

Acid Hydrolysis of Neutral Glycosphingolipids

Thesis submitted in fulfillment of the degree of Doctorate of Philosophy

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institution of higher learning, except where due acknowledgement is made.

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Abstract

Blood group glycolipids are important tools in the study of microbial receptor interactions and other biological phenomena. Presently blood group glycolipids of interest are isolated from biological samples. However, all glycolipids are not readily available due to the low frequency of some phenotypes in the general population. The ability to acquire the rare glycolipids from the degradation of common glycolipids would be a useful alternative to trying to obtain the molecules from biological sources.

This research set out to establish the ability of blood group glycolipids to be degraded into useful glycolipids in a controlled manner by acid hydrolysis and possibly metal catalysis. The initial experiments investigated the degradation/hydrolysis of the more readily available glycolipid globoside with a range of salts and acids to establish degradation concepts such as; temperature, type of acid, acid concentration, and the role of metal ions in glycolipid degradation. These concepts then led to a series of degradation experiments with the blood group glycolipids Le^b and ALe^b. These glycolipids were incubated with a range of acid concentrations and varying temperatures. Thin layer chromatography separation and chemical and immunochemical staining were the main methods used to identify the products of degradation.

It was established that metal ions were not directly involved in the catalysis of glycolipids in the short-term, however some metal ions were indirectly implicated in their degradation due to their ability to form acid solutions. Acid hydrolysis was established as the principle mechanism for glycan chain degradation. In general it was found that the glycan chain primarily lost its fucose groups (in no particular order) and was then followed by sequential degradation of the remaining glycan chain. The glycan chain also appeared to have a protective function on the ceramide moiety. Degradation of globoside established a simple sequential pathway of glycan chain reduction from the non-reducing end. Blood group glycolipids ALe^b and Le^b first lost their fucose side groups followed by sequential reduction of the glycan chain. Although not fully controllable, degradation of Le^b was able to produce Le^a, Le^d and Le^c. In contrast degradation of ALe^b did not produce any Le^a or Le^d. Instead A-type 1 and two novel A-like structures, 'linear A' and 'GalNAc-Le^a' were generated. Le^c was only produced from ALe^b in extremely acidic conditions.

This research established the ability to generate, by acid hydrolysis, a range of rare and "unnatural" novel glycolipids from more commonly available structures. It is of interest that the so-called unnatural glycolipids obtained from the acid hydrolysis of ALe^b may, in theory, occur naturally in the acid environment of the stomach, and as such could have the

potential to be implicated in disease. It is probable that by applying the principles learned here, a range of novel and natural structures suitable for use in the study of biological interactions can be obtained.

Abbreviations

ALe^b, A Lewis b, GalNAc α 1-3Gal(Fuc α 1-2) β 1-3GlcNAc(Fuc α 1-4) β 1-3Gal β 1-4Glc β 1-1Cer

A-type 1, GalNAc α 1-3Gal(Fuc α 1-2) β 1-3GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer

A-type 2, GalNAc α 1-3Gal(Fuc α 1-2) β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer

B-type 1, Gal α 1-3Gal(Fuc α 1-2) β 1-3GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer

B-type 2, Gal α 1-3Gal(Fuc α 1-2) β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer

Cer, Ceramide

Fuc, L-fucose

G-4-4, globoside, GalNAc β 1-3Gal α 1-4Gal β 1-4Glc β 1-1Cer

Gal, D-galactose

GalNAc, N-acetyl galactosamine

GalNAc-Le^a, GalNAc α 1-3Gal β 1-3GlcNAc(Fuc α 1-4) β 1-3Gal β 1-4Glc β 1-1Cer

GC, Gas Chromatography

Glc, D-glucose

GlcNAc, N-acetyl glucosamine

Le^a, Lewis a, Gal β 1-3GlcNAc(Fuc α 1-4) β 1-3Gal β 1-4Glc β 1-1Cer

Le^b, Lewis b, Gal(Fuc α 1-2) β 1-3GlcNAc(Fuc α 1-4) β 1-3Gal β 1-4Glc β 1-1Cer

Le^c, Lewis c / Type 1 precursor, Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer

Le^d, Lewis d / H type 1, Gal(Fuc α 1-2) β 1-3GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer

Linear A, GalNAc α 1-3Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer

Man, D-mannose

MS, Mass Spectrometry

NMR, Nuclear Magnetic Resonance

TLC, Thin Layer Chromatography

1 Introduction

Carbohydrates and their importance

Carbohydrates are the most abundant molecules present in any living system. They are made up of the three basic elements, carbon, oxygen and hydrogen. The simplest biological function of carbohydrates is storage of energy. Other complex roles of carbohydrates include the proper functioning of the immune system, fertilisation, pathogenesis, blood clotting and development.

Monosaccharides such as glucose are the building blocks for carbohydrates. Many units of monosaccharides joined together make up oligo- or polysaccharides such as cellulose (glucose units) and chitin (N-acetyl-glucosamine units). Other more complex carbohydrates include glycosaminoglycans and aminoglycosides (antibiotics).

Unlike proteins and nucleic acids, the monomers in a polysaccharide are held together by glycosidic bonds. The glycosidic bond is formed between the hemi-acetal group of a saccharide and the hydroxyl group of another saccharide as illustrated in Figure 1.

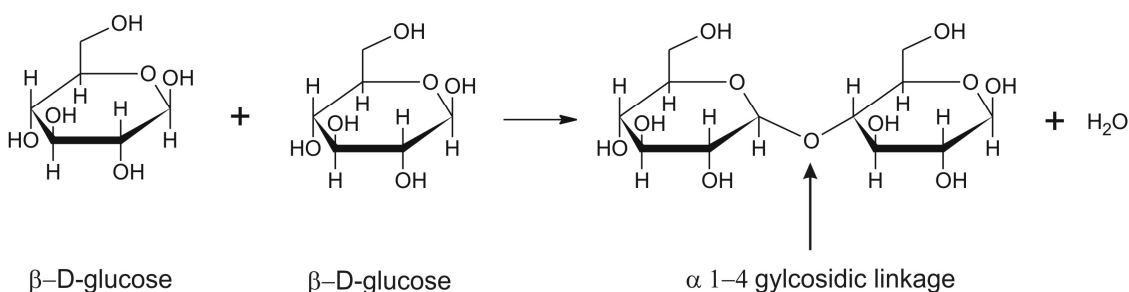


Figure 1: Formation of a glycosidic bond.

This bond is formed between a hemi-acetal group of one saccharide and the hydroxyl group of another saccharide.

The sugars can also form covalent bonds with other non-carbohydrate molecules to form glycoconjugates. Glycoconjugates such as glycolipids and glycoproteins consist of carbohydrates (sugar chains) covalently joined to either a lipid tail or protein molecule respectively. Sugars linked to proteins (glycoproteins) and lipids (glycolipids) have more specialised functions such as serving as antigenic sites, cell signalling and communication and cell adhesion (Lowe, 1994).

