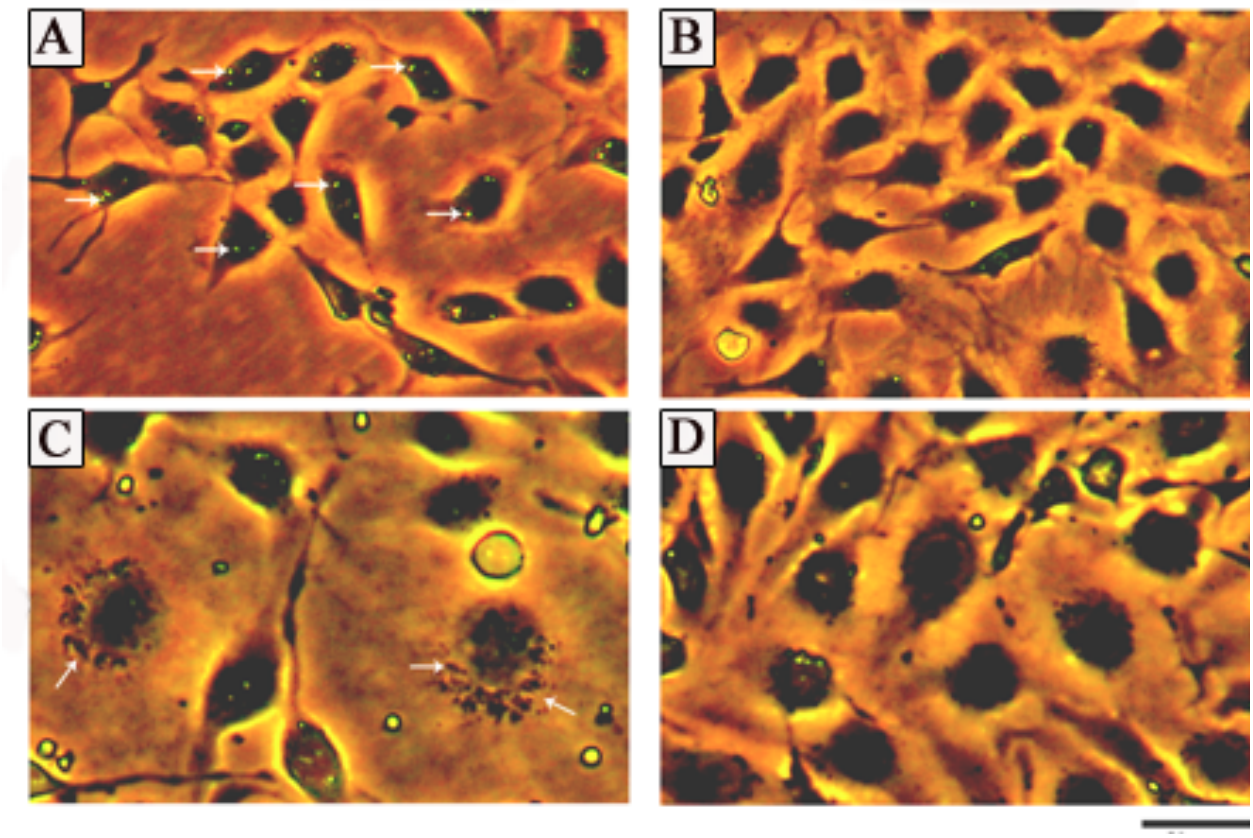

Figure 2.6. Representative pictures showing changes in morphology of MEF (A, B) and bEnd.3 (C, D) cells observed by light microscopy. Cells in fructose (A, C) present more cytoplasmic inclusions (white arrows) than cells grown in glucose (B, D). Scale bar = 50 μm.

In this study it is demonstrated that the cell surface glycochemistry and morphology of cells are not constant but rather are dependent on the carbohydrates provided in cell culture. I show that the closeness of the dependence between the cell type and its metabolism. This study emphasizes the biochemical principle that the genetic structure of living systems endows them with a pre-disposition to participate in biochemical processes along one path in preference to another, but the actual path chosen is strongly influenced by environmental factors. In the very important area of carbohydrate metabolism this and many other studies cited herein indicate that there is still much more to be understood and factored into our dietary regime, but there is a biochemical basis to rationalize potential problems and benefits associated with certain diets.

Concluding Remarks

This study shows the distinct effects different carbohydrate sources have on eukaryotic cellular properties such as their cell surface antigenicity, carbohydrate metabolic pools and morphology. It also shows that changes in these characteristics are cell-type dependent, and this is especially linked to the function and level of metabolic activity of the cell type. This observation contributes to our understanding of how diet quality and quantity can impact cellular characteristics involved in the development of disorders such as T2D and cancer. This can help us rationalize potential problems that can come from diets rich in particular sugars such as fructose and how to prevent diseases to a certain extent by diet management.

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REFERENCES

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CHAPTER 3

CHAPTER 3

EVALUATION OF THE BASIS FOR THE METABOLIC RESPONSES OF CELLS WHEN FRUCTOSE IS USED AS THE PRIMARY CARBON SOURCE.

Abstract

To determine the metabolic effects of high concentrations of fructose, gene expression analysis was performed on cells grown on fructose or glucose. An affinity matrix was designed to capture proteins that bind to fructose 6-phosphate to gain further insight into the microarray data. Gene expression related to C1 and glycerol 3-phosphate metabolism dominated in fructose-grown cells. The affinity matrix binding indicated fructose 6-phosphate can affect the activity of many enzymes by competition, especially with substrates containing a ribose 5-phosphate moiety. This structure similarity between furanoses in the presence of high amounts of fructose might affect the activity of a wide range of enzymes.

Introduction

Carbohydrates play roles in many biological phenomena critical to life[1-5] and malfunction in their metabolism is at the core of many diseases[6-9]. Glucose has been by far the most common source of carbon in our diet. However, due to changes in food technology, fructose is also a major simple sugar in our diet. It enjoys a high level of use as a sweetener. Heavy consumption of fructose has been suggested as a potentiating factor in diseases such as cancer[10], dementia[11], T2D and obesity[12], hypercholesterolemia[13] and hyperuricemia[14].

The fates of cells cultured in fructose are markedly different from those cultured in glucose. The biochemical pathways, level of nucleic acid synthesis, respiration rates and rates of division are all known to vary depending on which sugar is used as primary energy source[15,16,17,18]. The exposure of cells to fructose and its derivatives is usually controlled by the rate of isomerization of glucose 6-phosphate to fructose 6-phosphate. Large amounts of exogenous fructose can lead to cellular concentrations of fructose 6-phosphate that are well above the norm. In this case, the isomerase equilibrium between glucose 6-phosphate and fructose 6-phosphate would result in higher glucose 6-phosphate that would no longer be regulated by the availability of hexokinase. This could activate the PPP resulting in more ribose and deoxyribose derivatives[10]. Fructose also participates in many biochemical processes that glucose does not. The structural similarities between fructose 6-phosphate and substrates from other pathways allows for a new regulatory regime for these pathways based on simple competition. Fructose can also be acted on by fructokinase in the liver making fructose 1-phosphate, which gives rise to glyceraldehyde and dihydroxyacetone phosphate, bypassing the highly regulated enzyme phosphofructokinase.

A better understanding of the biochemical events that stem from growth of cells on fructose as a major carbon source is needed because high levels of fructose clearly lead to structural, physiological and biochemical changes in cells. At the level of gene expression and cellular chemistry, the observed high levels of nucleic acid synthesis requires high levels of C1 metabolism. This involves the conversion of serine to glycine, the decarboxylation of glycine to form methyl equivalents, the conversion of tetrahydrofolate (THF) to 5,10-methylene-THF by capture off these methyl equivalents, the S-adenosylmethionine biosynthesis from 5,10-

methylene-THF, the methylation of glycine to form sarcosine, dimethyl glycine and betaine to support C1 metabolism[19].

To study the potential effects of high levels of fructose on cellular metabolism, a low stringency affinity capture method was employed to cast a wide net for proteins that interact with fructose 6-phosphate. A fructose 6-phosphate analog was bonded to a solid matrix and cell extracts were washed onto the column. In the approach described here, 1-O-carboxymethyl-6-sulfato-D-fructose (**1**), an analog of fructose 6-phosphate with a linker attached to the 1-position, was covalently linked to a solid matrix via the carboxyl group. This analog is designed to have all of the important structural features of fructose 6-phosphate in both ring and acyclic forms. In this analog the phosphate group of fructose 6-phosphate is replaced with a sulfonate group[20]. This has been shown to be an effective substitution. It has the advantages of ease of synthesis and a resistance to loss of charge as occurs in the case of phosphates if phosphatases are present in the cell lysates. A bacterial cell lysate was passed over the matrix to adsorb the proteins that can bind to fructose 6-phosphate. The bound enzymes were then eluted from the matrix with increasing amounts of fructose 6-phosphate and the fractions analyzed. The nature of the proteins was evaluated by homology analysis based on gene sequence information on the proteins in each fraction.

I also measured mRNA levels that are affected by fructose. Microarray analysis of gene expression was used to provide a relatively unbiased evaluation of gene expression in bacterial cells cultured on fructose in comparison with the cells cultured on glucose.

This two-tiered approach allowed an assessment of the metabolic events surrounding exposure of cells to high concentrations of fructose from a gene expression perspective as well as from a classical biochemical perspective. In the latter approach, structural data that can be used

to categorize the enzymes that actually interact with fructose 6-phosphate is obtained. The characterization of the web of interactions that fructose 6-phosphate may have (when present in high concentrations) based on structure similarity is obtained.

Materials and Methods

1. Affinity chromatography

1.1. Preparation of Affinity Matrix

The complete sequence for the preparation of the affinity matrix is illustrated in Figure 3.1.

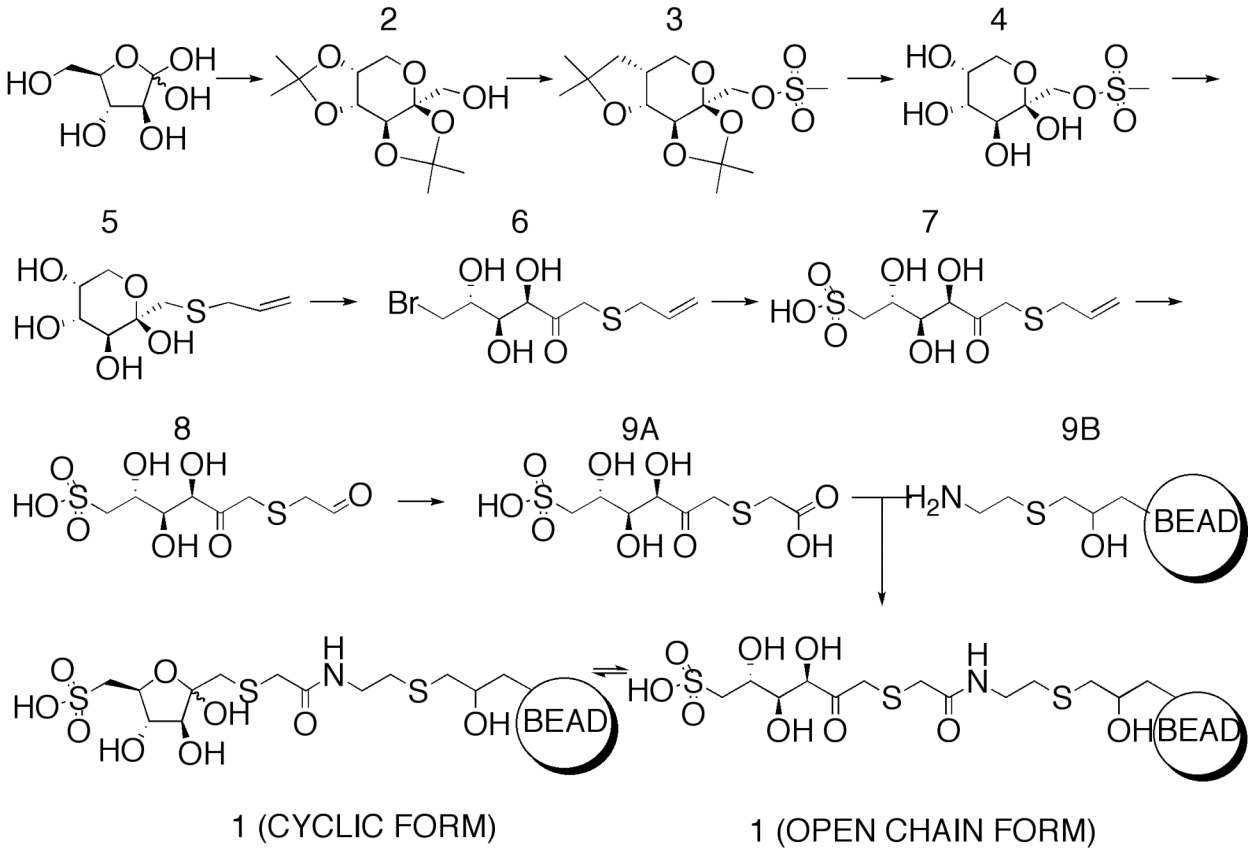


Figure 3.1. Route of synthesis to compound **1**.

2,3-4,5-di-O-isopropylidene-β-D-fructose (**2**). D-Fructose was converted to its 2,3-4,5-di-O-isopropylidene acetal according to established literature procedures[21] by treatment with acetone and sulfuric acid at room temperature. Briefly 100 g of fructose was added to 1 L of acetone containing 5% sulfuric acid and the mixture stirred for 5 hr at room temperature. Sodium bicarbonate (200 g) in 100 mL water was added and the mixture stirred until effervescence ceased. Acetone was removed by evaporation under reduced pressure and dichloromethane (1 L) was added. The lower dichloromethane layer was removed, washed again with water and concentrated to a syrup which soon crystallized. Spectra, rotation (-33^{o}, 1.5% solution in water) and mp (94- 95^{o}C) were consistent with literature values and structure.

2,3:4,5-Di-O-isopropylidene-1-O-(methylsulfonyl)-β-D-fructopyranose **(3).** The di-isopropylidene acetal **2** was converted to the known mesylate **3** by standard treatment with mesyl chloride and pyridine using established procedures[22,23]. Briefly 55 g of **2** was dissolved in 200 mL of pyridine and the solution cooled to 5^{o}C in ice. Methanesulfonyl chloride (50 g) was slowly added over a period of 30 min while maintaining the temperature below 10^{o}C. The mixture was stirred for 5 hr after addition. Sodium bicarbonate (60 g) in water (300 mL) and ice (100 g) was added to the mixture, which was stirred until effervescence ceased. It was extracted with 2 L of dichloromethane and the organic layer was recovered. The organic layer was washed once more with water, dried with anhydrous sodium sulfate, filtered and concentrated to give **3** as a syrup which eventually crystallized. Compound **3** was readily recrystallized from methanol. Optical rotation (-31.9°, 1%, isopropanol), mp (65-68°C). NMR spectra were consistent with structure.

1-Deoxy-1-thioallyl-D-fructose **(5).** The isopropylidene groups on compound **3** were first removed by acid hydrolysis (300 mL 10% sulfuric acid) for 1 hour at 80ºC followed by neutralization with calcium carbonate (100 g) with stirring for 2 hr. The mixture was filtered and concentrated under reduced pressure to give **4** as a syrup. NMR spectroscopy indicated the removal of the isopropylidene groups (no signals at 1-2 ppm in the proton spectra and the retention of the mesyl group (3H-singlet ~2.9 ppm). Intermediate **4** (21 g) was mixed with 1.5 equivalents of 2-propene-1-thiol and 3 equivalents of sodium carbonate in a 40% methanol/ water solution and stirred overnight at room temperature. The mixture was passed through a mixed bed ion exchange resin (H^+) and (OH^-) forms to remove all salts and then concentrated to a syrup. The loss of the mesyl group (~2.9 ppm) and the introduction of the 1-thiopropene group were readily confirmed by proton NMR spectroscopy. Signals for the vinyl protons on the thiopropene group were evident at 4.95, 5.05 and 5.95 ppm. The signals for the protons on C1 of fructose shifted upfield to 2.85 ppm.

Introduction of the sulfonate group and final construction of the affinity matrix. The sulfonic acid function was introduced using a strategy described earlier[20]. This involved selective conversion of the primary hydroxy group at C6 to a bromo group by treatment with carbotetrabromide and triphenylphosphine in pyridine followed by displacement of the bromo group with a sulfonate group using sodium sulfite in water. Success of this method is readily judged by the appearance of the protons on C6 at the unusual upfield position of 2.8 to 3.1 ppm in the proton NMR spectrum. Hence compound **5** (2 g) was reacted with carbotetrabromide (4 g) and triphenylphosphine (4 g) in pyridine (60 mL) for 1.5 hr at 80ºC. The pyridine was removed by evaporation at 50^{o}C under reduced pressure and the residue treated with water (20 mL) and

the water layer recovered by decantation and then by filtration after vigorous stirring and cooling. The aqueous solution was concentrated again at 50^{o}C under reduced pressure to remove traces of pyridine. It was then reconstituted in 30 mL of 5% sodium sulfite in water and heated at 80^{o}C for 3 hr to effect replacement of the bromo group with a sulfonate function. The product could be easily purified by adsorbing it onto an ion exchange resin (OH^-) form, washing with water to remove neutral materials and unreacted starting material and elution with 10% sodium chloride. Success in introduction of the sulfonate group was evaluated by NMR spectroscopy using the criteria introduced earlier[20] (signals between 2.8 and 3.1 ppm). The thiol allyl group (4.95, 5.05 and 5.95 ppm) was unchanged. The molecular composition was confirmed by high-resolution mass spectrometry (Figure 3.2, expected m/z 299.0259 measured 299.0269). The ligand was attached to the affinity matrix by two standard steps. Firstly, the alkene function was oxidized to an aldehyde (scission of the double bond) with ozone to give **8.** Secondly, the aldehyde group was oxidized to a carboxylic acid group with bromine water to give **9A**. Progress in the ozonation step was easily judged by monitoring the disappearance of the vinyl signals in the NMR spectrum during ozonation. These steps were carried out by dissolving 3 g of the sulfonated alkene in 70 % ethanol in water and treating the solution with ozone (60 mg/ h) overnight. The intermediate ozonide was decomposed by treatment with zinc metal (1 g in 66% ethanol in water) with stirring at room temperature for 4 hr. Treatment with bromine (1 g) in water (100 mL) at 5^{o}C for 14 hr effected the conversion of the aldehyde **8** to the acid **9A**.

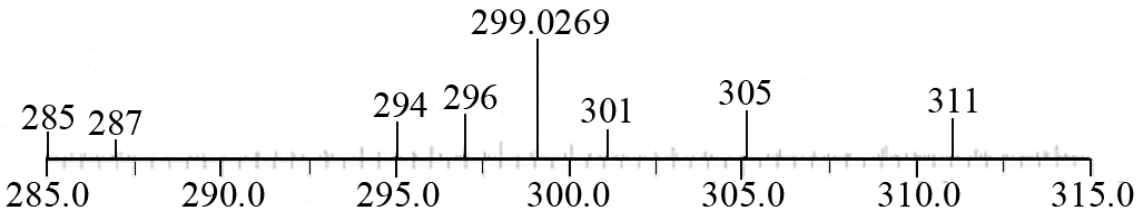


Figure 3.2. High-resolution mass spectrum of compound **7**.

Amino functions were introduced into epoxy-activated sepharose by treatment of this matrix with 2-amino ethanethiol (Figure 3.3). Epoxy-activated sepharose beads (3 g) were reacted with 2-amino ethanethiol (0.1 g) in water and stirred overnight at 4ºC. The ligand **9A** was coupled to the amino functionalized beads by standard carboxyl activating conditions using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). In this case 100 mg of the ligand **9A** was added to 1 g of amino-activated sepharose **9B** in the presence of EDC (150 mg) at pH 4. The mixture was gently stirred for 4 hr at 4ºC and then washed with water (10 mL) and 1:1 water methanol (10 mL) and finally again with water (100 mL).

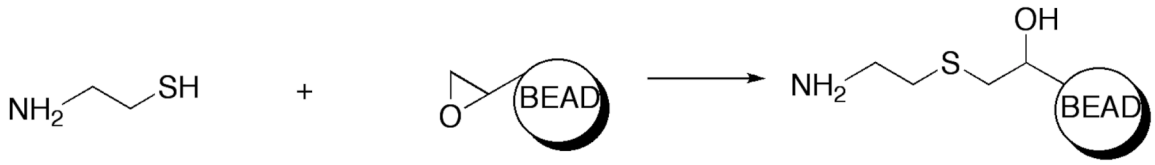


Figure 3.3. Introduction of the amino function in the epoxy-sepharose beads.