Other complex glycoconjugates include proteoglycans and glycosylphosphatidylinositol (GPI) anchors. Together these glycoconjugates play crucial biological roles within and

amongst cells. The carbohydrate portions of these glycoconjugates have diverse structures which can also store biological information (Lindhorst 2002). For example, the sequence of sugars on a glycolipid is responsible for the antigenic properties of erythrocytes and tissue cells (Schenkel-Brunner, 2000). Despite the large variety of monosaccharides available, only a small set is required in the biosynthesis of glycoconjugates. However, the number of ways these sugars can link stereochemically is almost unlimited (Lindhorst 2002). The major sugars that make up the blood group glycoconjugates are D-galactose (Gal), D-glucose (Glc), D-mannose (Man), N-acetyl glucosamine (GlcNAc), N-acetyl galactosamine (GalNAc), L-fucose (Fuc) and N-acetyl neuraminic acid (sialic acid) (Schenkel-Brunner, 2000).

Chemistry of blood group-related sugars

The monosaccharides that comprise the blood groups are six-carbon hexoses and have a carbonyl group each presented as an aldehyde. They are collectively termed aldohexoses. (except L-fucose which is a de-oxy aldohexose). Sialic acid is a nine-carbon monosaccharide and is also a common blood group antigen.

The monosaccharides can exist as isomers where they have the same general molecular formula but different structural formula. Isomers that are mirror images are termed enantiomers while diastereoisomers are non-mirror images. For example, D-Glc and L-Glc are enantiomers while D-Glc, D-Gal are diastereoisomers. Further, those monosaccharides that differ by the orientation of a single –OH at a chiral centre are called epimers; D-Man and D-Gal are epimers of each other.

In amino sugars such as GalNAc and GlcNAc, the hydroxyl group of carbon 2 is replaced by an acetamido group (Gilliver et al., 2003).

From a three-dimensional perspective, the functional groups (hydroxyl groups) of the monosaccharides in their pyranose rings adopt either an axial or equatorial position. In the former, the hydroxyl groups are perpendicular to the general plane of the ring while in the equatorial position the hydroxyl groups are in line with the general plane (Gilliver et al., 2003; Kennedy et al., 1988). The 3-D shape of the pyran rings are known to arrange in the lowest energy conformation and the equatorial orientation allows this. The hydroxyl functional groups are less crowded and more dispersed in the equatorial conformation.

As shown in Figure 1, a glycosidic bond is formed between two monosaccharides when the hemi-acetal group on carbon 1 of one monosaccharide bonds with the OH group of another sugar residue. A molecule of water is eliminated in the reaction and is therefore

termed a condensation reaction. The bond can be either an α or β glycosidic linkage. The hemi-acetal group is usually very reactive and glycosidic bonds form readily in the presence of an acid. These bonds can be broken down by strong aqueous acids. The formation of hemiacetals and acetals is shown in Figure 2.

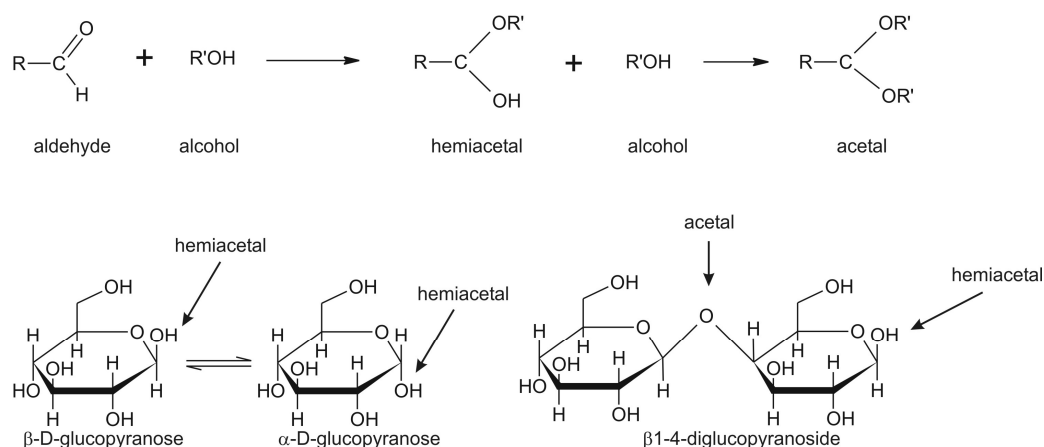


Figure 2: The formation of acetals.

Addition of one molecule of alcohol forms a hemiacetal; addition of two alcohol molecules forms an acetal. Monosaccharides can also alternate between the α and β anomers and the hemiacetal in each of the anomer is marked. The formation of a glycosidic bond 'locks' the ability to alternate between the anomeric forms thereby forming an acetal. However the carbon 1 which is not involved in the glycosidic bond can still alternate between the α and β forms hence is a hemiacetal. This position becomes 'locked' as this carbon 1 forms a glycosidic bond with yet another monosaccharide (Kennedy et al., 1988).

The anomeric carbon (carbon 1) in the pyran ring can exist as α and β anomers (isomers). In solution, the anomeric forms alternate so that a mixture is present at any given time. However upon forming a glycosidic bond with another pyran (monosaccharide) this anomeric carbon atom is 'locked' and cannot alternate between the two anomers. This is called an acetal. The carbon 1 of one of the monosaccharides is not involved in glycosidic bonding hence this pyran ring is 'free' to alternate between the α and β conformations and is called the (cyclic) hemi-acetal (Kennedy et al., 1988).

Glycobiology

This is a study of the role of sugars in biological processes. It is a relatively young science as the roles of many oligosaccharides in cell recognition, carbohydrate-receptor associations, therapeutic applications, and their interaction with protein molecules are still not fully understood. What is known is that the cell surfaces are inundated with carbohydrate moieties and the compositions and types of carbohydrates differ in different

cell types. This simple observation suggested that the presence of carbohydrates on cell surfaces may exhibit important functions in cell communication (Greenwell, 1997). It was observed in a study that altering the concentration of N-glycans and other surface oligosaccharides, present during varying stages of cell differentiation, resulted in a number of pathological conditions such as malignant transformation of cells and cancer (Lindhorst 2002). The carbohydrates present on cell surfaces then must have a role in cell communication and proliferation. Early investigations were mainly carried out on glycoproteins. Recently, it was shown that glycolipids also have a role in carrying out similar communication functions but have not been as widely investigated as the glycoproteins. These glycoconjugates are discussed in detail as follows.