1.2. Evaluation of the Ligands: Cell Preparation, Column Elution and SDS-PAGE

Escherichia coli cells were grown in LB medium at 37ºC under constant agitation overnight. The cells were harvested by centrifugation at 1700g for 15 min and cells in the pellet (1.5 mL of pellet/ per mL of 10 mM PBS solution) were disrupted by sonication. The lysate (13 mg of protein as measure by the bicinchoninic acid (BCA) method[24]) was applied to the column previously equilibrated with 10 mM PBS, in a proportion of 25% of lysate per column volume. The column was washed with 1 mL of 10 mM PBS three times to eliminate unspecific bound proteins. Increasing concentrations of fructose 6-phosphate (1, 2 and 5%) were passed through the column. One fraction of 1 column volume was collected for each concentration. The fractions

were desalted by gel filtration. Non-specific interactions between the bound proteins and the matrix were ruled out by carrying out the same process on three additional columns. One of these was the un-functionalized affinity support. A second contained functionalized support but was eluted with fructose (1, 2 and 5% as before). The third contained functionalized support but was eluted with ribose 5-phosphate (again 1, 2 and 5%). The un-functionalized column was eluted with fructose 6-phosphate (1, 2 and 5%). 5.37 mg of protein were recovered in the fraction eluted with 5% fructose 6-phosphate from the affinity matrix. The 5% eluted fractions from these 3 control columns and all samples (crude extract, pre-wash and post elution fractions) from the functionalized column eluted with fructose 6-phosphate were applied to a 10% Tris-HCl SDS gels and run at 100 V for 1.5 hr. For recovery of proteins for sequencing one gel with just the 5% fructose 6-phosphate eluent was stained with Coomassie brilliant blue solution and destained until bands were seen clearly (64 μg of crude extract and 134 μg of fructose 6-phosphate 5% fraction were applied to the gel). A second gel was run with a different cell extract as a control. Silver staining was used for the control experiments to evaluate specificity in conditions of high sensitivity.

Fourteen bands were identified in the gel stained with Coomassie blue solution. These were cut out and digested with trypsin and the peptides separated and sequenced with LC/MS. The *E. coli* database (NCBI) was used to score our potential protein candidates for each band. The number of unique peptides found for each match, the percentage of confidence, the percentage of total spectra coverage and sequence coverage are provided in Table 3.1.

2. Microarray studies

Escherichia coli DH5α was grown in minimal media M9 at 37ºC until logarithmic phase was reached. Cells were then grown in minimal media M9 containing either glucose or fructose at 25 g/L. Cells were grown for 12 hr at 37ºC and centrifuged at 4000g for 20 min. The supernatant was discarded and the cell pellet was treated with RNAprotect Bacteria reagent (Qiagen) to prevent RNA degradation. Total RNA was extracted with RNeasy Kit (Quiagen) according to manufacturer's protocol. RNA quality was monitored by electrophoresis in the Agilent BioAnalyzer. Gene expression analysis was performed with the *E. coli* chip from Agilent Technologies 8x15K with 15,208 genes represented. All analyses were performed in triplicate in three separate biological samples. Data analysis was performed using the Agilent's GeneSpring GX software. All genes related to pentose and C1 related pathways were presented in Tables 3.2 and 3.3.

Results and Discussion

The SDS-PAGE analysis of the crude lysate, fractions obtained from the pre-wash and various fructose 6-phosphate elutions (Figure 3.4A) indicated that a variety of protein species bound selectively to the matrix. A total of fourteen significant bands from the 5% fructose 6-phosphate fraction (the highest concentration and therefore the most tightly bound) (Figure 3.4A, lane 9) that were significantly pulled out of the affinity matrix were chosen for digestion and partial sequencing by mass spectrometry. The partial sequence information for each band was matched to protein candidates using several databases and these matches are shown in Table 3.1.

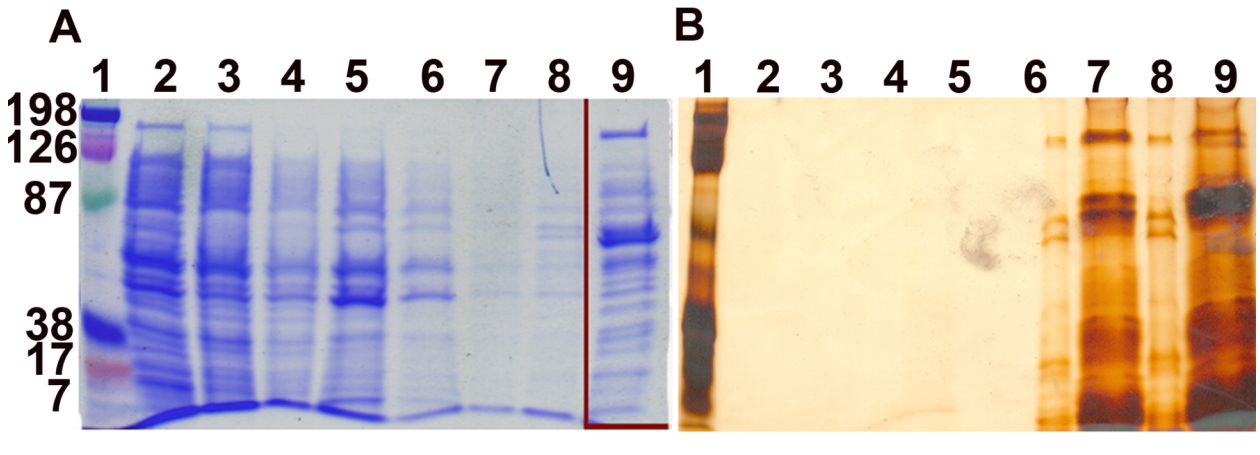


Figure 3.4. (A) SDS-PAGE (Coomassie brilliant blue staining) of the fractions of *E. coli* lysate purified from the affinity chromatography column. Lane 1: molecular weight standard, lane 2: crude extract (64 μg); lanes 3-6: 10 mM PBS pre-wash fractions; lane 7: fructose 6-phosphate (F6P) 1%, lane 8: F6P 2%; lane 9: F6P 5% (134 μg). Fourteen bands from the last fraction (lane 9) were sequenced by mass spectrometry and the protein candidates listed in Table 3.1. (B) SDS-PAGE (silver nitrate staining) of the 5% fractions eluted from the following conditions: Lane 1: molecular weight standard; lane 2: empty; lane 3: un-functionalized beads with F6P 5%; lane 4: affinity matrix eluted with fructose 5%; lane 5: empty; lanes 6 and 7: affinity matrix eluted with ribose 5-phosphate 5% (lane 6: 5μL and lane 7: 25 μL of sample); lanes 8 and 9: affinity matrix eluted with F6P 5% (lane 8: 5 μL and lane 9: 25 μL of sample).

Table 3.1. Protein candidates obtained by sequence analysis of the 14 bands eluted from the affinity column with fructose 6-phosphate 5%. Columns: 1: position on the gel (from top to bottom); 2: protein candidates of each band listed from highest to lowest score; 3: confidence interval for that match; 4: number of unique peptides found for that match; 5: percentage of total spectra covered; 6: percentage of sequence coverage; 7: substrates of the proteins described in column 2. Structural elements that overlap with fructose 6-phosphate are highlighted in blue. The table contains protein matches that exceed 95% confidence interval.

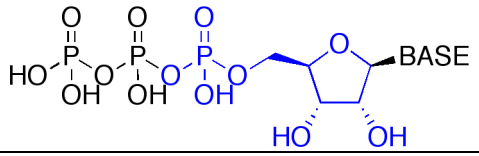
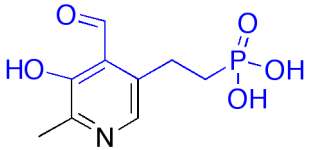
Position	Protein Candidate	Confidence (%)	# Unique Peptides	Spectra Coverage (%)	Sequence coverage (%)	Proteins' Substrates (Fructose 6-phosphate structure)
1	RNA polymerase, beta subunit gi|56384051	100	81	19	65.6	Ribonucleotides: polymerase 
	RNA polymerase, beta prime subunit gi|24054566	100	77	21	58.1	
2	Thioredoxin gi29143703	95	1	0.29	13.8	Fructose 6-phosphate: enolase
	ORF_f292: putative aldolase gi|16131721	95	1	0.29	10.6	
3	30S ribosomal subunit protein S1 gi|24051180	100	26	4.3	56.2	Ribonucleotides (ATP): 30S ribosomal subunit, chaperone protein dnaK, adenylate cyclase on PEP-PTS enzyme I
	Chaperone protein dnaK gi|21321902	100	32	5.7	53.9	
	PEP-protein phosphotransferase system enzyme I gi|1788756	100	22	4	52.3	
	Lysine decarboxylase gi|16131957	100	11	1.7	24	Pyridoxal 5-phosphate (as cofactor): lysine decarboxylase 

Table 3.1 (cont'd)

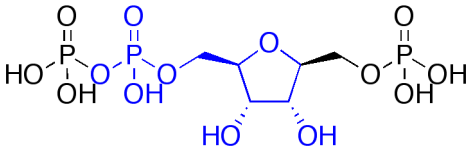
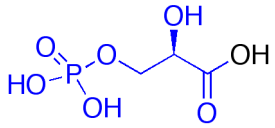
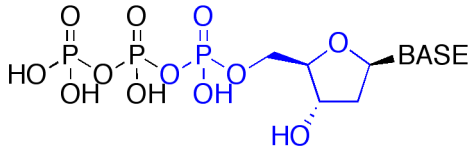
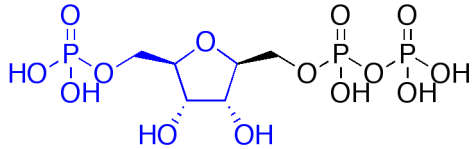
4	PEP-protein phosphotransferase system enzyme I gi|1788756	100	46	23	78.6	Ribonucleotides (ATP): PEP-PTS Enzyme I, chaperone Hsp60
	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein gi|24054822	100	35	9.4	74.3	
	Transcription pausing; L factor gi|24053641	100	30	6.4	56.0	
5	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein gi|24054822	100	44	37	86.9	Ribonucleotides (ATP): GroEL chaperone Hsp60 Phosphorybosyl pyrophosphate: PRPP amidotransferase 
	Amidophosphoribosyl transferase gi|24113684	100	10	1.5	26	
6	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein gi|24054822	100	24	6.2	58.9	Ribonucleotides (ATP, GDP): GroEL chaperone Hsp60, glycerol kinase, serine tRNA synthetase. Pyridoxal 5-phosphate (as cofactor): GAD α-protein Phosphorybosyl pyrophosphate: PRPP amidotransferase
	Glycerol kinase gi|16131764	100	25	6.5	58.0	
	Serine tRNA synthetase gi|24051120	100	20	4.4	52.6	
	PRPP amidotransferase gi|24113684	100	10	1.5	26	
	GAD alpha protein gi|466654	100	8	2	20	
7	GTPase (tRNA 5-methylaminomethyl-2-thiouridine) gi|2367268	100	17	5.0	51.5	Ribonucleotides (ATP): serine tRNA synthetase and GTPase
	Serine tRNA synthetase gi|24051120	100	21	4.2	56.5	
8	Enolase gi|24053192	100	11	2.3	44.2	2-phosphoglycerate: enolase 
	Peptide chain release factor RF-2 gi|2367172	100	14	5.8	46.8	Ribonucleotides (ATP, GTP): release factor RF-2

Table 3.1 (cont'd)

9	Methionine adenosyltransferase 1 (AdoMet synthetase) gi|56383766	100	16	4.7	52.3	Ribonucleotides (ATP): elongation factor EF-Tu, methionine adenosyltransferase
	Protein chain elongation factor EF-Tu gi|26250749	100	15	4.3	58.9	
10	Isoaspartyl dipeptidase gi|1790784	100	17	6.3	64.4	Deoxynucleotides: DNA-directed RNA polymerase  Pyridoxal 5-phosphate (cofactor): glutamate-1-semialdehyde aminotransferase
	DNA-directed RNA polymerase alpha chain gi|24114573	100	20	6.6	73.9	
	glutamate-1-semialdehyde aminotransferase gi|4146961	100	8	1.2	29	
11	Homoserine transsuccinylase gi|1790443	100	11	2.2	51.1	Fructose 6-phosphate: 6-phosphofructokinase
	6-phosphofructokinase gi|16131754	100	14	4.4	57.8	
	Acidic 34,893Da HtrM protein gi|466757	100	18	5.9	60.0	Deoxynucleotides: DNA polymerase
12	PRPP synthetase gi|16129170	100	15	4	53	ATP as inhibitor: D-lactate dehydrogenase 5-phospho-D-ribose-diphosphate: PRPP synthetase 
	Putative alpha helix protein gi|1790075	100	16	5.9	51.2	
	Lactate dehydrogenase gi|16129341	100	11	2.5	42	
13	Phosphoribosylpyrophosphate synthetase gi|1787458	100	15	4.0	53.0	5-phospho-D-ribose-diphosphate: PRPP synthetase
	3-oxoacyl-[acyl-carrier-protein] synthase III gi|26107632	100	13	4.2	60.9	
14	3-methyl-2-oxobutanoate hydroxymethyltransferase gi|1786326	100	7	2.3	51.1	Ribonucleotides (ATP): 50S and 30S ribosomal subunits proteins
	Sigma cross-reacting protein 27A gi|56383838	100	6	1.4	53.0	
	50S ribosomal subunit protein L1 gi|26250754	100	10	1.9	55.6	
	30S ribosomal subunit protein S3 gi|56415353	100	7	1.6	42	
					85	

The majority of bands corresponded to proteins that interact with substrates structurally similar to fructose 6-phosphate. These included ribonucleotides (RNA polymerase- band 1, tRNA synthetases- bands 6 and 7, ribosomal subunits- bands 3 and 14, peptide chain release factor RF-2- band 8), deoxynucleotides (DNA polymerase- band 10), ATP (GroEL- bands 5 and 6, kinase - bands 6, PEP-PTS enzyme I- bands 3 and 4, protein elongation factor EF-Tu- band 9, 6-phosphofructokinase- band 11, GTPase- band 7, chaperone dnaK- band 3, AdoMet synthetase- band 9, lactate dehydrogenase (as inhibitor)- band 12) and enzymes involved in nucleotide synthesis (amidophosphoribosyl transferase- bands 5 and 6, phosphoribosyl pyrophosphate synthetase- bands 12 and 13). In some cases the substrate is not structurally similar to fructose 6-phosphate but the cofactors required for the reaction share some similarity to it. Pyridoxal 5-phosphate is a case in point (lysine decarboxylase- band 3, GAD α-protein- band 6, glutamate-1-semialdehyde aminotransferase- band 10). A few other candidates share partial similarity to fructose 6-phosphate (2-phosphoglycerate in the case of enolase- band 8). A candidate that uses fructose 6-phosphate itself includes phosphofructokinase (band 11). No proteins were eluted from the un-functionalized matrix with fructose 6-phosphate or from the functionalized matrix with fructose. Many of the bands that eluted from the functionalized matrix with fructose 6-phosphate also eluted with ribose 5-phosphate albeit with lower efficiency (Figure 3.4B). There is significant structural overlap between ribose 5-phosphate and fructose 6-phosphate, which may explain why the binding patterns to the affinity matrix are similar (Figure 3.4B, lanes 7 and 9) when eluted with these furanose phosphate sugars.

Fructose 6-phosphate is a substrate for kinases (e.g conversion to fructose 1,6-biphosphate and fructose 2,6-biphosphate), aldolases (e.g. fructose-6-phosphate aldolase), transketolases and transaldolases (to form products like sedoheptulose 7-phosphate, xylulose 5-

phosphate and glyceraldehyde 3-phosphate) and amidotransferases for hexosamine biosynthesis. It also has considerable structural overlap with nucleosides, nucleotides, and nucleic acids and should interact strongly with enzymes involved in the synthesis, transport and use of these molecules. The sequence information obtained from the proteins that bound to the column represented many classes of enzymes. This supports the notion that there could be a variety of processes of critical importance that are affected by fructose 6-phosphate outside of its accepted metabolic roles (Table 3.1). The structural overlap between fructose 6-phosphate and the structures of the substrates used by the enzymes matched by the proteomics database is highlighted in Table 3.1 (column 7).

The global effect that fructose may exert on cell physiology would also have repercussions on gene expression. The gene expression studies using *E. coli* cells grown in either fructose or glucose as primary sugar showed that one carbon metabolism is the most affected in presence of fructose. The most relevant genes involved in the pathway in focus here are listed in Tables 3.2 (up-regulated genes) and 3.3 (down-regulated genes).

Table 3.2. Genes up regulated in cells exposed to fructose or glucose showing preferential increase of expression in genes associated with C1 metabolism when the fructose is the carbon source.

Position Fructose	Position Glucose	Gene	Description
1	393	proW	high-affinity transport system for glycine betaine and proline [b2678]
2	26	proV	glycine betaine/L-proline transport ATP-binding protein proV [c_3230]
3	190	proX	glycine betaine-binding periplasmic protein precursor [c_3232]
4	91	proV	ATP-binding component of transport system for glycine, betaine and proline [b2677]
5	45	proX	high-affinity transport system for glycine betaine and proline [b2679]
18	379	gadB	glutamate decarboxylase beta [c_1922]
65	1184	gcd	glucose dehydrogenase [b0124]

Table 3.3. Genes down regulated in cells exposed to fructose or glucose showing a larger decrease of genes involved in glyceraldehyde metabolism when fructose is the carbon source.

Position Fructose	Position Glucose	Gene	Description
3	87	glpA	sn-glycerol-3-phosphate dehydrogenase [b2241]
5	37	glpB	sn-glycerol-3-phosphate dehydrogenase [b2242]
7	30	glpC	sn-glycerol-3-phosphate dehydrogenase [b2243]
8	36	glpB	sn-glycerol-3-phosphate dehydrogenase [c2783]
23	40	glpQ	sn-glycerol-3-phosphate dehydrogenase [b2239]
27	8	glpT	sn-glycerol-3-phosphate permease [ECs3125]
32	4	glpT	sn-glycerol-3-phosphate permease [Z3498]
34	5	glpT	sn-glycerol-3-phosphate permease [b2240]
148	212	puuA	putative glutamine synthetase [b1297]
224	537	pps	phosphoenolpyruvate synthase [b1702]
231	339	Z5618	D-glucitol-6-phosphate dehydrogenase [Z5618]

Four of the five genes that showed the highest level of up-regulation in cells cultured in fructose were involved in glycine metabolism. It has been shown that when fructose increases the flux of pentose production it can also increase the metabolism of glycine in humans[19,25].

Glycine is formed by loss of a hydroxymethyl group from serine, which in turn is synthesized from 3-phosphohydroxypyruvate. It provides the C_2N subunits for the biosynthesis of purines[26]. It also provides C1 equivalents for the formation of 5,10-methylene-THF, which is used in the biosynthesis of thymine[27]. Glycine betaine, or trimethylglycine, is also an important metabolic product since it is a methyl donor for the biosynthesis of methionine from homocysteine. It can also be converted to choline, which is used in the synthesis of phospholipids[28]. An overview of C1 biochemistry involving glycine is shown in Figure 3.5. Cells grown in fructose also displayed a marked inhibition of glycerol-3-phosphate dehydrogenase, which is the enzyme that converts dihydroxyacetone phosphate to glycerol 3-phosphate. When dihydroxyacetone is not converted to glycerol 3-phosphate, it is redirected to form 3-phospho-hydroxypyruvate and consequently serine and glycine[25,29,30]. The ready availability of C1 equivalents was evidenced by the fact that genes involved in the regulation of glycine betaine (N,N,N-trimethyl glycine) production and transport featured prominently in cells cultured in the presence of fructose.

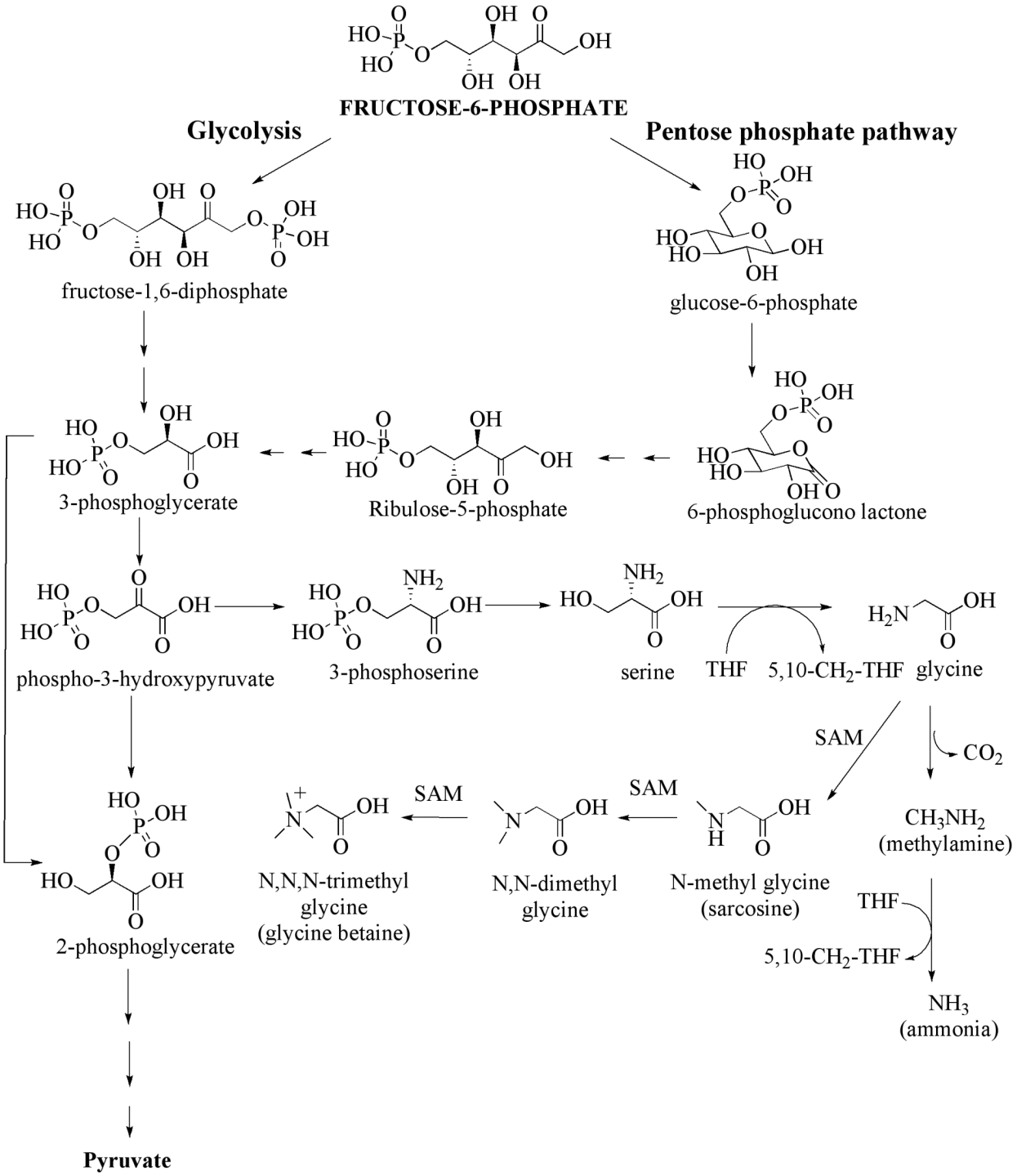


Figure 3.5. Metabolic Pathways affected by fructose. The route towards pyruvate formation is decreased and in direction of 3-phosphoserine/ glycine/ glycine betaine is increased.

It is clear from this study that the utilization of fructose as the major carbon source can strongly influence C1 metabolism. This revolves around the biosynthesis of glycine, which is determined by levels of glyceric acid 3-phosphate. The activation of pentose pathway by fructose[10] greatly augments glyceric acid 3-phosphate levels. Glycine betaine, sarcosine and glycine are important methyl donors for S-adenosyltransferase enzyme (SAM). SAM is the final methyl donor in the majority of methylation reactions within a cell, an important post-transcriptional modification that alters protein function and DNA expression. A hallmark of many diseases is the change of methylation patterns in these molecules. In cancer, for instance, DNA hypomethylation is associated with downregulation of enzymes of the glycine betaine pathway, such as glycine N-methyltransferase[19]. Sarcosine levels are unusually high in metastatic prostate cancer and are considered an important marker for cancer invasion and aggressiveness[31]. The knockdown of glycine N-methyltransferase can decrease the sarcosine concentration and attenuate the progression of the tumor. Furthermore, the addition of sarcosine in benign prostate cells can switch them into an invasive phenotype[31]. Choline, a methylated product formed from glycine betaine, is also found in high concentrations in breast cancers[32]. Altered methylation patterns have also been described in other pathologies, such as autoimmune diseases and cardiovascular disorders.

5,10-methylene-THF production is closely coupled to glycine biosynthesis[30] and should therefore be dependent on fructose intake and metabolism. There are known links between folate biochemistry and cancer establishment and progression[33-36]. This connection is not a simple one. Although well recognized, it is poorly understood. In general, folate levels are associated

with the occurrence of various cancers, including breast, cervical and colorectal cancers. Therapeutic approaches involving dietary supplements of folic acid have yielded mixed results[34-38].

The results presented here illustrate the tremendous effect fructose as a carbon source can have on determining flux through pathways involved in carbohydrate metabolism. Although the overall picture is quite complex, it is clear that the biochemical and physiological fate of the cells are strongly influenced by the type and amount of carbohydrate resources available for growth. This study taken with others point to the chemical underpinnings of the dramatic metabolic shifts that are observed when fructose is a major resource for cell growth and development. The link between carbohydrate processing and disease as it relates to the production and fate of fructose 6-phosphate and the metabolic processes at the root of cancer is especially important. This study is meant to add to the emerging biochemical framework that is necessary for understanding these processes and developing new therapies.

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CHAPTER 4

CHAPTER 4

THE METABOLIC AND BIOCHEMICAL IMPACT OF GLUCOSE 6-SULFONATE (SULFOQUINOVOSE), A DIETARY SUGAR, ON CARBOHYDRATE METABOLISM.

Abstract

Increase in activity of certain carbohydrate pathways (pentose phosphate pathway and glycolysis) is one of the hallmarks of cancer. Sulfoquinovosyl diacyglycerol (SQDG) is a sulfoglycolipid found in many edible plants and it possesses anti-cancer activity that is not demonstrated in the absence of the carbohydrate moiety (glucose 6-sulfonate or sulfoquinovose) from this molecule. The objective of this study was to explore which pathways glucose 6-sulfonate participates in using bacterial systems to further understand the metabolism of this sugar that confers its biological activity. Using ^{13}C-NMR spectroscopy and enzyme assays, it was found that glucose 6-sulfonate, unlike glucose 6-phosphate, cannot enter the pentose phosphate pathway but can competitively inhibit glucose 6-phosphate entry, hence decreasing pentose and nucleotide biosyntheses. In glycolysis, glucose 6-sulfonate only provides one pyruvate per monosaccharide molecule, decreasing the flux of this pathway by half when compared to glucose 6-phosphate (which yields two molecules of pyruvate per monosaccharide). The fact that glucose 6-sulfonate is a metabolic inhibitor of these two pathways may help explain the cytotoxic activity of sulfoquinovose against breast cancer cell lines. This adds to our knowledge of how vegetables rich in SQDG such as spinach, green onions and green tea can also act as metabolic inhibitors of pathways that are increased in diseases such as cancer. This further explains the chemopreventive properties of diets rich in these foods.

Introduction

There is a rising tide of evidence linking the long-term activity in certain carbohydrate processing pathways and the susceptibility to various diseases. In recent times the focus has been on the consumption of large amounts of simple sugars and, in particular, increased amounts of fructose[1]. High fructose consumption has been linked to a broad spectrum of diseases including T2D, hypercholesterolemia, AD and cancer[2-7]. Many of these disorders are associated with alterations in the metabolic activity through the main carbohydrate-processing routes. This has a profound effect on the availability of activated sugars for glycopolymer, glycoprotein, glycolipid and nucleoside syntheses in cells. The main carbohydrate routes generally affected are glycolysis, the pentose phosphate and the hexosamine biosynthetic pathways. These three pathways are interconnected and also coupled to other pathways, such as the TCA cycle. TCA intermediates are used for generating energy as well as for the biosynthesis of several amino acids and heme[8]. Products from glycolysis are also involved in serine and glycine biosyntheses. Acetyl-CoA is the major building block of lipid alkyl chains and sterols. An overview of the interconnectivities between these various carbohydrate-derived chemical processes and the products derived from them are presented in Figure 4.1.

Figure 4.1 shows that carbohydrate chemistry is central in cellular biochemistry and should therefore be an important determinant of the state of health of organisms. Evidence shows that the level of activity in certain branches of this web in Figure 4.1 is connected with the occurrence of certain diseases. For instance, increased flux through the HBP is linked to T2D, AD and cancer[9,10,11]. Hyperactivity in the PPP has been linked to cancer and excessive production of nucleic acids[12]. Heavy use of the glycolytic pathway is associated with cancer and

the characteristic over acidification of cells due to high lactic acid production[13,14,15]. Decreased activity through TCA cycle is found in cancer, T2D and AD[16,17,18]. Understanding the dynamics of the processes between the segments of the various pathways and determining ways to apportion the activities between the various branches represent critical goals for developing a metabolism-based approach to disease management.

Another important way of regulating the level of activity in certain segments of the highly interconnected chemical processes is through the use of chemical inhibitors to reduce flux into particular pathways. A specific inhibitor that targets only one or two chemical steps might be employed. A broader strategy utilizing a carbohydrate compound of dietary significance that is capable of generating intermediates that inhibiting several steps or skewing the activity through one or more pathways in a favorable fashion is also very valuable.

Glucose 6-sulfonate (sulfoquinovose) is an important sugar present in human diet. It is found in all plants to varying extents as the free sugar, a glyceryl glycoside, or in combination with diacylglycerol to form sulfoquinovosyl diacylglycerol (SQDG). It is present in high amounts in spinach, green tea, and other green leafy plants[19,20,21]. When these various forms are factored in, the availability of glucose 6-sulfonate from some plants can be 10 times larger than some amino acids[22]. In these amounts it can exert a profound influence on carbohydrate metabolism in humans.

Not much is known about the metabolism of glucose 6-sulfonate in mammals although it has been shown that microbial flora in the digestive tract of guinea pigs are responsible for most of the degradation of SQDG[23]. Guinea pigs are herbivores and food processing takes place under anaerobic conditions in a complex microbial environment. The degradation begins with the

deacylation by lipases to form the glycerol glycoside of glucose 6-sulfonate. The study could not identify the next steps but the rapid elimination of sulfate was observed. This is to be expected because sulfate is used in a dissimilatory function as a terminal electron acceptor and assimilatory function for synthesis of cysteine, methionine and other sulfur containing compounds by rumen microorganisms. These processes, however, do not represent the pathways used in humans for the catabolism of sulfoquinovose and its derivatives.

Studies on its metabolic fate in bacteria shows that sulfoquinovose probably follows a catabolic path in which it is converted to fructose 6-sulfonate (a parallel to the conversion of glucose 6-phosphate to fructose 6-phosphate) and from then follows a pathway that is similar to the glycolytic breakdown of fructose 6-phosphate. There is one importance difference because sulfolactate is formed from one half of the molecule instead of glyceraldehyde 3-phosphate[24]. There is not much known about the metabolism of glucose 6-sulfonate to other pathways and which intermediates are actually formed in the process. As illustrated in Figure 4.1, there are several points at which glucose 6-sulfonate or metabolic products (such as glucosamine 6-sulfonate[25]) from sulfoquinovose can modulate activity through several other elements of carbohydrate metabolism. This potential modulation opens up a path for potentially tailoring metabolic activity to confine it within certain regimes as a strategy for disease management.

The evaluation of the status of a complex biosynthetic web is facilitated by non-destructive analytical methods that do not perturb the chemical processes in evaluation and also allow a quantitative or semi-quantitative analysis of the extent of reaction in the different pathway segments in real time. The method used should allow the identities and relative amounts of the products being formed. Nuclear Magnetic Resonance (NMR) spectroscopy is a well-established non-destructive analytical method that can give information on the identity and

quantity of analytes provided the isotopic abundance of the nucleus being probed is high enough. Studies based on the carbon-13 enriched analytes are especially valuable and have been used to evaluate metabolic events in very complex systems[26-29]. This technique was employed in the current study to follow the catabolism of fructose 6-phosphate, glucose 6-sulfonate, and fructose 6-phosphate in the presence of glucose 6-sulfonate. Using ^{13}C-NMR spectroscopy, we are able to readily identify signals for 6-phosphogluconic acid, glucose 6-sulfonate, glucose 6-phosphate, fructose 6-phosphate, serine, aspartate, glutamate, glutamine, acetate, lactic acid, alanine and ethanol.

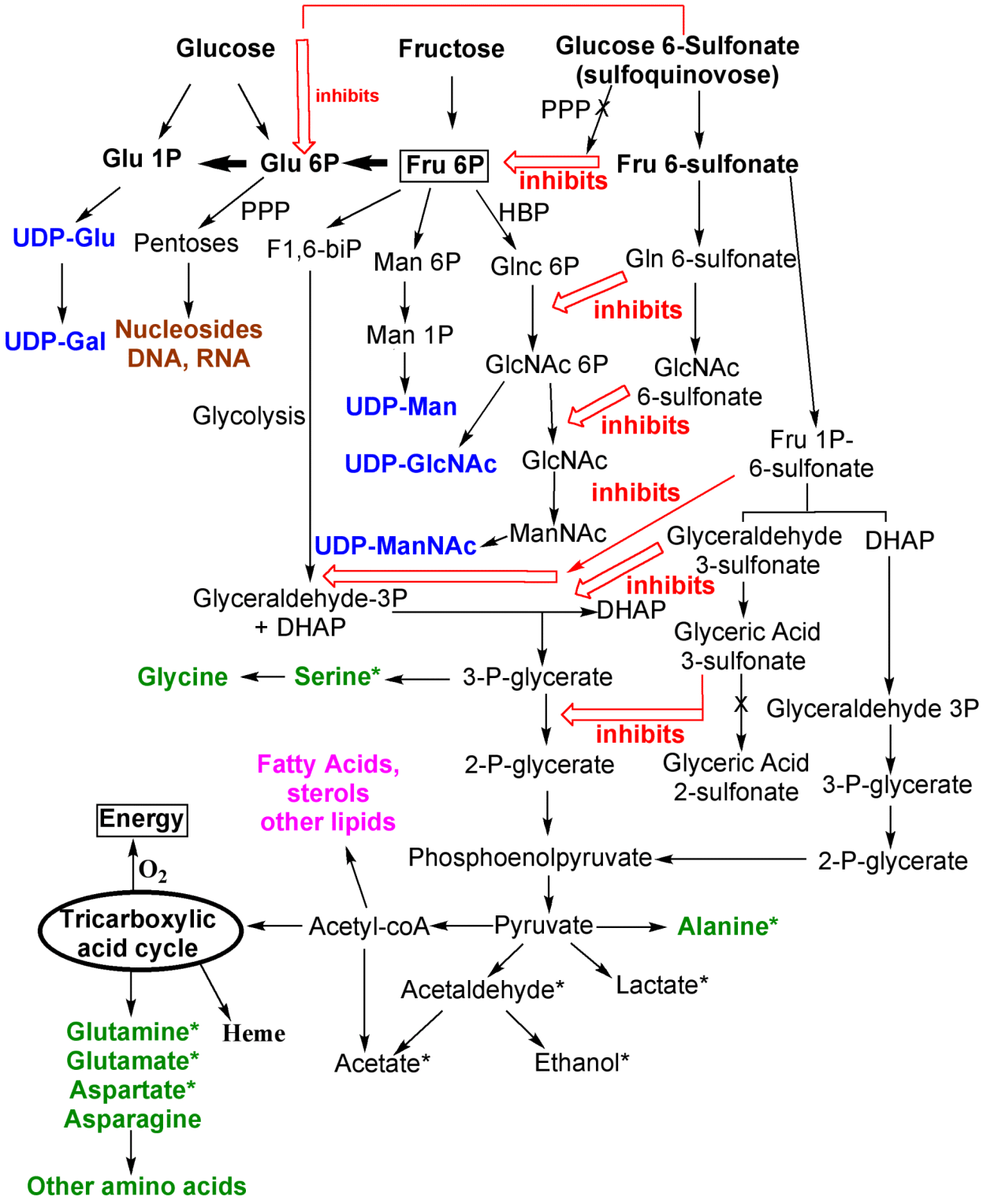

Figure 4.1. Metabolic web. The colors indicate the different classes of biosynthetic pathways. Biosynthesis of: lipids (pink); amino acids (green); nucleotides (brown); UDP-sugars (blue). Red

represents which carbohydrate pathways could potentially be inhibited by glucose 6-sulfonate or its derivatives. *Represents the molecules detected in the ^{13}C-NMR studies.

Materials and Methods

1. NMR Studies

1.1. Cell culture

Escherichia coli cells were grown in minimal media M9 containing 4% glucose at 30°C until logarithmic phase was reached. Cells were then precipitated by centrifugation at 3000 rpm for 20 min and the pellet disrupted at 4°C by sonication in 50 mM Tris-HCl buffer pH 7.6. This procedure for obtaining the bacterial lysate was employed for all NMR experiments. The volume of the lysate was reduced and used directly in subsequent studies.

1.2. Synthesis of ^{13}C-1-fructose 6-phosphate

^{13}C-1-Fructose 99% (250 mg) (Sigma) was converted to ^{13}C-1-fructose 6-phosphate by incubation with 3 units of hexokinase (Sigma-Aldrich) in 50 mM Tris-HCl 13.3 mM $MgCl_2$ (600 μL) buffer for 48 hr at 37°C. The conversion was monitored by ^{1}H- and ^{13}C-NMR.

1.3. Synthesis of ^{13}C-1-glucose 6-sulfonate (^{13}C-1-sulfoquinovose)

The overall scheme of biosynthesis of ^{13}C-1-glucose 6-sulfonate is illustrated in Figure 4.2. ^{13}C-glucose was first converted to its methyl glucoside by Fisher glycosidation[30]. ^{13}C-1-glucose (Sigma) (250 mg), methanol (100 mL) and sulfuric acid (0.2 mL) were refluxed

overnight to form compound **2** (^{13}C-1-methyl-glucose). Sodium bicarbonate (1 g) was added to the methanol solution of **2** and the mixture stirred, filtered and concentrated under reduced pressure to give crude **2**, which was used without further purification. Compound **2 (**250 mg) was mixed with pyridine (5 mL), triphenylphosphine (0.6 g) and carbotetrabromide (0.6 g). The mixture was stirred overnight at room temperature to form the bromo compound **3.** Compound **3** was dried, ressuspended in water (10 mL) at 4°C to precipitate triphenylphosphine and triphenylphosphine oxide. The supernatant was decanted and passed through a mixed bed ion exchange resin column (H^+/ OH^-) and concentrated under reduced pressure. The residue compound **3** was mixed with sodium sulfite (0.5 g) in water (10 mL) and stirred for 3 hr at 80°C to form the sulfonate compound **4**, which was passed through a strong acid ion exchange resin (H^+ form) to remove pyridine and other cation ionic impurities. The solution was dried and mixed with 2 M HCl in water (10 mL) and heated for 2 hr at 120°C to give compound **1** (^{13}C-1-6-deoxy-6-sulfo-D-glucopyranose).

The acid was removed before 1 was used for biological assays by stirring in methanol containing excess sodium bicarbonate, checking for neutrality, filtering and concentrating.