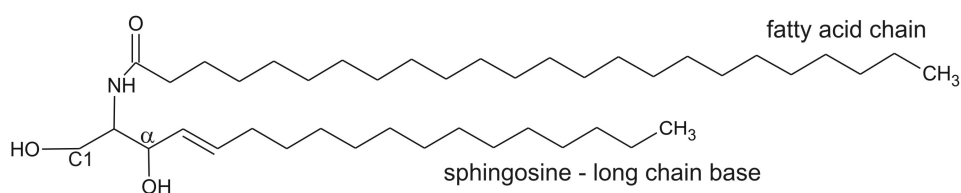
Glycolipids

A sequence of sugars (linear or branched) covalently linked to a ceramide bi-lipid tail is termed a glycolipid. A ceramide molecule consists of a long chain base called sphingosine and a fatty acid chain. The sphingosine is an amino alcohol with a long unsaturated hydrocarbon chain and a fatty acid chain (which is either saturated or unsaturated) linked to the amino group. Together they form a sphingolipid. The fatty acid usually contains 16-26 carbon atoms where the α -carbon atom is most often hydroxylated. This hydroxyl group is oxidized to form a β -glycosidic linkage with the carbon 1 of the β -glucose residue upon glycosylation (Gilliver et al., 2003). This concept is illustrated in Figure 3.

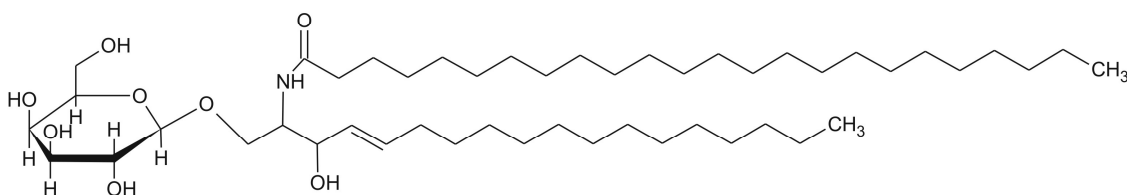
Ceramides have varying hydrocarbon chain lengths, varying levels of saturation and varying number of hydroxyl groups. In general, there are over 60 variations of long chain bases and each can be chemically linked to about 20 different types of fatty acids (Gilliver et al., 2003). The terms glycolipid and glycosphingolipid are used interchangeably.

Glycolipids are amphiphilic, meaning that they contain both a hydrophilic (oligosaccharide chain) and a lipophilic (hydrophobic ceramide unit) entity. They are present in the cell membrane of eukaryotes with the oligosaccharide chains 'sticking' out of the phospholipid bilayer of cell membranes and with the sphingolipid portion inserting itself into the lipid bilayer. The carbohydrate moiety is thought to ensure firm attachment of the ceramide into the lipid bilayer (Koscielak et al., 1978).

The oligosaccharide chains of glycolipids have a vast structural variety and play a more important role of mediating the transfer of information into and out of the cells (Lowe, 1994). This idea was suggested when a noticeable difference in the cell-surface carbohydrate composition was observed during the varying stages of cell differentiation process (Lowe, 1994).



Ceramide or sphingolipid structure



Glycolipid or glycosphingolipid (Galactosyl cerebroside)

Figure 3: Chemical structure of a ceramide and a simple glycosphingolipid.
(Lindhorst 2002; Schenkel-Brunner, 2000)

Cerebrosides are the simplest known glycolipids consisting of one galactose residue bound to the sphingolipid as shown in Figure 3.

Globoside (G-4-4), one of the main glycolipids found in the erythrocyte membrane, is a more complex glycolipid consisting of four sugar residues bound to each other by glycosidic linkages and has the structure $\text{GalNAc}\beta 1-3\text{Gal}\alpha 1-4\text{Gal}\beta 1-4\text{Glc}\beta 1-1\text{Cer}$ as shown in Figure 4. During biosynthesis of oligosaccharides, the initial two steps of glycosylation of the sphingolipid are the same for all glycolipids (Lindhorst 2002). After this, more sugar residues can bind in a variety of ways to form structurally diverse glycolipids. The types and sequence of the sugar moieties of glycolipids vary widely but in general there are seven collective groups namely globo, isoglobo, muco, lacto, neolacto, ganglio and gala chains (Schenkel-Brunner, 2000). The presence of several hydroxyl groups on the sugar structures allows for glycosylation at many different sites and this property allows for the formation of branched structures (Lindhorst 2002). Glycosidic linkages also give rise to alpha (α) and beta (β) stereoisomers.

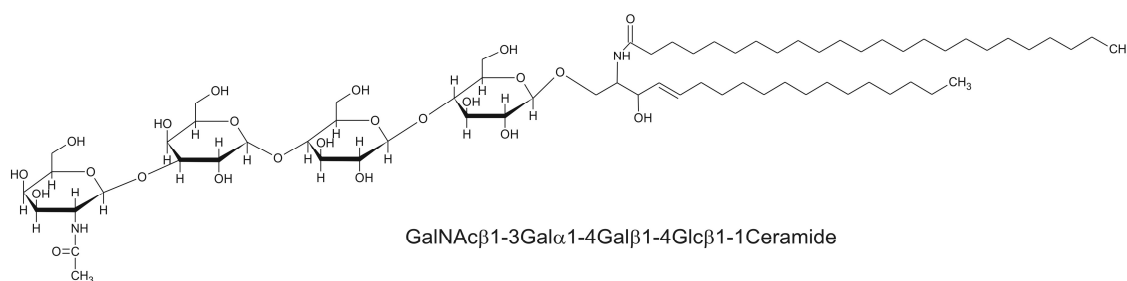


Figure 4: The sugar sequence present on a globoside glycolipid.

The many different types of glycolipids, their origins and methods of purifying and analyzing them is discussed extensively elsewhere (Carter et al., 1965; Geyer et al., 1998).

Glycoproteins

The blood group active substances were initially isolated from glycoproteins found in the saliva and human ovarian cyst fluid. The sugars in glycoproteins are linked to the polypeptide backbone by either an N-linked or O-linked glycosidic bond. They usually have high molecular masses and the carbohydrate content can be between 1% to more than 85% (Schenkel-Brunner, 2000). The N-linked sugars are mostly found in the glycoproteins of serum and cell membranes while the O-linked sugars are found in the exocrine secretions and mucins (Schenkel-Brunner, 2000). Carbohydrates can also be bound to proteins by the ethanolamine phosphate group to form glycosylphosphatidylinositols (GPI) that anchor proteins in the cell membrane (Lindhorst 2002).

Biosynthesis of glycoconjugates

Glycoconjugates are the key elements of some major blood group systems. Unlike proteins, glycoconjugates are not synthesized based on the 'one gene, one protein' concept. Instead the sugar units are added to the protein or lipid entity or to growing carbohydrate chains in a coordinated stepwise fashion by highly specific glycosyltransferases. These glycosyltransferases are coded for by genes. The transfer of each glycosyl unit forms the acceptor substrate for the next transfer step (Schenkel-Brunner, 2000). Each of the sugars to be transferred is activated in the form of UDP-galactose, UDP-N-acetylgalactosamine, GDP-mannose, GDP-fucose and CMP-N-acetylneuraminic acid. The transfer of sugars by the specific glycosyltransferases requires the presence of certain metal ions and takes place in the Golgi apparatus.

This is diagrammatically shown in Figure 5.

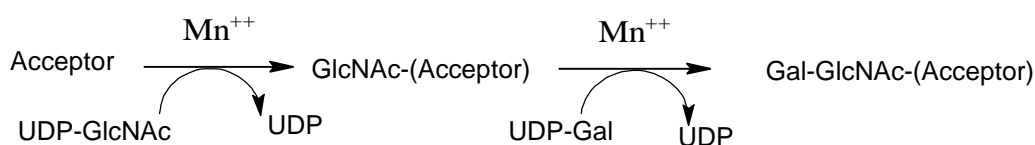


Figure 5: Transfer of sugar nucleotides to an acceptor by glycosyltransferases. The activated sugars are usually transferred in the presence of metal ions (Schenkel-Brunner, 2000).

Hence one gene codes for one glycosyl transferase (protein) and, as a result, there is a group of different genes that control the biosynthesis of a complete carbohydrate chain (Schenkel-Brunner, 2000). The transferase genes involved in the biosynthesis are usually independent of each other.

Biosynthesis of N-linked carbohydrate chains

There are two stages involved in the synthesis of N-linked carbohydrate chains. The first stage involves the assembly of a complex oligosaccharide unit in the membrane of the rough endoplasmic reticulum and proceeds by the sequential transfer of N-acetyl glucosamine, mannose and glucose residues onto dolichol pyrophosphate which acts as a transitional carrier molecule. The sugar chain formed as a result has an average composition of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (Schenkel-Brunner, 2000).

In the second step, this sugar-chain is transported from the lipid molecule (dolichol pyrophosphate) onto the protein. The acceptor region in the polypeptide chain for the oligosaccharide unit would ideally be Asn-X-Thr or Asn-X-Ser where X can be any amino acid except proline and aspartic acid. The increased nucleophilicity ('electron-hating' therefore negatively charged) of the amido electron pair brought about by the formation of the hydrogen bridge between the amido group of the aspartic acid and the hydroxyl group of serine/threonine helps to aid the transfer of the carbohydrate unit (Schenkel-Brunner, 2000).

Biosynthesis of O-linked carbohydrate chains

The O-linked carbohydrate chains are bound to the polypeptide backbone through α -GalNAc to the hydroxyl groups of serine and threonine amino acid residues or Glc to a ceramide tail in glycolipids (Schenkel-Brunner, 2000). The GalNAc residue of the glycoprotein is transferred onto the Ser and Thr amino acids by specific

N-acetylgalactosaminyl-transferases which are present in the cis-Golgi cisternae. The addition of further sugar residues does not occur immediately and usually take place in the trans-Golgi reticulum by the same enzymes involved in the synthesis of the N-linked carbohydrate chains (Schenkel-Brunner, 2000).

The O- and N-linked carbohydrate chains in glycoproteins are shown in Figure 6.

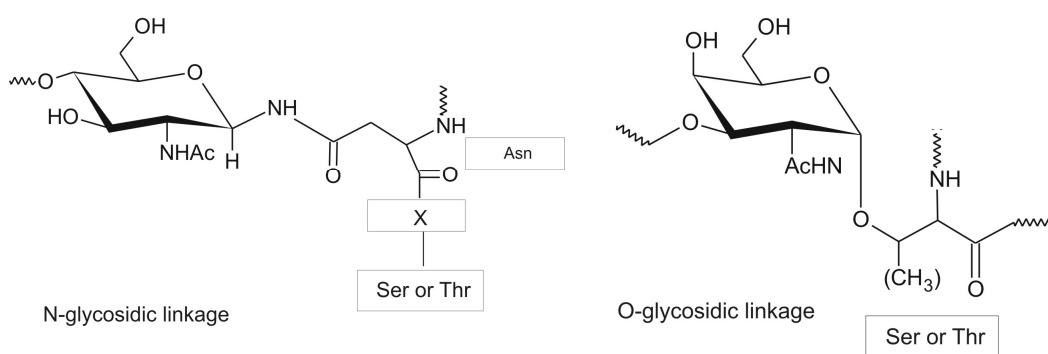


Figure 6: N- and O-glycosidic linkages in glycoprotein.

The 'X' in the N-glycosidic linkage can be any amino acid except proline and aspartic acid (Lindhorst 2002; Schenkel-Brunner, 2000).

Carbohydrate blood groups

Carbohydrates, proteins or carbohydrate-protein complexes make up the blood group systems. They usually associate with other molecules such as lipids to form specific blood group substances. Any polymorphic 3-D carbohydrate structure expressed on the outer membrane of erythrocytes to which an antibody is available is termed an antigen and its formation is the result of the interaction of many glycosyltransferases (Gilliver et al., 2003; Schenkel-Brunner, 2000). In addition, the type and sequence of sugar residues embedded on the erythrocytes are the antigenic structures that determine the ABO blood type of an individual. Many of these structures are also found in human tissues and secretions (Lindhorst 2002; Szulman, 1966). Individuals with the A and B blood types have the terminal glycosyl residues; GalNAc and Gal respectively, on their H oligosaccharide chain. Those who are type AB have both type A and B oligosaccharides. Individuals with O blood type lack the terminal GalNAc and Gal residues and consist of only the unconverted precursor H-structure which is common to both A and B blood types. This is represented in Figure 7.

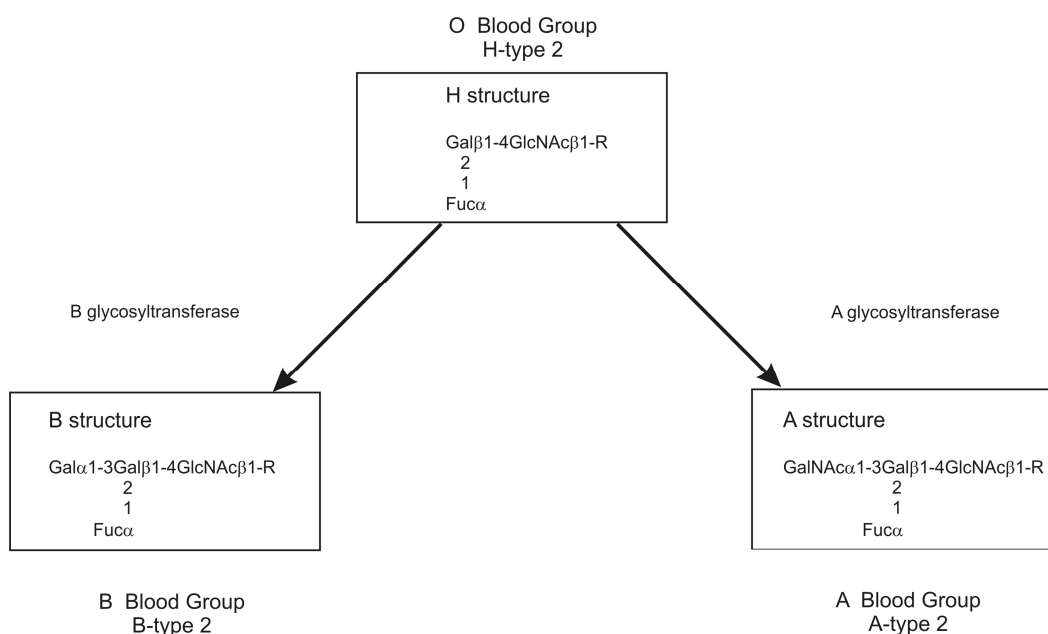


Figure 7: Formation of ABO antigens by glycosyltransferases.