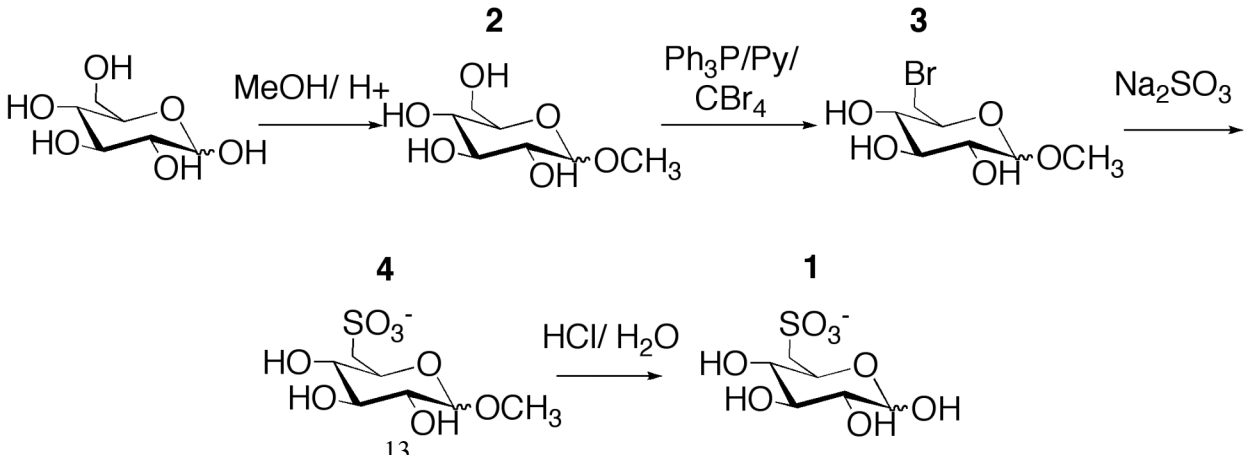

Figure 4.2. Synthetic route to ^{13}C-1-D-glucose 6-sulfonate (1).

1.4. NMR Analyses

NMR spectra were measured at ambient temperature on a Varian Anova 600 MHz equipped with a 5 mm Pulse-Field-Gradient switchable broadband probe operating at 599.804 MHz (^{1}H) and 150.83 MHz (^{13}C). One-dimensional ^{1}H were referenced to the solvent residual peak and ^{13}C were referenced to standards of known frequency. Semi quantitative ^{13}C was obtained with a relaxation delay of 5 s and inversed-coupled gated. ^{1}H NMR spectra was obtained using a spectral width of 8000 Hz over 64000 data points and ^{13}C in a spectral width of 36200 Hz over 94000 points and multiplied by an exponential function corresponding to a 0.50 Hz broadening prior to Fourier transformation. Spectra were obtained in buffered water as indicated containing 13% D_2O in order to obtain a stable lock signal.

1.4.1. ^{13}C-1-Fructose 6-phosphate studies

^{13}C-1-fructose 6-phosphate (20 mg) was mixed with glutamine (40 mg), *E. coli* lysate (300 μL) and 50 mM Tris-HCl pH 7.6 (400 μL). The reaction incubated at 37°C and monitored by ^{13}C NMR at 0, 1, 2, 3, 4, 5, 24 and 144 hr.

1.4.2. ^{13}C-1-Glucose 6-sulfonate (sulfoquinovose) studies

^{13}C-1-glucose 6-sulfonate (20 mg) was mixed with glutamine (40 mg), *E. coli* lysate (300 μL) and 50 mM Tris-HCl pH 7.6 buffer (400 μL) and the reaction incubated at 37°C and monitored by ^{13}C NMR at 0, 1, 2, 3, 4, 5, 24 and 144 hr.

To analyze if ^{13}C-1-glucose 6-sulfonate is an inhibitor of enzymes involved in the metabolism of fructose 6-phosphate, 20 mg of ^{13}C-1-fructose 6-phosphate and 20 mg of ^{13}C-1-glucose 6-sulfonate were mixed with glutamine (40 mg), *E. coli* lysate (300 μL) and 50 mM Tris-HCl pH 7.6 buffer (400 μL) and the reaction incubated at 37°C and monitored by ^{13}C NMR at 0, 1, 2, 4, 5 and 24 hr.

2. Glucose 6-phosphate Dehydrogenase (G6PDH) Enzyme Activity Assay

Glucose 6-phosphate (6.25, 12, 25, 50 and 100 μM) was mixed with 488 μM NADP and 150 mM HEPES buffer pH 7.2 up to a volume of 250 μL. The absorbance was monitored at 334 nm. Once the background was established, 0.25 U of G6PDH was added and the conversion to 6-phosphogluconolactone monitored at 334 nm by the coupled formation of NADPH. All the experiments were done in triplicate. The reaction rate in each time point was calculated as follows: OD (observed) * volume (250 μL) / 6270 * minutes (0.4). K_m was calculated from the linear regression obtained from the Hanes-Woolf plot.

To evaluate if glucose 6-sulfonate is a substrate for G6PDH, 50 100, 200 and 400 μM of glucose 6-sulfonate, 488 μM of NADP and 150 mM HEPES buffer pH 7.2 were mixed up to a total volume of 250 μL and the reaction monitored by the same method described above.

To evaluate the potential inhibition of G6PDH by glucose 6-sulfonate, 50 μM of glucose 6-phosphate, 488 μM of NADP and 150 mM HEPES buffer pH 7.2 were mixed to glucose 6-sulfonate at 50, 100, 200 and 400 μM up to a total volume of 250 μL. To calculate K_I for this inhibitor, the following formula was used:

$$v = \frac{V_{max}\,[S]}{[S] + K_m\,(1 + [I]/K_I)},$$

Where [S] is the concentration of glucose 6-phosphate, K_m of G6PDH, [I] the concentration of glucose 6-sulfonate under those conditions of reaction rate (*v*). Solver in Excel was used to minimize the sum of errors squared.

3. Glucose 6-sulfonate Cytotoxicity Assay

MCF-7, LCC9 and 184B5 cell lines were cultured in IMEM media in 10% FBS, 1% Penicillin/ Streptomycin. The first two are breast cancer lines while the last one is a non-tumorigenic epithelial breast cell line. All cell lines were plated at 96-well plates at densities experimentally determined. Glucose 6-sulfonate at 11, 22, 44 and 88 mM was added to the cell lines and incubated for 48 hr at 37°C at 5% CO_2. These concentrations were chosen to result in ratios between glucose (present in the media at 11 mM) and glucose 6-sulfonate of 1:1, 1:2, 1:4 and 1:8. Cells were fixed with 50% trichloroacetic acid for 1 hour, washed under water and stained with 0.4 % (w/ v) of sulforhodamine B for 30 min[31]. Cells were washed with 1% acetic acid and resuspended with 10 mM Tris Base buffer and the absorbance measured at 540 nm. All samples were done in triplicate.

Results and Discussion

^{13}C-1-Glucose 6-sulfonate was successfully synthesized as evidence by the NMR profile. A mixture of purified isomers of 1-methyl-glucose 6-sulfonate is shown in Figure 4.3, with the following NMR signals: ^{1}H-NMR (D_2O, 600 MHz) δ 4.97 (d, 1H, *J* = 4.01 Hz) H1α; 4.72 (d, *J*

= 4.01Hz) H1β; 3.91 (t, 1H, *J* = 11.61 Hz) H3α; 3.58 (t, 1H, *J* = 9.29 Hz) H4α; 3.46-3.53 (m, 2H) H3β and H4β; 3.38 (s, 3H) CH_3α; 3.32 (s, 3H) CH_3β; 3.17-3.30 (m, 2H) H5 and H6 (α + β); 2.98-3.12 (dd, 2H) H6'α+β.

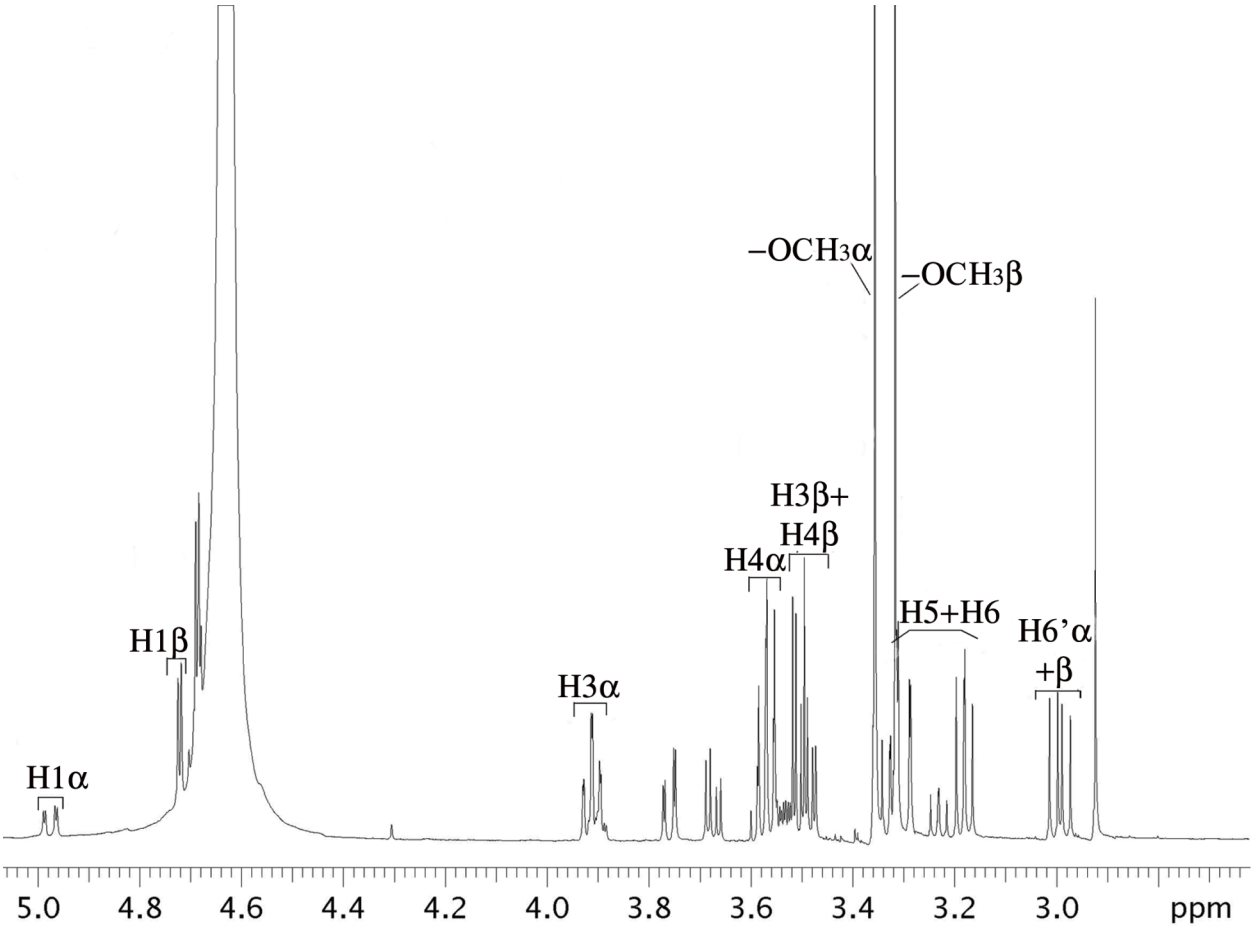

Figure 4.3. ^{1}H-NMR Chemical Shits of a mixture of isomers of ^{13}C-1-methyl-glucose 6-sulfonate.

Both ^{13}C-1-glucose 6-sulfonate and ^{13}C-1-fructose 6-phosphate synthesized by chemical or enzymatic reactions were used for profiling the metabolites originated after 1, 2, 4, 5 and 144

hr of incubation with the cellular pool of enzymes obtained from *E. coli*. Small peaks started to appear in the NMR profiles after 5 hr of incubation, but the most significant changes in the profile were established after 144 hr; therefore, the signals listed in Table 4.1 refer to this last time point (144 hr).

The incubation of the cell lysate with ^{13}C-1-fructose 6-phosphate generated large quantities of ethanol, alanine, lactic acid and significant amounts of acetic acid, glutamine, glutamate, aspartate and serine and 6-phosphogluconate (Table 4.1). The production of ethanol, alanine, lactic acid, acetic acid, alanine and serine are directly related to the entry of fructose 6-phosphate into glycolysis. Glutamine, glutamate and aspartate are derived from entry of pyruvate into the Krebs cycle, in which intermediates can be used for amino acid biosynthesis as well.

Entry into the PPP is detected by the signal at 181 ppm, which corresponds to the formation of 6-phosphogluconolactone from glucose 6-phosphate. High instantaneous concentrations of fructose 6-phosphate can cause a shift towards the production of high quantities of glucose 6-phosphate due to the equilibrium constant of the phosphoglucose isomerase ($K_{eq[Glu6P/Fru6P]}$~3)[32]. Elevated concentrations of fructose 6-phosphate induces a higher activity down the PPP[33]. Fructose can induce an increase of 250% in the transketolase activity (an enzyme from the non-oxidative stage of the PPP) in comparison to glucose in pancreatic cancer cells[33]. This important link shows that fructose causes a stronger induction of nucleotide biosynthesis, and therefore cell division and proliferation, in comparison to glucose.

When ^{13}C-1-glucose 6-sulfonate is used as carbon source, it produces many of the same metabolites as ^{13}C-1-fructose 6-phosphate, with direct evidence of the biosynthesis of alanine,

lactic acid, pyruvate, glutamine, glutamic acid, acetic acid, aspartate, asparagine and cysteine (Table 4.1). Many of these metabolites are derived from the activity of glycolytic enzymes. Our ^{13}C-NMR data corroborates the literature[24] that shows that glucose 6-sulfonate does go through glycolysis by the detection of methyl derivatives such as lactic acid, acetic acid, pyruvate and alanine in the region 15-30 ppm. However, the formation of 6-phosphogluconolactone (which is detected at 181 ppm) was not observed in this case, indicating that glucose 6-sulfonate cannot be metabolized by G6PDH, which is the enzyme responsible for this conversion on the first step of PPP.

Table 4.1. Resonances obtained from different treatments after 144 hr of incubation. Compounds that are uniquely found in the respective treatments are marker with an asterisk.

Fructose 6-phosphate		**Glucose 6-sulfonate (sulfoquinovose)**	
Chemical shift ^{13}C	**Compound identified**	**Chemical shift ^{13}C**	**Compound identified**
16.5	Ethanol*	19.6	Alanine
19.7	Alanine	23.6	Acetic acid
22.5	Lactic acid*	26.7	Cysteine*
23.2	Acetic acid	27.1	Pyruvate*
32.4	Glutamine	30.4	Glutamine
33.6	Glutamic acid*	36.7	Asparagine*
38.5	Aspartate	37.6	Aspartate
63.8	Serine	62.8	Serine
67	Fructose 6-phosphate	98.9	Glucose 6-sulfonate
180.6	6-Phosphogluconolactone*		

When cell lysates are mixed with both ^{13}C-1-fructose 6-phosphate and ^{13}C-1-glucose 6-sulfonate, the majority of the products formed are the same as if fructose 6-phosphate was incubated alone (Table 4.2). Glucose 6-sulfonate does not participate nor impede in fructose 6-phosphate metabolism at the concentration used as observed by the formation of intermediates of

the PPP (6-phosphogluconolactone) and glycolysis (alanine, lactic acid) (Table 4.2). A comparison of the metabolic routes and intermediates observed when fructose 6-phosphate or glucose 6-sulfonate is the sole carbon sources incubated with cell lysates is depicted in Figures 4.4 and 4.5.

Table 4.2. Resonances obtained from different treatments after 24 hr of incubation.

Fructose 6-phosphate + Glucose 6-sulfonate	
Chemical shift ^{13}C	**Compound identified**
19.6	Alanine
22.8	Lactic acid
25.9	Cysteine
35	Glutamic acid
36.2	Asparagine
41	Aspartate
63.9	Serine
66.2	Fructose 6-phosphate
99	Glucose 6-sulfonate
183.3	6-Phosphogluconolactone

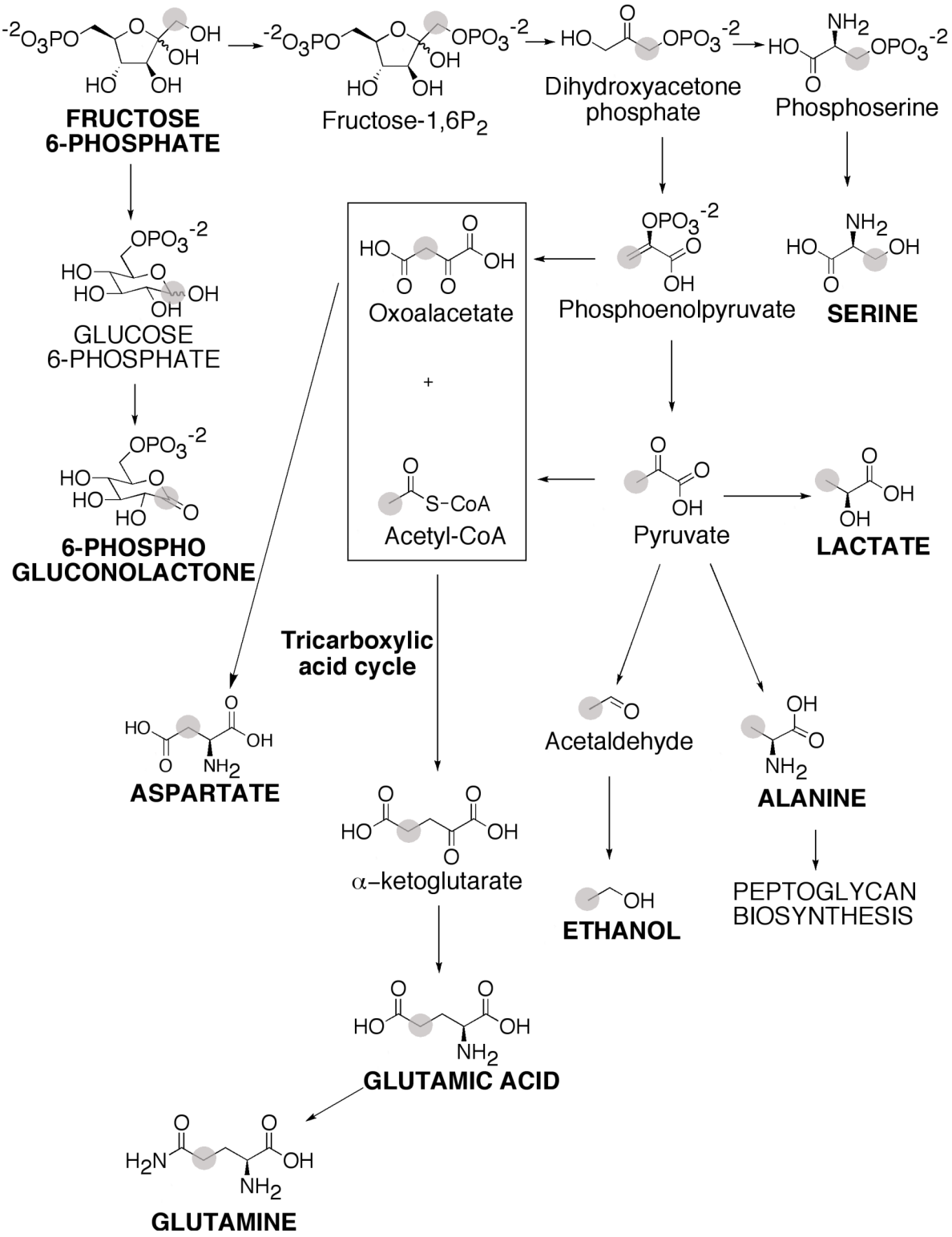

Figure 4.4. Fructose 6-phosphate metabolic pathways observed by ^{13}C-NMR after 144 hr of incubation with cell lysate.