The actions of specific glycosyltransferases determine the ABO blood type of an individual. Those that lack these specific enzymes have the blood type O while individuals who are blood type AB have both A and B glycosyltransferases hence have both types A and B glycoconjugates on their erythrocytes, tissues and mucous secretions (the latter only if a secretor). R represents the inner core structure.

Other carbohydrate blood group systems include the Lewis, H, Ii, P, Sd, Cad, etc. Again they are not direct products of a single gene; rather they are formed when specific glycosyltransferases transfer specific glycosyl residues to available precursor molecules in the biosynthetic pathway.

The terminal, non reducing β -D-Gal residue of the H structure is glycosidically linked to either the carbon 3 or 4 of the β -D-GlcNAc sub-terminal residue to form the type 1 or type 2 chains respectively. Types 1 and 2 chains are present in secretions while type 2 chains are more confined to the membranes of the erythrocytes. This difference in the linkages is readily recognized by antibodies and therefore represent the immunodominant part of these antigenic determinants (Lemieux, 1978).

ABO(H) blood group system

This system of classification originated from the pioneering work of Karl Landsteiner in 1900 and it involves the grouping of individuals into four blood groups. He stated that there two erythrocyte antigens, A and B and individuals can exhibit either the A or B or A and B antigens on their red blood cells. On the other hand, individuals can also lack these antigens and hence belonged to the H or O blood group. The H structure is common to both the types A and B blood groups as the unconverted substrate. The serum of individuals of the A blood type contains the B antibodies and the B type individuals have A antibodies in their sera. Those who are type AB lack these antibodies while the H/O type individuals exhibit both anti-A and anti-B antibodies (Schenkel-Brunner, 2000).

The frequencies of ABO blood groups of the Caucasian Australian population are shown in Table 1.

Table 1: ABO blood group phenotypes of the Caucasians in Australia.

Blood type	Frequency (%)
O	40.3
A	42.5
B	14.9
AB	2.3

Source: (CSL poster, 2007).

The ABH antigens have also been found in the saliva. The ability to secrete the blood group antigens in saliva and other mucosa tissues is due to the presence of *Secretor* gene. This gene encoded fucosyltransferase can be found to be present in 69.3% of a given Australian Caucasian population.

Lewis system

The Lewis blood group system was established by Mourant in 1946 (Schenkel-Brunner, 2000). Lewis antigens are glycolipids (and glycoproteins) and like the ABO antigens, they are present on the erythrocytes and as well as in body secretions (mucous) and tissues of humans and a few species of non-human primates (Schenkel-Brunner, 2000).

The Lewis antigens expressed on the erythrocytes are acquired from the plasma and not synthesized by the erythrocytes. White blood cells and platelets also adsorb the Lewis

antigens from the plasma in a similar manner. It was shown that together with Lewis a (Le^a) and Lewis b (Le^b), the precursor molecule, Lewis c (Le^c) and the H type 1 (Le^d) are also present in the plasma and readily adsorbed by the red blood cells (Greenwell, 1997; Hirsch et al., 1980; Koscielak et al., 1973). Lewis glycosphingolipids are produced in intestinal mucous cells and are present in minute amounts in the erythrocyte membrane.

The mucosa of the small intestine and pancreas are excellent sources of Lewis glycolipids while Lewis glycoproteins are found in saliva, amniotic fluid and ovarian cyst fluid. In addition, Lewis oligosaccharides present in milk, urine and meconium are rich sources of both Lewis glycolipids and glycoproteins (Schenkel-Brunner, 2000).

In a given Australian European population, 94.7% of the individuals are positive for Lewis while the remaining 5.3% are negative (CSL poster, 2007). The *Secretor* and *Lewis* genes are independent of each other and their phenotype frequencies based on the general Caucasian population are shown in Table 2.

Table 2: Lewis and Secretor phenotypes of a given European population (Australian).
+ and – indicate the presence or absence of the Lewis (Le) and Secretor (Se) genes respectively.
Source: CSL poster 2007.

RBC phenotype	Lewis	Secretor	Frequency (%)
Le(a+b-) Le(a-b+)	+		94.7
Le(a-b-)	-		5.3
Le(a-b-) Le(a-b+)		+	69.3
Le(a-b-) Le(a+b-)		-	30.7
Le(a-b+)	+	+	65.6
Le(a+b-)	+	-	29.1
Le(a-b-)	-	+	3.7
Le(a-b-)	-	-	1.6

The Lewis biosynthetic pathway

All Lewis antigens have the common Gal β 1-3GlcNAc which is a type 1 oligosaccharide chain also known as Le^c or type 1 precursor.

The Lewis biosynthetic pathway in Figure 8 illustrates the formation of complex Lewis antigens from the type 1 precursor molecule under the influence of *Lewis* (*Le*), *Secretor*

(Se) and ABO genes. Le^c, when modified by product of the Se gene is converted to Le^d by the addition of a fucose residue. Similarly, Le^c is converted to the Le^a structure by a Lewis fucosyltransferase enzyme coded for by the Le gene.

Under the control of the A/B genes, Le^d is then converted to either an A-type 1 or B-type 1 moiety which can be further transformed into the ALe^b or BLe^b structures respectively by fucosyl transferases that are encoded by the Le gene. Alternatively, Le^d is converted to the Le^b structure by a Lewis glycosyltransferase. Le^a is not a precursor of Le^b and Le^b is not a precursor for the ALe^b/BLe^b glycolipids.

The frequencies of the different Lewis antigens formed during biosynthesis are also shown and correspond to the values in Figure 8.

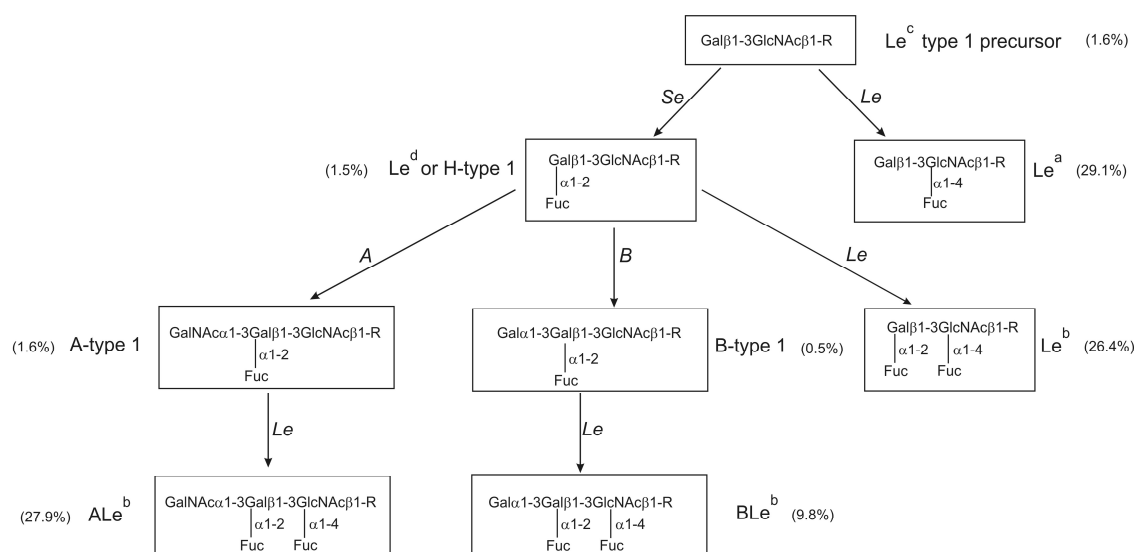


Figure 8: Biosynthetic pathway for Lewis antigen formation.