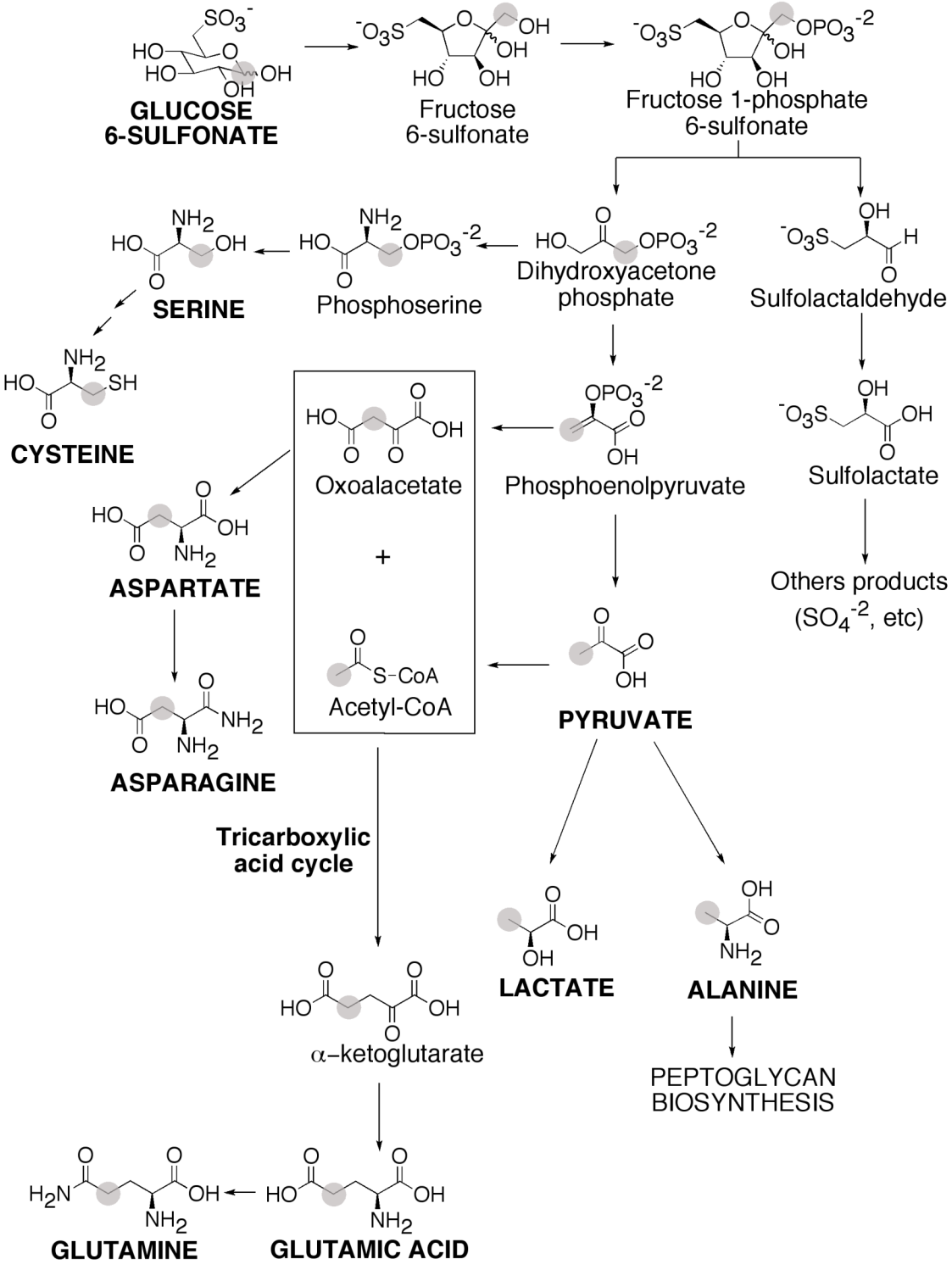

Figure 4.5. Glucose 6-sulfonate (sulfoquinovose) metabolic pathways observed by ^{13}C-NMR after 144 hr of incubation with cell lysate.

To better understand the possible role of glucose 6-sulfonate as an inhibitor of the pentose phosphate pathway, an enzymatic assay using glucose 6-sulfonate as substrate was performed. When glucose 6-sulfonate is incubated alone with the enzyme G6PDH (the enzyme from the first step of PPP), less than 1% of enzyme activity is detected. This result confirms that the absence of a signal for 6-phosphogluconolactone in the NMR study is because glucose 6-sulfonate is not a substrate for G6PDH and, therefore, cannot form 6-sulfogluconolactone and enter pentose biosynthesis.

When analyzing if glucose 6-sulfonate can act as a competitive inhibitor of glucose 6-phosphate for its utilization by the enzyme G6PDH, glucose 6-sulfonate incubated in increasing concentrations with glucose 6-phosphate was found to inhibit enzyme activity. The K_I of this competitive inhibitor was high (K_I= 2.84 mM) in comparison to the K_m for glucose 6-phosphate (K_m= 56.45 μM). This is confirmed by the fact that glucose 6-sulfonate does not impede nor participate in fructose 6-phosphate metabolism in the concentrations used when both substrates are provided for a cell lysate and the reactions monitored by ^{13}C-NMR (Table 4.2).

In physiological concentrations that are exclusively associated with the ingestion of glucose 6-sulfonate through diet, this molecule is not a relevant inhibitor of the PPP. The percentage of SQDG in spinach, for instance, is 1.7% of the dry weight[34,35], meaning that the ingestion of 100 g of spinach by a person weighing 75 kg would result in blood levels of 19 μg/mL (assuming that 60% is water). For achieving concentrations of 2.84 mM to act as an effective inhibitor of G6PDH, the concentration sulfoquinovose in the blood would need to be 690 μg/mL. Although these numbers show that the use of sulfoquinovose as an inhibitor of

pentose biosynthesis cannot be achieved through only its ingestion in diet, it is a promising result for the potential use of glucose 6-sulfonate as a pharmacological drug.

When analyzing the quaternary structure of G6PDH[36], it is noticeable that three residues are especially important to stabilize the charge and accommodate the phosphate group of glucose 6-phosphate in the active site. These residues are His178, Tyr179 and Lys182. This is supported by site directed-mutagenesis studies showing that the substitution of these residues, in particular H178N, decreases the ability of the enzyme to discriminate between glucose and glucose 6-phosphate. It is proposed that His178 interacts with the phosphate moiety through hydrogen bonds and charge-charge interactions. Apparently, the substitution of this amino acid increases the distance between the three residues and to the phosphate moiety, which affects the binding of glucose 6-phosphate to the enzyme. If the distance between the H-bond donor ligands and the phosphate group is longer than 9Å, the interaction is disrupted[36].

The structure of glucose 6-sulfonate contains the sulfonate group directly attached to the C6 of the glucopyranose, without an oxygen molecule bridging the bond between SO_3^- and the C6 in glucose 6-phosphate. The lack of this oxygen increases the distance between the three residues mentioned above (His178, Tyr179 and Lys182) and also between these residues and the substrate's charged group. As mentioned before, if this distance is longer than 9Å, the interaction is interrupted. Therefore, the increased distance between the SO_3^- group and the enzyme residues should be an important factor in decreasing the interaction between the G6PDH and glucose 6-sulfonate. This can help explain why glucose 6-sulfonate was unable to serve as substrate to G6PDH to form 6-phosphogluconolactone.

Another characteristic apparently important for glucose 6-phosphate interaction with G6PDH is the divalent charge of its phosphate group (PO_4^{-2}). It is reported that double charged anions such as phosphate (PO_4^{-2}), carbonate (HCO_3^{-2}) or sulfate (SO_4^{-2}) are able to inhibit the enzyme activity by occupying the site pertinent to the phosphate moiety in the active site. Single charged ions (Cl^-), however, inhibit the enzyme very poorly[37,38]. Glucose 6-sulfonate possesses a valence of 1, due to the replacement of the phosphate (PO_4^{2-}) by the sulfonate (SO_3^-) group. This is probably another reason contributing to the inability of this enzyme to use glucose 6-sulfonate as substrate and why this inhibitor has a high K_I.

The pentose phosphate pathway is responsible for the biosynthesis of riboses, purines and pyrimidines, which makes this pathway one of most important control points of nucleic acid biosynthesis through the regulation of the availability of substrates involved in their formation[39]. An important reaction that regulates the flux through this pathway is the one catalyzed by G6PDH. The suppression of the G6PDH activity can cause inhibition of proliferation and cell death[40]. On the other hand, a hyper activation of PPP is characteristic of cancer cells[39]. Therefore, drugs that can inhibit this pathway by competitively blocking enzyme activity, and therefore, blocking the elevated level of nucleic acid biosynthesis, are promising molecules for cancer therapy.

The ability of glucose 6-sulfonate to be metabolized through glycolysis but not PPP makes it an attractive molecule for evaluation on biological systems that require high rates of glycolysis as well as high rates of nucleotide biosynthesis. Because of that, the potential activity of glucose 6-sulfonate as cytostatic or cytotoxic agent was evaluated in different breast cancer

cell lines. Glucose 6-sulfonate at 1, 2, 4 and 8 times the molar concentration of glucose in the media was added to two breast cancer cell lines and one non-tumorigenic breast line. Figure 4.6 shows that glucose 6-sulfonate has a cytostatic effect on all lines from 11 to 44 mM and starts to show an intense cytotoxic effect in one cancer cell line (MCF-7) between 44 and 88 mM.

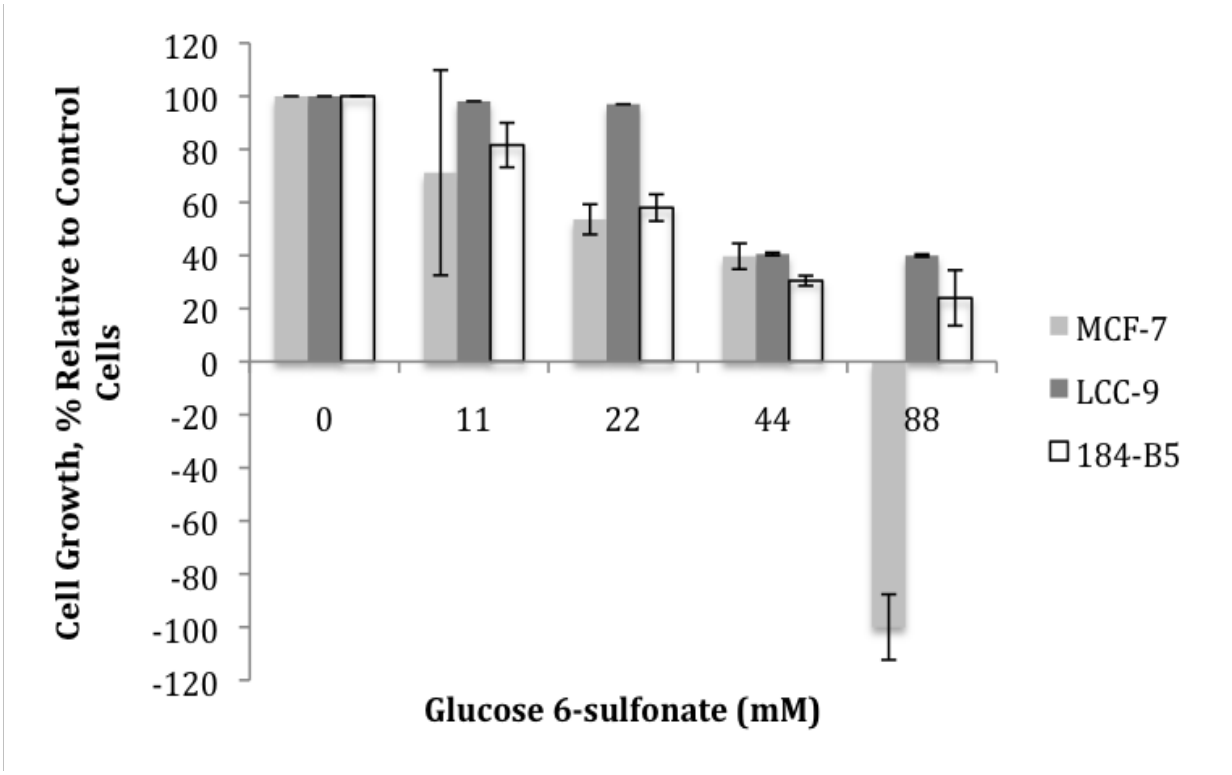

Figure 4.6. Cell growth in presence of glucose 6-sulfonate. Cells were treated for 48 hours with 0, 11, 22, 44 and 88 mM of glucose 6-sulfonate and their cell growth was indirectly calculated by a spectrophotometric meaurement (540 nm) of their protein content. In the Y-axis, negative cell growth represents cell death.

Sulfoquinovosyl diacylglycerol (SQDG) is described to have many biological properties, acting as an anti-viral[41], anti-tumoral[42-49], anti-angiogenic[50] and anti-bacterial[51] molecule. In most of these studies, the carbohydrate portion (glucose 6-sulfonate) from SQDG is shown to be

essential for the attributed activity and our findings contribute to explain why glucose 6-sulfonate moiety is important for the described activities of SQDG. The inability of a cell to have a proper rate of nucleic acid biosyntheses (as shown by the absence of 6-phosphogluconolactone formation when glucose 6-sulfonate is the carbon source) and energy (through glycolysis) help explain why SQDG possesses cytotoxicity and the other biological activities. Due to the remarkable activity that molecules containing glucose 6-sulfonate have, further knowledge of the metabolic pathways glucose 6-sulfonate *per se* is involved is crucial in order to explore this molecule and its derivatives as potential drugs.

The data described in Table 4.1 also shows evidence for glucose 6-sulfonate metabolism through glycolysis by the detection of compounds such as ethanol, acetic acid and lactic acid. The involvement of glucose 6-sulfonate in the glycolytic pathway has been previously described and our data corroborate these previous findings[24]. An important point about glucose 6-sulfonate metabolism through glycolysis is that glucose 6-sulfonate could possibly act as an inhibitor of glycolysis as well. While each molecule of fructose 6-phosphate can form two molecules of pyruvate, one molecule of glucose 6-sulfonate only provides one molecule of pyruvate. Because of the inability of a mammalian cell to break the carbon-sulfur bond between the sulfonate and C6 of glucopyranose, only half of the molecule can be converted to glyceraldehyde 3-phosphate. A proposed route of metabolic conversion for glucose 6-sulfonate and its ability to act as an inhibitor of carbohydrate-related pathways is illustrated in Figure 4.1.

In this study, I report a natural component of the diet, glucose 6-sulfonate (sulfoquinovose), which is obtained by consumption of plants, algae and certain cyanobacteria as food or in supplements, to have inhibitory properties over the pentose phosphate pathway and glycolysis. This study adds to the body of knowledge in the field of biochemical nutrition of why

the consumption of vegetables containing high amounts of sulfonate sugars can have chemo protective activities. This can be clear seem in many epidemiological studies showing that consumption of these vegetables are also associated with a lower rates of disorders of carbohydrate metabolism, such as cancer and type 2 diabetes[52,53].

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CHAPTER 5

CHAPTER 5

GLUCOSAMINE 6-SULFONATE (2-AMINO-2,6-DIDEOXY-6-SULFO-D-GLUCOSE): HOW CAN DIET HELP INNATE DEFENSE?

Abstract

A broad-spectrum antibacterial agent, glucosamine 6-sulfonate, was shown to possess anticancer activity *in vitro* especially against a highly metastatic breast cancer cell line. Since this compound is likely biosynthesized from glucose 6-sulfonate, a natural sugar in the diet, we also investigated the possibility that glucosamine 6-sulfonate occurs naturally *in vivo*. Glucosamine 6-sulfonate is found as a natural component in mammalian blood in the μg/ mL range. I propose that glucosamine 6-sulfonate could also be a potential anticancer agent reasonably tolerated since it occurs naturally in humans. In addition, this compound might already contribute to innate immunity against blood-borne pathogens and pre-cancerous cells.

Introduction

Glucosamine 6-sulfonate[1] (2-amino-2,6-dideoxy-6-sulfo-D-glucose, **1**, Figure 5.1) is an analogue of glucosamine 6-phosphate and inhibits the transfer of the amino group of glutamine to fructose 6-phosphate by the enzyme glucosamine-6-phosphate synthase (GlmS or glutamine: fructose-6-phosphate amidotransferase (GFAT)[1]. It has been demonstrated that glucosamine 6-sulfonate is so similar in structure to glucosamine 6-phosphate that it can serve as substrate for the glucosamine-6-phosphate acetyltransferase (GAT, EC 2.3.1.4), which converts glucosamine 6-phosphate to GlcNAc 6-phosphate. The ability of **1** to inhibit these two enzymes that are critical for glucosamine synthesis resulted in it having broad-spectrum antibacterial activity by

inhibiting bacterial cell wall synthesis in both Gram-negative and Gram-positive organisms[1]. Since some antibacterial agents (e.g. adryaminic, daunorubicin)[2] have been shown to be useful in cancer therapy, we investigated in this study if glucosamine 6-sulfonate possesses anticancer activity *in vitro* against two breast cancer cell lines. I used a non-metastatic and a highly metastatic cell lines to test its spectrum of activity.

A consideration of many anticancer agents is their significant toxicity. I investigated whether glucosamine 6-sulfonate is found *in vivo* since a natural component of the diet might exhibit less toxicity. If we analyze the potential natural precursors for glucosamine 6-sulfonate, the probability of its occurrence in mammalian systems is high. The idea is that glucosamine 6-sulfonate would originate from glucose 6-sulfonate, which is a component of the naturally occurring plant lipid SQDG[3] where it is covalently linked to diacylglycerol. Glycosidase action on SQDG should lead to free glucose 6-sulfonate when plant materials are included in the diet[4]. The percentage of SQDG in spinach has been reported to be 1.7% of the dry weight[5,6]. This means that ingestion of 100 g of spinach by an individual weighing 75 kg would result in blood levels of 19 μg/mL assuming that 60% of the individual was water and that the sugar was released all at once and evenly distributed. This number does not include free sulfoquinovose that might be present in the cytoplasm of the plant cell. If phosphoglucose isomerase (EC 5.3.1.9), the enzyme that converts glucose 6-phosphate to fructose 6-phosphate[7], acts on glucose 6-sulfonate to form fructose 6-sulfonate and this were a substrate for GFAT, then it should be expected that glucosamine 6-sulfonate would be a naturally occurring metabolite. It has been shown that glucose 6-sulfonate can be converted to fructose 6-sulfonate at least in bacteria

through the demonstration that glucose 6-sulfonate (sulfoquinovose) can participate in the glycolytic pathway after its conversion to fructose 6-sulfonate[8]. Besides, the demonstrated tolerance of enzymes in the hexosamine biosynthesis pathway for substitution of phosphate groups by sulfonates[1] makes it reasonable to propose that fructose 6-sulfonate would serve as a substrate for GFAT leading to the enzymatic production of glucosamine 6-sulfonate (Figure 5.1). This means that glucosamine 6-sulfonate might be produced *in vivo* if organisms are exposed to glucose 6-sulfonate. This has special significance because it would serve as a mechanism for modulating the activity through the hexosamine biosynthesis pathway by inhibiting the formation of glucosamine 6-phosphate. As well established in the literature, hexosamine biosynthesis is very important for bacteria growth[9,10] and is increased in cancer[11]. If glucosamine 6-sulfonate is naturally produced, one may speculate that it might be one of the mechanisms by which some vegetables (such as spinach) may have antibacterial and anticancer properties.

In this study, I address two important questions of whether (1) glucosamine 6-sulfonate has anticancer activity and (2) if it occurs naturally in mammals by analyzing the blood of cows and humans. To accomplish the last aim, I developed a method for derivatization and identification of glucosamine 6-sulfonate in complex mixtures using HPLC, UV spectroscopy and mass spectrometry. The significance of a positive finding is great because it would then be an effective antibacterial and anticancer molecule that occurs naturally in mammals. This has significant clinical implications from the standpoint of safety especially if the concentrations that inhibit the growth of microbes and cancer cells are only slightly higher than the concentration found in people.

Materials and Methods

1. Evaluation of glucosamine 6-sulfonate anticancer activity

MDA MB.231 and MCF-7 breast cancer cell lines were cultured in DMEM medium with 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin in 5% CO_2 at 37ºC. For cytotoxicity evaluation, cells were plated in 96 well plates in densities experimentally determined and the concentrations of glucosamine 6-sulfonate **[1]** previously synthesized were added as follows: 200, 300, 400, 500, 750 and 1000 μg/mL. Cells were grown for 48 hr at 37ºC in 5% CO_2 and cell confluence determined by cell counting in a Nikon inverted microscope. The IC_{50} for each cell line was determined by a non-linear regression. All samples were performed in duplicate.

2. Design and detection of glucosamine 6-sulfonate in vivo

2. 1. Development of Method: for detection of glucosamine 6-sulfonate, we used glucosamine 6-sulfonate that was previously synthesized by our group as a standard[1]. Glucosamine 6-sulfonate (10 mg) (standard) was derivatized by mixing it with methanol (100 μL), water (100 μL), 3,5-dinitrobenzoyl chloride (3,5-DNB-Cl) (30 mg) and sodium bicarbonate (30 mg) with vigorous stirring for 48 hr at room temperature. Acetic acid (50 μL) was added, the mixture dried and eluted through a reverse phase column (250 mg C18 resin) in 2:1 water: methanol (total volume 500 μL). This solution (20 μL) was injected on an HPLC column (Aminex HPX-97H) using 0.018 M H_2SO_4 as eluent. The UV spectroscopy measurements were obtained in aqueous solution in the range of 240-400 nm. The mass spectrometry profile was obtained by electrospray ionization in negative ion mode.

2.2. Detection of glucosamine 6-sulfonate in vivo: for investigation of the possible presence of glucosamine 6-sulfonate *in vivo*, cow (10 mL) and human (10 mL) blood were used. Both samples were clotted at 4°C and centrifuged at 3500 rpm for 20 min. Each was mixed with ethanol 100% (40 mL), sonicated for 20 min and centrifuged at 3500 rpm for 20 min. The supernatants were collected, dried and derivatized and analyzed under identical conditions as described for the standard.

Results and Discussion

The inhibition of cell division in cancer cell lines by glucosamine 6-sulfonate is demonstrated in Figure 5.2. In MDA MB231 cells, the calculated IC_{50} is 538 μg/mL and for MCF-7 could not be detemined.

As observed in Figure 5.2 and by the IC_{50} values, the MDA MB231 cell line is more sensitive to the effects of this inhibitor of hexosamine biosynthesis and we speculate that this may be related to the metastatic characteristic of this cell line. It is described that the addition of O-GlcNAc plays an essential role in breast cancer metastasis[12]. Therefore, it is possible that glucosamine 6-sulfonate, by inhibiting hexosamine biosynthesis, decreases the rate of O-glycosylation, which may affect cell proliferation.

In addition, the conversion of glucose 6-sulfonate to glucosamine 6-sulfonate may help explain why the ingestion of glycolipids (such as SQDG) found in vegetables has biological properties as an anti-viral[13], anti-tumoral[14,15,16], anti-angiogenic[17], anti-bacterial[18] and anti-inflammatory[19] molecule.

The *in vivo* occurrence of glucosamine 6-sulfonate was monitored in cow and human blood

samples derivatized with 3,5-DNB-Cl and analyzed by HPLC with UV monitoring. The peak corresponding to the desired derivative (3,5-DNB-glucosamine 6-sulfonate) was identified based on its retention time compared to the derivatized synthetic standard and by mass spectrometry (m/z 436) and UV spectrometry (Figure 5.3). Based on UV absorbance measurements compared to a standard it is estimated that the concentrations of **1** in the cow and human blood samples was approximately 2 to 4 μg/ mL (Figure 5.4).

As shown in Figure 5.4, the concentration of glucosamine 6-sulfonate in human blood is approximately 2 μg/ mL. The human blood sample was taken from the subject more than 16 hr after ingestion of any plant material and the levels could possibly be much higher if assayed earlier. The kinetics of elimination from the blood stream as a function of time after ingestion of known amounts of sulfolipid in several subjects is a study that should be performed in the future.

While the glucosamine 6-sulfonate concentrations found in the blood samples are in the low microgram per mL range, I began to observe inhibition in Gram-negative bacteria at 470 μg/ mL in my earlier study. Bacteria require large quantities of glucosamine for cell wall biosynthesis. Peptidoglycan, the major component of bacterial cell walls, is essentially a polymer of glucosamine that is cross-linked and functionalized[9,10]. The high rate of synthesis that is necessary to sustain this production should require significantly higher levels of inhibitor than most biochemical processes. Because glucosamine 6-sulfonate is a metabolite naturally found in blood in significant levels, this molecule could have great potential as an antimicrobial agent that would exhibit much smaller side effects than any non-naturally occurring molecule. It is possible that spikes in the concentration of this substance in blood soon after the ingestion of foods containing large amounts of SQDG or glucose 6-sulfonate serves as part of an innate defense mechanism against blood-borne pathogens.

While the anti-bacterial activity observed started at a concentration of 470 μg/ mL, the anti-cancer activity started at 200 μg/ mL. This is of great significance since the concentrations found naturally in human blood after 16 hr after ingestion of a meal containing SQDG was 2 μg/mL. After 3 hr after ingestion, almost 100% of glucose is absorbed and the plasma blood levels are back to pre-prandial levels[20]. If glucose 6-sulfonate is absorbed in a similar time frame there is a chance that the post-prandial concentration of this sulfonated sugar to be 100 times higher, which is close to the beginning of the inhibitory concentration found in this study. Future work should include toxicity studies to demonstrate if glucosamine 6-sulfonate could be used as a therapeutic drug with fewer side effects than non-naturally occurring molecules in blood.

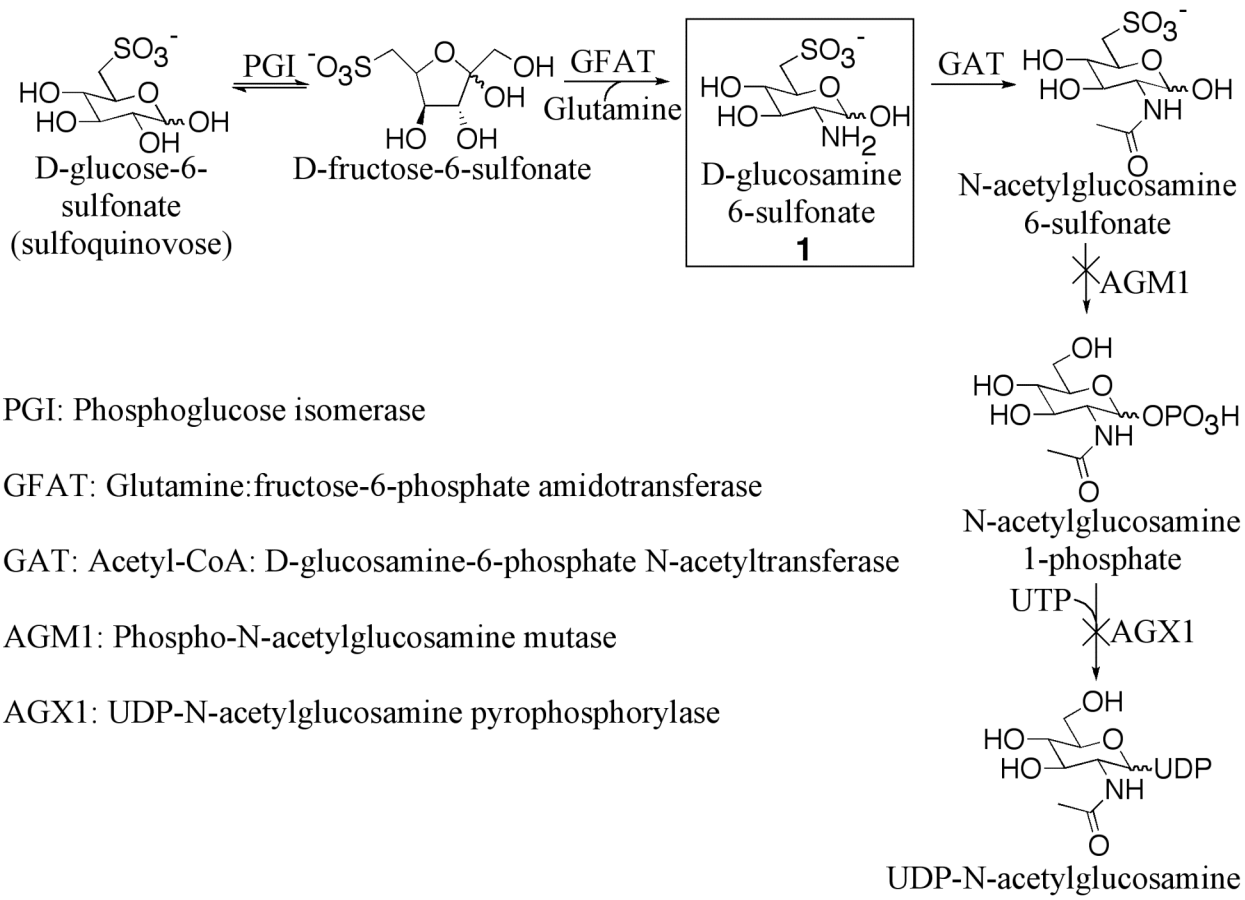

Figure 5.1. Potential metabolic route of biosynthesis of glucosamine 6-sulfonate *in vivo*.

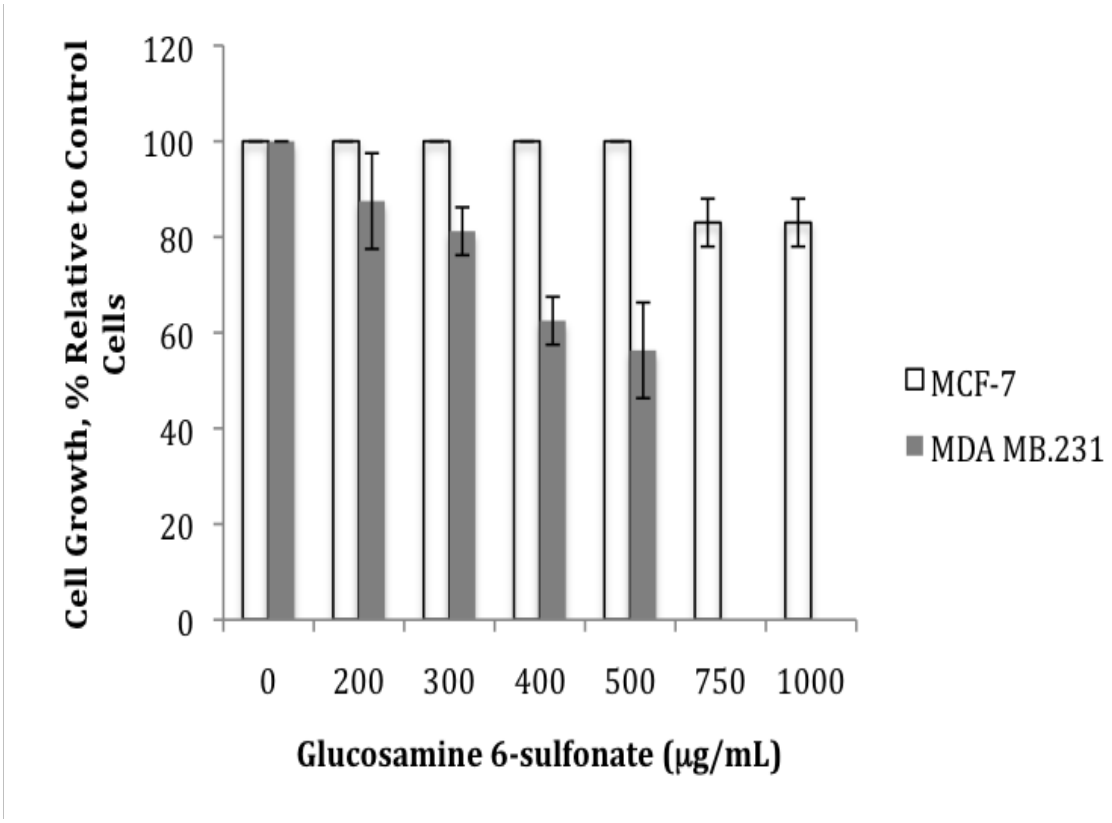

Figure 5.2. Growth inhibition of MDA MB.231 and MCF-7 with glucosamine 6-sulfonate. Cells were incubated with 200, 300, 400, 500, 750 and 1000 μg/mL of glucosamine 6-sulfonate for 48 hours and their growth calculated by cell counting on an inverted microscope. Controls for each line were prepared without the inhibitor.

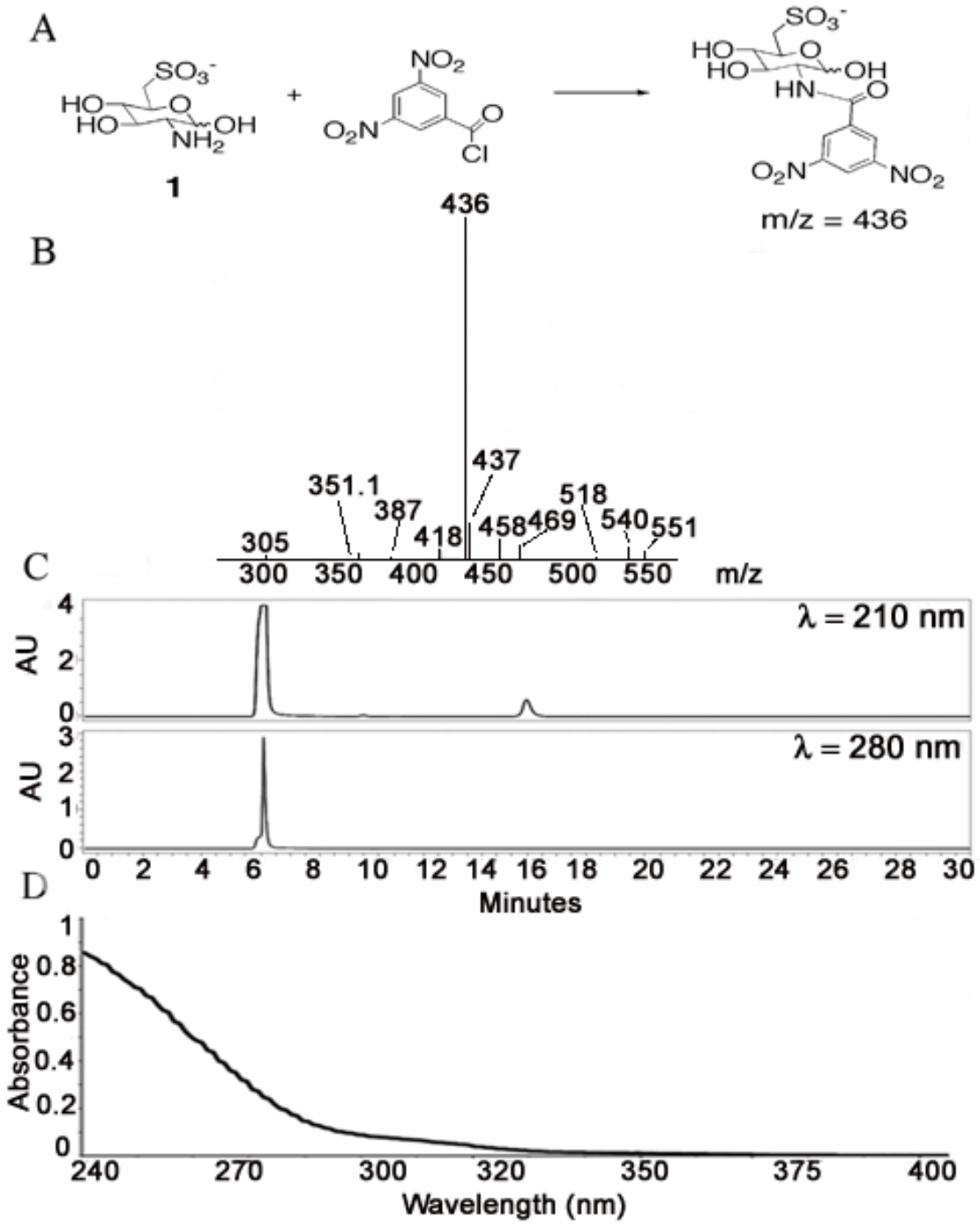

Figure 5.3. (A) Route of derivatization of the synthetic glucosamine 6-sulfonate (standard). (B) Electrospray mass spectrum of the dinitrobenzoyl derivative of **1**. (C) HPLC trace at 210 and 280 nm showing peak corresponding to derivative at 6.2min. (D) UV spectrum of 3,5-DNB-glucosamine 6-sulfonate.

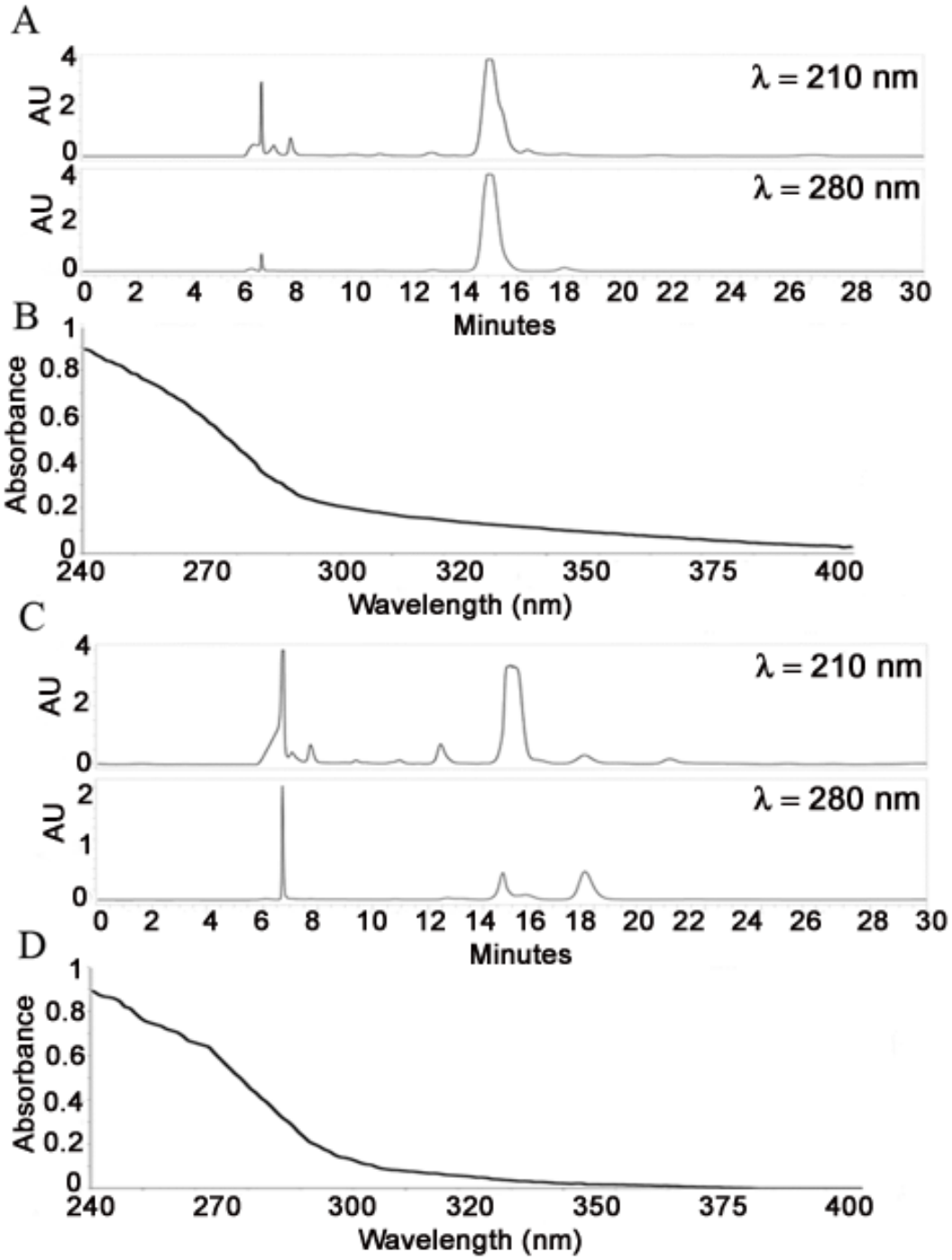

Figure 5.4. (A) HPLC profile and (B) UV spectrum of 3,5-DNB-glucosamine 6-sulfonate from cow serum. (C) HPLC profile and (D) UV spectrum of 3,5-DNB-glucosamine 6-sulfonate found in human serum. The peak eluting just after 6 min was collected in order to obtain the UV spectra.

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REFERENCES

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CHAPTER 6

CHAPTER 6

SYNTHESIS AND EVALUATION OF AN N-ACETYLGLUCOSAMINE BIOSYNTHESIS INHIBITOR.

(Adapted from *Carbohydrate Research* (2011) 346: 2294-2299).