The percentage frequencies (Australian Caucasian population) for each of the antigens are also shown. (Frequency data obtained from Table 3).

The frequencies of the Lewis antigens shown in Table 3 are the result of the independent frequencies of the ABO, Lewis and Secretor genes. The data shows that the B-type 1, H-type 1 (Le^d), type 1 precursor (Le^c), A-type 1 and BLe^b antigens are those present in lowest quantities in a European population. Hence the ability to obtain these molecules by the simple removal of appropriate sugar residues of the more common molecules by chemical methods may be a convenient and economical means of obtaining these valuable structures. The aim of the current project was to investigate the potential of degrading the Lewis antigens into structures that are not readily available.

However, the degradation mechanisms of acid on glycolipids need to be clearly understood. To date, there are no reported studies on the production of precursor molecules by the way of acid hydrolysis of end-product Lewis antigens of the biosynthetic pathway.

Table 3: Frequencies of Lewis blood group antigens in the Australian Caucasian population. The antigens are listed from least to most common. Source: (CSL poster, 2007).

Phenotype			Frequency (%)	Dominant glycoconjugates							
				B-6-1	Le ^d	Le ^c	A-6-1	BL ^e	Le ^b	AL ^e	Le ^a
ABO	Le	Se									
B	a-b-	S	0.5	ü							
O	a-b-	S	1.5		ü						
ABO	a-b-	NS	1.6			ü					
A	a-b-	S	1.6				ü				
B	a-b+	S	9.8					ü			
O	a-b+	S	26.4						ü		
A	a-b+	S	27.9							ü	
ABO	a+b-	NS	29.1							ü	

Non secretor = NS
Secretor = S
Lewis = Le
ABO = all ABO groups

Analysis of polysaccharides in glycolipids

In order to completely understand the activity and function of a glycolipid and the role it plays in nature, one needs to carry out its full structural analysis. In practical terms, this is time consuming and there could be many limitations. Some drawbacks of such structural studies include the cost and availability of appropriate equipment required for analysis and the difficulty in interpreting complex sets of results. Consequently, many complex glycolipids have not been completely structurally defined.

The structural analysis of glycolipids includes the component and sequence determination of the primary structure. Both chemical and biological methods may be used to determine this. Unravelling the primary structure gives an insight into the secondary, tertiary and

quaternary structure of the polysaccharide of interest. However, to determine the intermolecular linkages, monosaccharide components that make up a polysaccharide and the sequence in which they are joined, one needs to employ a variety of methods and techniques together to obtain structural information. Some of them include;

- § Nuclear Magnetic Resonance spectroscopy (^1H NMR and ^{13}C NMR).
- § Mass Spectrometry
- § Thin Layer Chromatography (TLC)

Glycolipids can be separated on silica plates by TLC and the separation is based on size and polarity of the glycolipid. Glycolipids with larger oligosaccharide chains move much slower up the TLC plate than those bearing shorter oligosaccharide chains. For example, a 4-sugar glycolipid would migrate further than a 6-sugar glycolipid on a TLC plate. This is because larger glycan chains bear more sugar residues and, as a result, have a greater tendency to interact with the hydroxy groups present on the silica stationary phase thereby slowing down the rate of migration of the entire glycolipid on the TLC plate. The ceramide tails of glycolipids consisting of hydroxyl groups also interact with the stationary phase thereby slowing down the migration of the entire glycolipid. Hence, any degradation of the oligosaccharide chain or the ceramide moiety will result in a characteristic change in the migration.

The number of sugar residues borne on a glycolipid molecule can be identified by its migration on the TLC plate by comparison with known standards. Both Lewis a (Le^a) and H-type 1 glycolipids consist of 5 identical sugars making up their glycan chains, the only difference being a fucose residue bound to different sugar residues within the chains. These structures have slightly different migratory distances from the point of origin on a TLC plate. A detailed description of techniques used in TLC for separating glycolipids is also discussed elsewhere (Kundu et al., 1981). TLC separation of ceramides has also been described by Karlsson's group (Karlsson et al., 1971) Antigenic glycolipid structures can be further identified upon immunostaining assays (see below).

Chemical stains such as anisaldehyde can be used to visualise the migration of glycolipids in the form of bands. Anisaldehyde also has the additional ability to develop characteristic colours that reflect certain chemical qualities of particular lipid-containing compounds. For example glycolipids stain as green bands, ceramides stain as blue bands, glycolipids containing fucose residues stain

light-green while degraded glycerophospholipids bands stain a red-violet colour (Karlsson et al., 1987).

§ TLC-Immunostaining

Many polysaccharides have antigenic properties. Employing specific antibodies to recognise a specific sequence of monosaccharides in an oligosaccharide chain can give important information on the polysaccharide (glycan) structure.

The term immunostaining involves using an antibody based method to detect active carbohydrate/oligosaccharide sequences present in glycolipids and glycoproteins. There is a range of monoclonal antibodies available that can detect glycolipids. Overlay antibody-binding assays on TLC plates have been used to provide structural information on the epitopes of glycolipids and was one of the main methods of detection used in the current project.

Briefly, the glycolipids of interest are separated on a TLC plate and an antibody solution is overlaid on the glycolipid-developed area on the TLC plate and incubated for a period of time. Then the plate is flooded with a secondary antibody (conjugated with phosphatase enzyme) followed by incubation with a colorimetric enzyme substrate solution which makes the bands detected by the monoclonal antibody visible.

TLC-immunostaining has proven to be a simple and rapid method of detection of antigenic oligosaccharide sequences in glycolipids (Ishikawa et al., 2000). It is also able to handle small sample sizes and as well as those which contain trace levels of non-glycolipid impurities such as salts.

Other methods of obtaining structural information on glycoconjugates not discussed here include electrophoresis and separation based on molecular size and shape such as size exclusion HPLC.

Degradation of glycolipids

Detailed information about the identity and quantity of components that form the glycoconjugates requires degradation of the polymer. The degradative methods used for the characterisation of the oligosaccharide sequence of glycolipids can be adapted from those employed for general carbohydrate and glycoprotein analysis. These methods are well documented and a few are listed as follows;

Total acid hydrolysis

The acid hydrolysis conditions for degrading any polysaccharide must ensure that only the glycosidic bonds are cleaved with little or no degradation of the monosaccharide components. The strength of the individual glycosidic bonds are dependent upon a number of conditions some of which include polysaccharides containing furanose, pentoses and de-oxyhexoses (e.g. fucose, a 6-deoxyhexose) are more readily hydrolysed than those with pyranoses and amino sugars. Degradation of the monosaccharide units is inevitable upon total acid hydrolysis. Alternative methods have been developed to prevent non-specific degradation of these components. These methods employ the use of trifluoroacetic acid (CF_3COOH) instead of mineral acids such as HCl and H_2SO_4 . Methanolysis method also causes minimal damage to the cleaved monosaccharide residues; this method yields monomethyl glycosides on the anomeric carbon which, in turn, could have a slight influence on the polarity of the entire molecule (White et al., 1988). However, all hydrolysis processes degrade the residues to some extent and there is always some information loss of substituent groups and the position and configuration of intermolecular linkages.

Partial acid hydrolysis

Partial acid hydrolysis includes stopping the hydrolytic reaction before the reaction goes to completion. One advantage of using this method is that the structure of the simpler oligosaccharides released is easier to determine than if they were in a polymer. Polysaccharides made up of homologous components have their glycosidic linkages hydrolysed at the same rate and the products are smaller homopolysaccharides. On the other hand, polysaccharides consisting of a variety of different monosaccharides do not have the same rate of cleavage of their glycosidic linkages. It is now known that furanoside linkages are hydrolysed at a rate 10-1000 times greater than pyranosides (White et al., 1988). The (1-6) linkages are more stable than the (1-4) linkages in mineral acids. The opposite is true for reactions carried out in acidified acetic anhydride (White et al., 1988). Using these pieces of information, an acid hydrolysis method can be engineered to cleave glycosidic linkages between varying types of monosaccharide residues in any given heteropolysaccharide. In this way, more concise information on the identity of the polysaccharide is obtained.

Methylation analysis

This analysis is carried out to determine the linkage and sequence of monosaccharide units in a polysaccharide chain. Methylation involves reacting the free hydroxyl groups in

the polysaccharide to form methyl derivatives that are stable to acid hydrolysis conditions. Upon hydrolysis of the glycosidic linkages, the hydroxyl groups produced at the positions indicate where the linkages were on each of the monosaccharide residue. Methylation can be carried out with methyl iodide in the presence of silver oxide/carbonate (Purdie method) or dimethyl sulphate in aqueous alkali (Haworth method) (White et al., 1988). The methylated polysaccharide is hydrolysed with either H_2SO_4 or CF_3COOH . The volatile components or products of the hydrolysis mixture can then be analysed by gas chromatography (GC). A mass spectrometer linked to the GC can further identify these volatile derivatives (White et al., 1988).

For each of the methods, there are always some molecules with glycosidic bonds that have not completely cleaved (under mild conditions) while others may have completely degraded under more acidic conditions.

Alkaline degradation

This method of analysis does not provide much information about the overall structure of the polysaccharide. Alkaline degradation takes place by hydrolysing the ester groups which are attached through hydroxyl or carboxylic acid groups of the monosaccharide and the monomers are removed from the reducing end of the polysaccharide also known as the 'peeling' reaction (White et al., 1988). Further, the 1-3 glycosidic linkages degrade about 10 times faster than 1-4 linkages in cold, dilute alkali while the 1-2 linkages are stable to cold alkali but are degraded at elevated temperatures (White et al., 1988).

Alkaline degradation was introduced as an isolation technique for blood group substances (glycoproteins) when it was discovered that the fucose linkages were extremely labile to acid hydrolysis. Alkaline degradation preserved the fucosyl linkages and cleavage of glycosidic linkages proceeded through a β elimination procedure. This process is discussed in detail by Montreuil and Pazur (Montreuil et al., 1994; Pazur, 1994).

Chromium trioxide oxidation

Chromium trioxide, CrO_3 , is a strong oxidizer used in organic synthesis. It is very soluble in water, forming chromic and dichromic acids, H_2CrO_4 and $\text{H}_2\text{Cr}_2\text{O}_7$.

Chromium trioxide was once occasionally used for dehydrogenation of secondary hydroxyl groups to ketone groups in sugars. It was later discovered that CrO_3 in acetic acid oxidizes acetals, $\text{R}^1\text{CH}(\text{OR}^2)\text{OR}^3$ of aldehydes to esters, R^1COOR^2 , in which one of the OR groups forming the acetal is retained while the other is oxidized to a ketone (Angyal et al., 1970). Glycosides, being acetals, are also oxidized in the same way, the aglycone (the alcohol

obtained from a glycoside) being retained and the ring being ruptured (Angyal et al., 1970). The products are 5-ketogluconic acids from pyranosides and 4-ketogluconic acids from furanosides. This reaction is unique in that it is specific to β -pyranosides but favours both anomeric forms (α and β) of furanosides (Angyal et al., 1970; Laine et al., 1975). It has also been shown that CrO_3 can almost completely oxidize β -glycosidically linked glucose, galactose, mannose, N-acetylglucosamine and N-acetylgalactosamine units in simple glycerol- and sphingolipids (Laine et al., 1975). On the other hand, α -glycosidically linked monosaccharides resisted oxidation (Angyal et al., 1970).

Enzymatic hydrolysis

Enzymatic cleavage of glycosidic bonds is useful in identifying the various types of carbohydrate linkages. Enzymatic degradation of polysaccharides is a controlled form of hydrolysis. Substantial information about the structure, bond and bond orientation in polysaccharides is obtained from enzymatic cleavage as enzymes are always specific to the substrate and the linkages they cleave (White et al., 1988).

Many enzymes are now commercially available. The enzymes have to be used in their most pure form to obtain unambiguous results as the presence of other enzymes can lead to erroneous interpretation of results. In addition, minute differences in linkages in carbohydrate chains within the same macromolecule can also make structural interpretations difficult.

Enzymes that degrade polysaccharides are divided into 5 groups and they are as follows; (White et al., 1988)

- § *endo-Polysaccharide hydrolases.*
- § *exo-Polysaccharide hydrolases*
- § *Glycoside hydrolases*
- § *Polysaccharide lyases*
- § *Glycopeptidases*

A comprehensive discussion based on the enzymatic cleavage of polysaccharides can be found elsewhere (McCleary et al., 1986).

Enzymatic degradation of blood group substances

Some studies have shown that certain enzymes have the potential to alter the serological properties of A, B and H blood group antigens. Small scale expensive experiments in

converting the A and B blood groups into the underlying common precursor, O, has been investigated by many researchers as shown in Table 4.

Table 4: Bacterial enzymes used for the modification of blood group substances.