Abstract

The structural rationale, synthesis and evaluation of an inhibitor designed to block glucosamine synthesis by competitively inhibiting the action of glutamine: fructose-6-phosphate amidotransferase and subsequently reducing the transformation of any glucosamine 6-phosphate formed to UDP-GlcNAc is described. The inhibitor 2-amino-2,6-dideoxy-6-sulfonato-D-glucose (D-glucosamine 6-sulfonate) is an analog of glucosamine 6-phosphate in which the phosphate group in the latter is replaced with a sulfonic acid group. The inhibitor is designed to function by three different modes, which together reduce UDP-GlcNAc synthesis. This reduction was confirmed by evaluating the effect of the inhibitor on bacterial cell wall synthesis and by demonstrating that it inhibits acetylation of glucosamine 6-phosphate competitively and by acting as a surrogate substrate. Inhibition of glucosamine production or suitably activated glucosamine in bacteria leads to disruption of the peptidoglycan structure, which results in softening, bulging, deformation, fragility and lysis of the cells. These modifications were documented for bacteria treated with the inhibitor by scanning electron microscopy. They were observed for inhibitor concentrations in the 20 mg/ mL range for *E. coli* and *B. subtilis* and the 5 mg/ mL range for *R. trifolii*.

Introduction

Amino sugars such as D-glucosamine (2-amino-2-deoxy-D-glucose), galactosamine and mannosamine (the *galacto*- and *manno*- isomers respectively) are characterized by a nitrogen atom being attached directly to the carbon chain of a carbohydrate molecule. These are all hexose sugars and are members of the hexosamine group. D-glucosamine is the most common amino sugar and it is an integral part of all living systems. It is now known that amino sugars make up the most abundant form of organic matter in the oceans[1]. Many bacteria can utilize glucosamine as the only source of carbon and are able to transform it to fructose 6-phosphate and initiate the glycolytic pathway[2]. Amino sugars and their derivatives are present in many glycolipids and most glycoproteins known showing the crucial importance of hexosamine biosynthesis for cell survival[3-8]. They are also major components of bacterial and fungal cell walls[9].

The conversion of glucose to glucosamine is a critical biochemical pathway. Fructose 6-phosphate is the species that primes glucosamine biosynthesis. The main limiting step of this pathway is catalyzed by the enzyme glucosamine synthase (GlmS) or GFAT in eukaryotes. This enzyme catalyzes the conversion of fructose 6-phosphate in presence of glutamine into glucosamine 6-phosphate. It is of universal importance to prokaryotes and eukaryotes. Because of its wide occurrence in bacteria and fungi, the production of inhibitors for this enzyme is an appealing strategy in the development of new antibiotics and antifungals.

Hexosamines in mammals have great clinical significance. These compounds are now known to be involved in the establishment and development of many diseases, such as T2D, AD, rheumatoid arthritis and cancer[10-14]. Although the molecular basis of these diseases is well

appreciated the development of cures is still a significant challenge. A limited number of therapies with a low percentage of cures are currently available. The development of new drugs that function by interfering with the hexosamine pathway is an active strategy. The main enzyme of the HBP is called GFAT and it has two structural domains. One of these is for binding glutamine (N-terminal or glutaminase domain) and another for binding fructose 6-phosphate (C-terminal or isomerase domain)[15,16,17]. Glucosamine is incorporated into other biomolecules through its activated form UDP-GlcNAc. This is formed by the successive action of 3 enzymes on glucosamine 6-phosphate (Figure 6.1). The first is acetyl-CoA: D-glucosamine-6-phosphate N-acetyltransferase (GAT), which converts the amino group to an acetamido group using acetyl-CoA as the acyl donor. The second step is the transfer of the phosphate group from the 6-position to the 1- position by phospho-N-acetylglucosamine mutase (AGM1) to form GlcNAc 1-phosphate. The third and last step is by the action of the enzyme UDP-GlcNAc pyrophosphorylase (AGX1) which catalyses the process in which uridine triphosphate (UTP) reacts with GlcNAc 1-phosphate to form UDP-GlcNAc and pyrophosphate.

A structural analog of glucosamine 6-phosphate in which the phosphate group is replaced with a similar charged group presents many modes of inhibiting UDP-GlcNAc synthesis. It would bind to GFAT, inhibiting the formation of the glucosamine 6-phosphate. It would also inhibit GAT by again competing with glucosamine 6-phosphate binding. If the similarity is close it would be converted to an acylderivative which could bind to AGM1 but not lead to the formation of GlcNAc 1-phosphate. Such an inhibitor could function by three different modes. To this end, 2-amino-2,6-dideoxy-6-sulfonato-D-glucose (D-glucosamine 6-sulfonate, compound **1**), an analog of D-glucosamine 6-phosphate in which the phosphate group was replaced with a sulfonate group, was synthesized and evaluated for its ability to inhibit glucosamine synthesis.

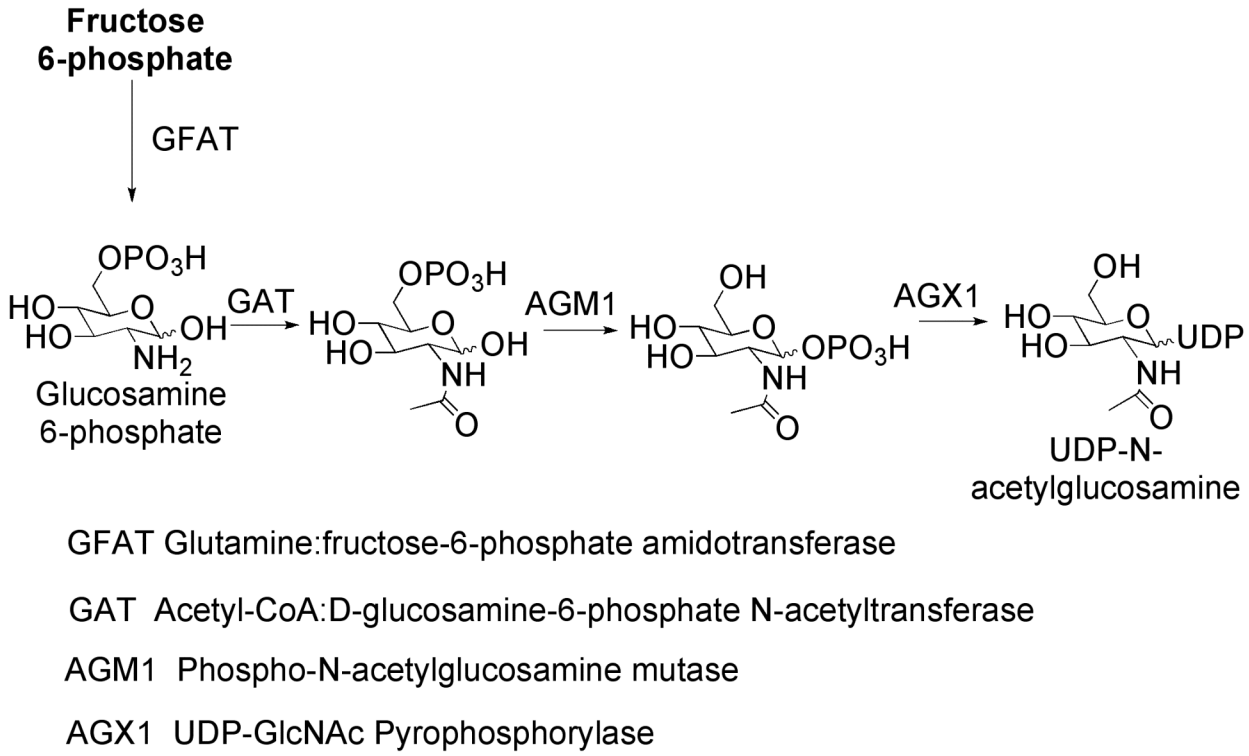
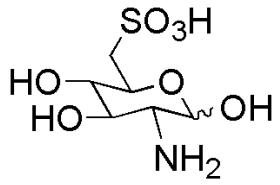


Figure 6.1. Biosynthetic steps in the conversion of fructose 6-phosphate to UDP-GlcNAc.



1

The synthetic method used in the synthesis of **1** is illustrated in Figure 6.2.

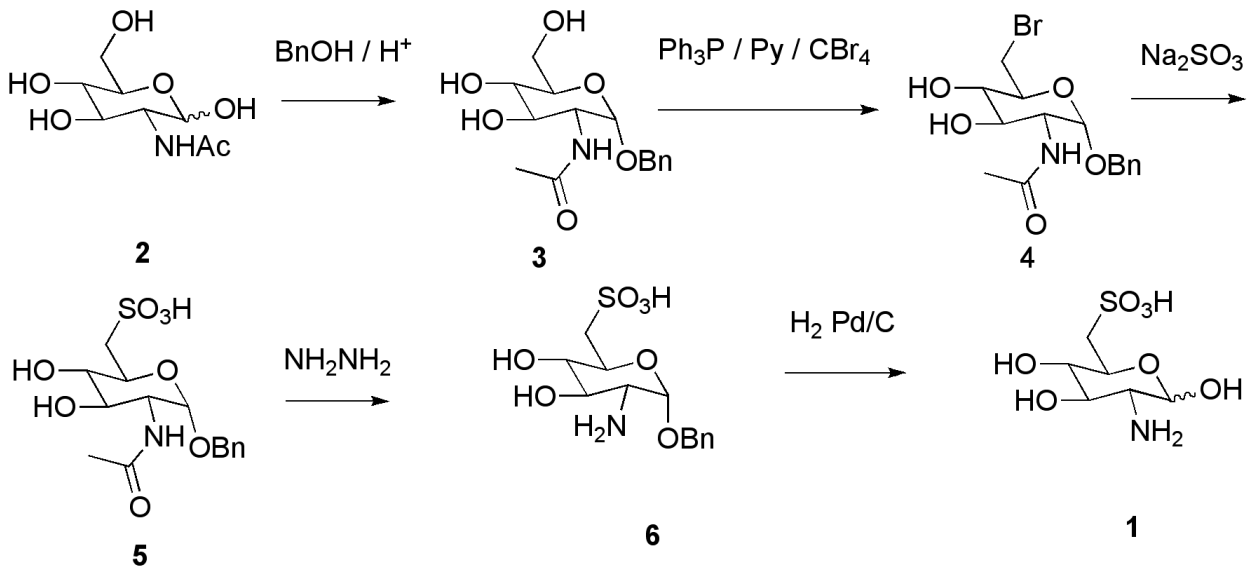


Figure 6.2. Synthetic route to D-glucosamine 6-sulfonate.

Materials and Methods

*1. Structural comparison of D-glucosamine 6-sulfonate (**1**) and D-glucosamine 6-phosphate*

D-glucosamine 6-phosphate contains a phosphate group ($O\text{-}PO_3^{2-}$) attached to carbon 6 of the sugar structure. The glucosamine 6-phosphate analogue contains a sulfonic acid group (SO_3^-) replacing this $O\text{-}PO_3^{2-}$ group, with the sulfur atom being directly attached to the C6 of the aminosugar. In its mono-ionized form the phosphate group is the same in charge and similar in shape and size to the sulfonic acid group but with an extra atom connecting the carbon. The analog would easily fit the active site.

The structure of the binding site of glucosamine-6-phosphate synthase of *E. coli* (PDB ID 2vf5[23]) is shown in Figure 6.3. The active site of the isomerase domain contains a P-loop, which is composed by residues 347-352. This loop is responsible for stabilizing the phosphate group in the correct orientation to allow the transfer of ammonia to the C2 of fructose 6-phosphate. The P-

loop residues interact with the oxygen atoms of the phosphate group through hydrogen bonds between the hydroxyl groups of the Ser347, Ser349 and Thr352 and the amino group of the main chain of Ser349 and Gln348[16]. These interactions do not require the presence of any metals or other components that would make of these strong interactions; therefore, the replacement of the phosphate group by a sulfonate group should give a molecular species that binds comparably well compared to the phosphorylated species.

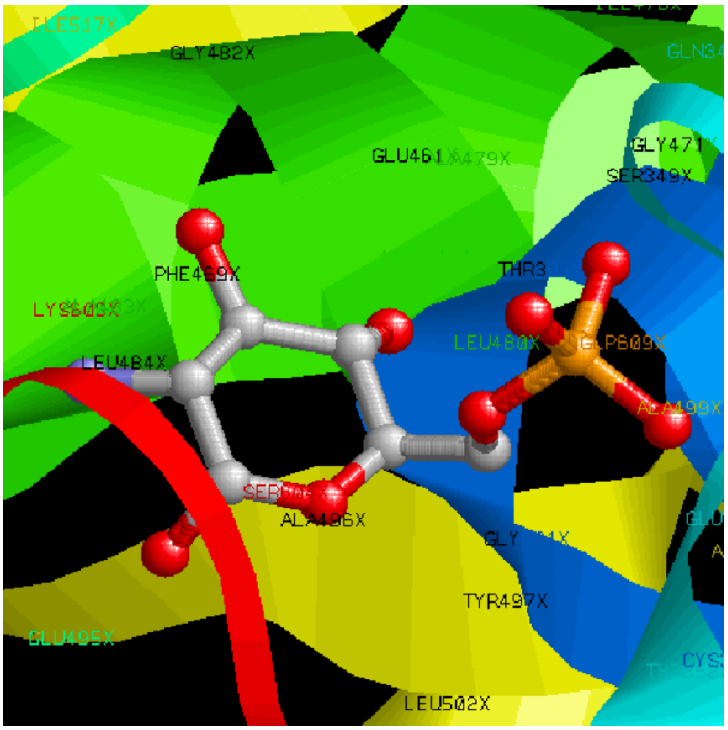


Figure 6.3. View of the active site of the isomerase domain of *E. coli* in presence of the glucosamine 6-phosphate.

2. Synthesis of 2-amino-2,6-dideoxy-6-sulfonato-D-glucose (D-glucosamine 6-sulfonate).

Benzyl 2-acetamido-6-bromo-2,6-dideoxy-α-D-glucopyranoside: 2-acetamido-2-deoxy-D-gluco pyranose was converted to a mixture of the α and β-benzyl glycosides as described by Kushida and Hichiro[24]. This consisted of heating 10 g 2-acetamido-2-deoxy-D-glucopyranose in 200 g

dry benzyl alcohol in the presence of 2 g Amberlite IR120-H at 60°C for 3 hr. The resin was filtered off and the excess benzyl alcohol removed under reduced pressure at 60°C. One gram of the 5:1 α: β mixture of benzyl glycosides so formed was converted to a corresponding mixture of benzyl 2-acetamido-6-bromo-2,6-dideoxy-D-glucopyranosides without further purification using triphenylphosphine and pyridine as described by Galemmo and Horton[25]. The product was purified by chromatography on silica using 2:1 acetone dichloromethane. Yield 0.42 g (40 %) of the pure α anomer. MP 183-184 (^{1}H and ^{13}C-NMR data match that reported).

2-Amino-2,6-dideoxy-6-sulfonato-α,β-D-glucopyranose: Benzyl 2-acetamido-6-bromo-2,6-dideo xy-α-D-glucopyranoside (0.4 g) was dissolved in 5 ml water. Sodium sulfite (0.5 g) was added and the mixture heated at 80°C for 3 hr. The solution was cooled and poured down a column (20 g) of strong base anion exchange resin (Dowex Monosphere 550A, OH form). The resin bed was washed with water (200 ml) and then with 5% sodium chloride (100 ml). The sodium chloride wash was concentrated almost to dryness and then treated with 200 ml methanol at room temperature. The mixture was stirred for 10 min and filtered. The filtrate was concentrated to a syrup which was dissolved in 0.5 ml hydrazine and heated at 90°C to remove the acetyl group. Excess hydrazine was removed on a rotary evaporator at 60°C under high vacuum followed by successive evaporation of several 10 ml volumes of water at the same temperature. The resulting solid was dissolved in 1 ml water and a few drops of HCl added to adjust the pH to between 5 and 6. Methanol (10 ml) was then added followed by 0.4 g of 10% palladium on carbon. The mixture was hydrogenolyzed at 30 psi pressure for 5 hr to remove the benzyl group yielding the desired product (110 mg) after filtration and evaporation of solvent. This was purified on an XAD-7 column (10 cm X 1 cm) using 70% ethanol in water as the eluent. Evaluation of the

product by ^{1}H-NMR spectroscopy (D_2O) at this stage revealed the expected loss of the signal at ~ 2.1 ppm for the N-acetyl group as well as the signals between 7.3 and 7.5 ppm for the benzyl group and the appearance of multiplets at 2.9 to 3.1 ppm characteristic of the protons on the sulfonated C6 position. The product existed almost exclusively as the alpha anomer when heated in acid and cooled but this mutarotated to a 2:1 mixture of the alpla and beta anomers after standing for a while. IR [CaF_2 film, cm^{-1}] 3408, 2911, 2592, 1873, 1734 ^{1}H NMR: 5.29, d, J= 3 Hz, H1α; 4.84, d, J= 8Hz, H1β; 4.12, t, J= 8 Hz, H3α; 3.76, t, J= 8 Hz, H4α; 3.71, t, J= 8 Hz, H3β; 3.58, t, J= 8 Hz, H3β; 3.2 – 3.5, m, H5 and H6α and β; 2.90 – 3.01, m, H6´α and β. ^{13}C-NMR (mixture of anomers): 89.2, 93.0, 72.4, 72.0, 70.0, 69.7, 68.2, 68.1, 56.8, 54.4, 52.2. High Resolution MS: Calculated mass for molecular formula $C_6O_7NSH_{12}$: 242.0334; measured mass: 242.0345. The high-resolution mass spectrum of **1** is shown in Figure 6.4.

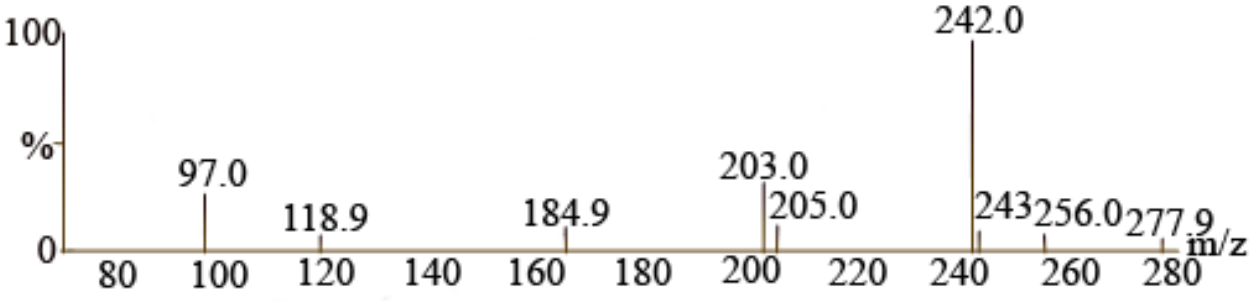


Figure 6.4. Negative ion electrospray mass spectrum of **1** showing strong molecular ion.

3. Evaluation of glucosamine-6-sulfonate activity in bacterial systems

3.1. Activity Assay

D-Glucosamine 6-sulfonate was evaluated for its ability to inhibit cell wall formation in three bacteria strains (*Escherichia coli* DH5α*, Rhizobium trifolii* ANU843 and *Bacillus subtilis* PY74). The first two strains are Gram negative and the last one is Gram positive. This allowed

the efficacy of the compound as a GlcNAc synthesis inhibitor over a broad spectrum of bacteria to be evaluated. The strains cultured in liquid media were treated in log phase with the concentrations of the analogue varying from 0 to 20 mg/ mL. Their growth rates were evaluated after 6, 18 and 24 hr of incubation by monitoring optical density at 600 nm. The results are expressed as the concentration that inhibits 50% of the bacteria growth (IC_{50}) in mg/ mL after 24 hr of treatment.

3.2. Scanning Electron Microscopy

The bacteria after 24 hr of treatment with the inhibitor were shadowed with gold and their morphology visualized by scanning electron microscopy (JEOL 6300F with field emission, Oxford EDS).

4. Glucosamine-6-phosphate Acetyltransferase (GAT) Activity Assay

To verify if the analogue has inhibitory activity over the GAT enzyme we performed the assay as previously described[26]. Yeast cells were lysed in 0.6 M sorbitol, 0.02 M HEPES-KOH buffer and used as source of enzyme. 50 mM Tris-HCl pH 7.4, 1mM EDTA, 1 mM glucosamine-6-phosphate, 0.5 mM acetyl-CoA, 0.5 mM of 5,5'-dithio-*bis* (2-dinitrobenzoic acid), 75 µL of yeast lysate and 20, 10, 5, 2.5 mg/ mL of inhibitor were mixed up to 250 µL per well to observe if it has any inhibitory activity over the GAT enzyme. The possibility of the inhibitor acting as a substrate of GAT was also investigated by evaluating the result without glucosamine 6-phosphate and with the same concentration of inhibitor as was used for glucosamine 6-phosphate.

Results and Discussion

The D-glucosamine 6-sulfonate concentration that resulted in a 50% reduction in growth of the bacterial cells (IC_{50}) was obtained by optical density measurement at 600 nm. The results are as follows: *B. subtilis* PY74, 2.88 mg/ mL; *R. trifolii* ANU843, 0.56 mg/ mL; *E. coli* DH5α, 4.72 mg/ mL. Complete inhibition of cell growth was observed for *R. trifolii, B. subtilis* and *E. coli* by the inhibitor at concentrations 5, 20 and 20 mg/ mL respectively.

To evaluate whether the bacterial growth inhibition observed was connected to the disruption of cell wall synthesis, the ultra structure of the cell walls were assessed by scanning electron microscopy (Figure 6.5). Glucosamine is the primary building block of peptidoglycan in the cell wall. This is a complex macromolecule that gives bacteria cells their strength and rigidity and that results in the characteristic cigar shapes of many bacteria. Inhibition of peptidoglycan synthesis results in a loss of shape and in cells with fragile walls that might be enlarged and with spherical or irregular shapes[18,19]. Cells treated with glucosamine 6-sulfonate show a high frequency of lysis and many have bloated irregular shapes (Fig 6.5 B, C, E, F, H, I) when compared to control cells (Fig 6.5. A, D, G). These results are consistent with the inhibition of the peptidoglycan synthesis. This is in accord with the expectation that the enough glucosamine would not be available for this because of the several modes by which glucosamine 6-sulfonate would inhibit GlcNAc synthesis.

The glucosamine 6-phosphate analogue can potentially bind to several enzymes on the hexosamine pathway. It could inhibit GFAT as a competitive inhibitor and also act as a competitive and allosteric inhibitor of GAT. It is also possible for glucosamine 6-sulfonate (**1**) to act as a substrate for GAT thus diverting acyl equivalents away from the synthesis of N-acetylglucosamine. The sulfonated GlcNAc thus formed cannot be converted to UDP-GlcNAc.

To evaluate the latter possibilities, GAT activity was measured in an assay in which the transfer of acetyl groups from acetyl-CoA to glucosamine 6-phosphate was evaluated by measuring coenzyme A formation during the process using 5,5'-dithio-*bis*(2-nitrobenzoic acid). This forms the highly colored 2-nitro-5-mercaptobenzoic acid on reaction with Coenzyme A by disulfide exchange. This colored substance can be readily monitored at 412 nm. An extract of lysed yeast cells was used as the source of the enzyme. The results are presented in Figure 6.6. Compound **1** inhibits the acetylation of glucosamine 6-phosphate by about 60% at 2 and 4 times the substrate concentration and almost completely at 8 times the substrate concentration. The very small difference observed between the two lower inhibitor concentrations hints that there is another aspect to the interactions besides a simple competition. This was made clear when the natural substrate was removed and compound **1** was used as a substrate. It was a very effective acceptor for acetyl groups from acetyl-CoA under catalysis by GAT at a level of 40% of the native substrate. The GAT enzyme also presents an allosteric site for binding GlcNAc 6-phosphate, which is its natural inhibitor[20,21]. This could explain the complete inhibition by **1** at very high concentrations. The glucosamine 6-sulfonate is not capable of being transformed to UDP-GlcNAc 6-phosphate because the sulfonate group cannot be removed by the same mechanism that the phosphate group can.

The glucosamine 6-phosphate analogue was design to act as a competitive inhibitor for the isomerase active site and for the enzymes GAT and AGM1, leading to a decrease in the production of GlcNAc 6-phosphate within the cell. The fragility of cell walls resulting in cell deformation and (at higher concentrations) cell lysis was expected and detected in all strains incubated with the inhibitor (Fig 6.5). Gram-negative strains should be even more susceptible to the inhibitory effects of these inhibitors since they also depend on GlcNAc production for the

synthesis of lipid A, a main constituent of the lipopolysaccharide (LPS). LPS form regular crystalline arrays in the outer membrane[22]. These features make bacteria cells good systems for the evaluation of the efficacy of these inhibitors. In summary, 2-amino-2,6-dideoxy-6-sulfonato-D-glucose inhibited bacterial cell wall biosynthesis consisted with expectations. This compound should be a valuable tool in elucidating the contribution of amino sugars to biochemical processes and in developing antimicrobial and therapeutic agents.

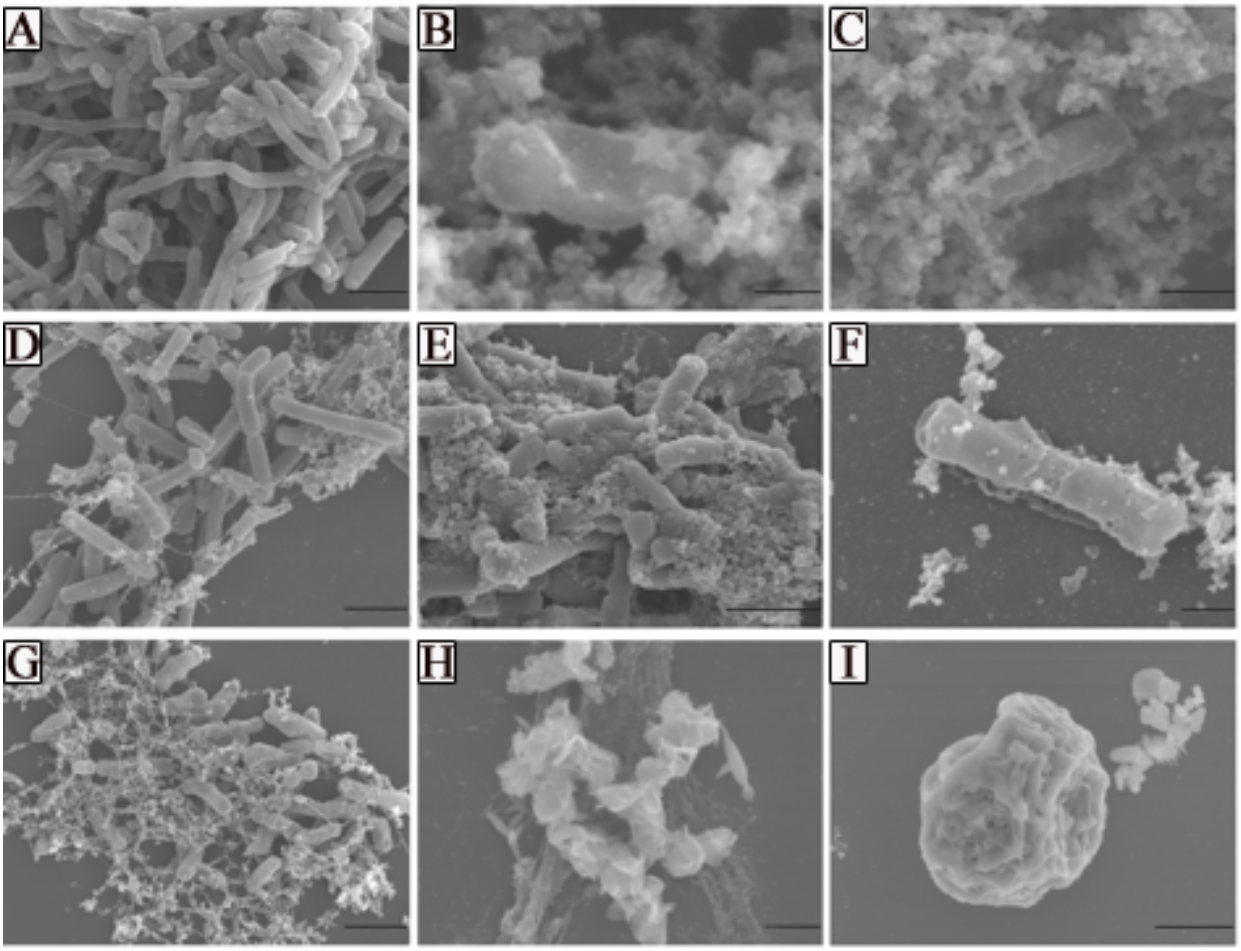


Figure 6.5. Representative scanning electron microscopy images of the bacterial strains treated with increasing concentrations of glucosamine 6-sulfonate for 48 hours. A-C: *E. coli*: (A: Control Cells, B-C: 1. 5 mg/ mL of inhibitor); D-F: *B. subtilis* (D: Control cells; E-F: 20 mg/ mL inhibitor); G-I: *R. trifolli* (G: control cells; H-I: 5 mg/ mL inhibitor). Scale Bar: A, D, E, G: 2 μm; C, I: 1 μm; B, F, H: 500 nm.

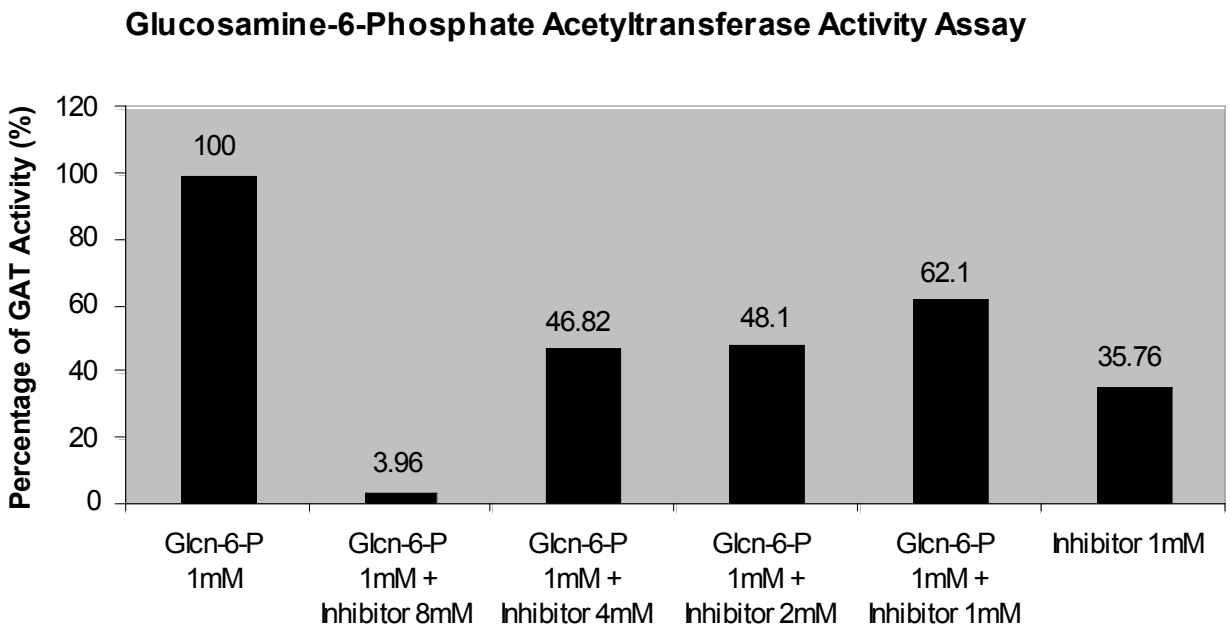


Figure 6.6. Glucosamine-6-phosphate acetyltransferase (GAT) activity in presence of 1mM of glucosamine 6-phosphate and 1, 2, 4 and 8 mM of glucosamine 6-sulfonate. Enzyme Activity was measured 412 nm by the release of TNB, which indicates the consumption of Acetyl-CoA for the acetylation reaction catalyzed by this enzyme. Controls: Glucosamine 6-phosphate (Glcn 6-P): 1 mM; Inhibitor (Glucosamine 6-sulfonate): 1 mM.

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CHAPTER 7

CHAPTER 7

CONCLUSIONS AND FUTURE PERSPECTIVES

This work brings into focus new and old findings on how far reaching the effects of short and long term exposure to different types and concentrations of carbohydrates are with respect to cellular properties and consequently tissue physiology. The findings here support both the overall and the specific hypotheses proposed for this work. My overall hypothesis was that the type of sugar used as carbon source by cells can modify the relative flux through glycolysis, PPP and HBP, resulting in shifts in a variety of cellular properties. This was tested by perturbing the relative flux through these three pathways with different carbon sources (fructose and glucose) or by the addition of competitive inhibitors (glucose 6-sulfonate and glucosamine 6-sulfonate) of these carbohydrate pathways.

My first specific hypothesis was that treating cells with fructose instead of glucose would increase the flux through the HBP, leading to an increase of hexosamine-containing antigens. This was confirmed by an increase in A blood antigen (increase in GalNAc content) and hexosamine contents in fructose-fed cells. These changes were cell-type dependent and correlated with the degree of cellular metabolic activity. Cells with higher metabolism provide a high turnover of substrates and products that are more likely to be detected as changes in carbohydrate dependent antigens. Additional cellular features such as morphology were also shown to change in the same fashion as cell surface antigenicity and carbohydrate pools.

My second specific hypothesis was that fructose-fed cells would increase the flux through to biosynthetic processes in comparison to glucose, such as the metabolism of glycine and its

derivatives. Microarray analysis showed that genes correlated with glycine metabolism were increased significantly in fructose-fed cells in relation to glucose. This reinforces the idea that fructose could also affect the balance of methyl equivalents available for one carbon metabolism, fact that would increase the methylation of proteins and lipids within the cell.

The imbalances between carbohydrate pathways that result in changes in cellular properties observed here add to the body of evidence showing that a correlation between carbohydrate pathways and the development of metabolic diseases. Many epidemiological studies, some of which are described here, show that diet is an extremely important factor in predisposing us to the development of certain metabolic diseases. Examples of such are T2D, obesity, neurological and autoimmune disorders and certain types of cancer. The detailed biochemical and molecular connections between diet and the development of these disorders, however, are largely unknown. This work should add significantly to the growing body of knowledge that will form a basis for explaining these connections on a molecular basis.

Carbohydrate metabolism is a very complex process and there is a tendency to try to study it by separation of this complex matrix into different (seemingly independent) pathways. The secret to understanding the bridge between these diseases and the core of complex carbohydrate metabolism is an actual understanding of what happens at the interfaces of pathways. The alteration in the balance between intermediates through these complex carbohydrate metabolism webs ultimately results in changes in cell surface antigens, carbohydrate pools and glycosylation patterns, which are all very important in defining cell characteristics that are altered in these diseases. The abundance of certain sugars and their linkages in cell antigens holds another important set of information. It tells us what glycosyl synthesis and transfer processes are dominant in the diseased and normal state. Methods such as

NMR spectroscopy that allow us to take large panoramic views of the various processes across compound classes hold great promise for addressing this complexity.

This work also provides a basis for rationalizing the Warburg phenomenon. This is the observation that cancer cells have low mitochondrial activity and obtain energy by fermentative processes. High concentrations of sugars will lead to high flux through the carbohydrate metabolic pathways; however, all of the output cannot be used by the TCA cycle and oxidative respiration in the mitochondria. A selection for cells that use anaerobic respiration will consequently be more represented in the populations. One common observation in cancer cell growth is that this shift in energy generation mode leads to cells growing anaerobically to overgrow others. The large concentrations of unused sugars that are not burned in the mitochondria as acetyl-CoA equivalents convert to other sugar types and are polymerized on glycoproteins and glycolipids to form cancer markers. This example describes the main characteristic of cancer cells. Management of the carbohydrate metabolic web by controlling the type/ concentration of sugar intake or using inhibitors to regulate flux through specific pathways may be an important strategy for preventing the establishment of these type of disorders.

There is the saying that prevention is better than cure. It is known, for instance, that 30-40% of all cancers can be prevented by lifestyle changes in diet and exercise. In relation to diet, the two top issues that lead to diseases like cancer are hypercaloric diet and a high intake of carbohydrates[1]. One approach to controlling the regime within which carbohydrate metabolism occurs is by regulating dietary intake of sugars. Regarding diet management, many approaches can be taken to regulate type and amount of sugar intake. Firstly, to prevent instantaneous high concentrations of simple sugars available for cells, disaccharides should be preferred over monosaccharides. The first one is digested more slowly than the simple sugars. This avoids a

momentarily abrupt increase in free monosaccharide levels.

Another tactic to increase sugar burnt through oxidative phosphorylation if high concentrations of carbohydrates are present is by increasing oxygenation in cells. This can be done by aerobic exercise. This activity increases the oxygen concentration in all tissues and with that oxidative respiration can be increased to keep up with burning high amounts of carbohydrates. This can also avoid a persistent increase in concentrations of intermediates originated from carbohydrate metabolic pathways and the resulting changes in cellular properties. Therefore, a stable diet regime, a regular exercise plan, in which you can keep under control the concentration of the metabolites so imbalances between pathways cannot arise and, consequently, disease is prevented, would be the best option to avoid these disorders.

Another way of managing the flux through the various carbohydrate pathways is by increasing the ingestion of foods that contain metabolic inhibitors of these routes. Such example is glucose 6-sulfonate (or sulfoquinovose), which is found in different concentrations in all vegetables, and in especially high amounts in green leaves and green tea. My third specific hypothesis was that glucose 6-sulfonate, a naturally occurring sugar, would decrease the flux through glycolysis and the PPP by competitive inhibition with glucose 6-phosphate. These ideas were confirmed by ^{13}C-NMR, enzymatic assays and in cancer cells, and it was also shown that glucose 6-sulfonate is also a competitive inhibitor of G6PDH. This compound, for instance, provides an opportunity for managing the onset of disease. A program to study diet management should focus on the possible clinical benefits of compounds such as this and its derivatives.

Another observation from this work is that glucose 6-sulfonate obtained from diet can also be converted *in vivo* as shown here to other compounds such as glucosamine-6-sulfonate (the novel analogue described in this work) that blocks hexosamine biosynthesis. This also opens new

avenues because diseases of hexosamine metabolism, such as rheumatoid arthritis, are related to an increase activity of this pathway. This would be an important potential way of managing or even preventing these diseases that are related to antigen shifts in cell properties due to increase flux through a particular carbohydrate pathway.

My fourth hypothesis was that glucosamine 6-sulfonate, a metabolic drivative of glucose 6-sulfonate, can decrease the flux through the HBP by competitive inhibition with glucosamine 6-phosphate, as shown in bacteria and in cancer cells. This highlights the idea of how the HBP can be used as a potential route for the development of drugs that contain anti-bacterial and anti-cancer properties.

As shown in this work, sulfoquinovose and its derivatives (glucosamine 6-sulfonate) can act as a competitive inhibitor of intermediates of glycolysis, PPP and HBP, slowing down the rate of biosynthesis from these pathways. This is very important for disease prevention since it is established that increase in these pathways is at the very core of metabolic diseases. As known, increase in PPP generates higher rates of DNA and RNA synthesis, which is found in cancer[2]. Therefore, slowing down the PPP is also an interesting way of stopping cells to take this route to disease. As mentioned earlier, glycolysis is also elevated in cancer. There is already some movement in the direction of combating cancer by interfering with glycolysis with the "anti-glycolytic" drugs[3].

The importance of the diet (and carbohydrate metabolism) in defining cellular characteristics is a fundamental point to understand the level of influence the genotype can have on defining a cell or a disease state. This investigation also shows that no chemical process that can be independent of the type/ concentration of substrate you provide to that reaction in a reactor (the cell). This brings up the issue of how much a cell is defined by its genotype. The

general idea that the genetic information is the only determinant of the set of cellular properties is quite misleading. Genes contain the information for the biosynthesis of the catalysts of reactions (enzymes). Enzymes can convert a substrate to a product in a rate that is determined primarily by the amount of substrate that is available for that reaction. This is supported by both the rate law and collision theory for chemical reactions. The genome provides the set of enzymes that can be used by a cell but the concentrations and identities of the different substrates (which can vary enormously through factors such as diet) for the various reactions of the cell are the actual determinants of how cellular properties are going to be defined. In other words, the genome can determine which set of reactions a cell will have by determining the enzymes it will contain, but it does not define a locked, invariant set of conditions by which a cell will be defined. In summary, environmental conditions are as important a factor in defining cellular characteristics as the genome of an individual.

In conclusion, prevention does seem to hold much more promise than a cure. The picture is complex but there is an appreciation for and even some degree of understanding of many of the elements that contribute to diseases that are initiated or are characterized by a breakdown of carbohydrate metabolism. There will always be cases in which the dominant cellular chemistry (genetic disposition) will be a major driving force in determining whether an individual will succumb to a disease or not. There is the hope that in many instances our emerging understanding of how specific carbohydrate intake, exercise and metabolism defining natural chemical therapies can forestall the onset of these diseases.

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