Substance	Enzyme used	Reference
O	<i>Bacillus fulminans</i>	(Iseki et al., 1953)
A	<i>Clostridium tertium</i>	(Iseki et al., 1953)
B	<i>Bacillus cereus</i>	(Iseki et al., 1956)
O and AO	<i>Bacillus fulminans</i>	(Yosizawa, 1957)
A,B, H	<i>Clostridium tertium</i>	(Howe et al., 1958)
A,B, H	<i>Clostridium tertium</i>	(Schiffman et al., 1958)
A, B, H, Le ^a	<i>Trichomonas foetus</i>	(Watkins, 1962)
A,B, H	Bacterial glycosidases	(Hoskins, 1968)
B	<i>Pseudoalteromonas</i> spp	Cited by (Olsson et al., 2004)
B	<i>Coffea canephora</i>	Cited by (Olsson et al., 2004)
A	<i>Clostridium perfringens</i>	Cited by (Olsson et al., 2004)
A	<i>Ruminococcus torques</i>	Cited by (Olsson et al., 2004)
A	<i>Acremonium</i> sp	Cited by (Olsson et al., 2004)
A and B	Bacterial glycosidases	(Liu et al., 2007)

The main limitation of using enzymes to degrade blood group antigens has been the large quantities of enzymes required for conversion into the type O as reported by Liu and Olsson (Liu et al., 2007; Olsson et al., 2004). Only recently have investigators isolated enzymes from two bacterial glycosidase gene families that are able to remove the A and B antigens at neutral pH to convert these antigens into the type O antigen (Liu et al., 2007). These methods have been reported to be economical.

Other methods

Other means of degrading polysaccharide are as follows and will not be discussed in detail but could be found elsewhere (Lindberg et al., 1975).

§ Deamination

§ Weerman and Lossen Rearrangements

§ Degradation based upon β -elimination which includes;

Degradation of polysaccharides containing uronic acids

Degradation preceded by oxidation

Degradation by way of sulfone derivatives

Acid hydrolysis of carbohydrates

Acid hydrolysis involves the addition of water across the glycosidic bond (Biermann, 1989). Hydrolysis of glycosidic linkages is normally carried out in an aqueous environment with an acid catalyst. In certain instances alkaline catalysts are also employed to release oligosaccharide chains from glycoconjugates.

Further, acidified solvents such as methanolic hydrogen chloride (for methanolysis) and organic acids such as anhydride-acetic acid-sulphuric acid (for acetolysis) and formic acid (for formolysis) are also capable of cleaving glycosidic bonds at elevated temperatures.

The rate of hydrolysis of the glycosidic bonds in a polysaccharide depend on the type of glycosidic linkages, the ring structure of the residues, the anomeric configurations of the glycosidic bonds and the nature of monosaccharide residues in a glycosidic linkage (Pazur, 1994; Wolfrom et al., 1963). Because the glycosidic bonds and monosaccharide residues have varying lability in acidic conditions, acid hydrolysis methods can be engineered to selectively cleave bonds to modify polysaccharide structures.

Glycosidic linkages are formed between the hydroxyl group of carbons 2, 3, 4 or 6 of one sugar residue and the hydroxyl group of the anomeric carbon (usually carbon 1) of another sugar residue giving rise to (1-2), (1-3), (1-4) or (1-6) linkages respectively. The (1-6) linkage has been found to be the most stable in acid followed by (1-4), (1-3) and (1-2) which is the least stable (Shallenberger, 1982).

As mentioned earlier, the anomeric configuration of a glycosidic bond can also determine the stability of linkages. Wolfrom reported that, generally, α linkages in disaccharides of D-glucopyranose are more labile to cleavage in acidic conditions than the corresponding β linkages with the exception of α -D-(1-6) linkage which is more stable than the β -D-(1-6) bond (Wolfrom et al., 1963). No information could be found with respect to the relative acid lability of linkages of sugars in the L form.

The following structural aspects of a polysaccharide need to be taken into consideration; From (Adams, 1965a, , 1965b; BeMiller, 1967; Biermann, 1989; Kennedy et al., 2004; Lindberg et al., 1975; Rege et al., 1963; Shallenberger, 1982; Wolfrom et al., 1963)

- § furanoside linkages are much more labile than pyranoside linkages in acidic conditions
- § glycosidic linkages involving de-oxy sugars are extremely vulnerable to acid hydrolysis; glycosidic linkages of 6-de-oxyhexoses are hydrolysed about 5 times faster than those of hexoses

- § alpha(α) glycosidic bonds are generally much more labile to acid hydrolysis than beta(β) glycosidic bonds and
- § the presence of uronic acid and N-acetylated groups on polysaccharides makes the latter more resistant to acid hydrolysis; upon acid hydrolysis, the N-acetylated amino sugars are deacetylated.

Partial acid hydrolysis processes are also well documented and are usually carried out for structural analysis purposes. With this method, the glycosidic linkages can either undergo random cleavage or could be more specifically directed towards a particular bond in the oligosaccharide chain to obtain a pool of oligosaccharides with uniform structure (Pazur, 1994).

In the methanolysis method, glycosidic linkages are cleaved by the addition of a methanol molecule across the glycosidic linkage to form methyl glycosides. It is absolutely critical for the reagent to be water-free in order to form protective methyl glycosides at the point of hydrolytic cleavage (Biermann, 1988; Chambers et al., 1971). The presence of water creates free forms of sugars. As a result, the products form a complicated mixture of free sugar and methyl glycosides. Methanolysis may be the preferred form of hydrolysis as it causes minimal damage to the monosaccharides liberated (Biermann, 1988). Chambers et al (Chambers et al., 1971) have carried out a systematic and quantitative investigation of products obtained with varying volumes of methanolic HCl concentrations. They found that methanolysis extensively deacetylates the acetylated amino sugars and monosaccharides can remain reasonably stable in 1M-2M methanolic HCl upto 24 hours at temperatures ranging from 85°C -100°C. The exoskeleton of insects, chitin, is a repeating sequence of β 1-4GlcNAc units. Methanolysis of this polysaccharide removes the acetyl groups (deacetylation) but the glycosidic bond is still resistant to hydrolysis by 1M methanolic HCl at 85°C. On the other hand, the sugars of glycoproteins, glycopeptides and oligosaccharides are readily degraded at methanolic acid concentrations higher than or equal to 4M (Chambers et al., 1971).

Acetolysis (acetic anhydride-acetic acid) and formolysis (formic acid) methods are modified forms of acid hydrolysis and is discussed in detail elsewhere (Biermann, 1988).

Acid hydrolysis provides information on both the sequence and anomeric configurations of sugar residues of the polysaccharide in question. Often the rates of hydrolysis of different glycosidic linkages do not differ considerably but at the same time there are many other polysaccharides whose glycosidic linkages do differ in lability (Lindberg et al., 1975). However, the results obtained from the acid hydrolysis method is qualitative as the

products are formed in a lower yield which are difficult to quantify (Lindberg et al., 1975).

Polysaccharides, under controlled acid hydrolysis, cleave into monosaccharides or oligosaccharides at the points of weak glycosidic acid-labile linkages. It is usually the examination of these products that give some valuable information about the structure and to a limited extent, the sugar sequence, of the polysaccharide in question.

In addition, oligosaccharides undergo acid hydrolysis until all of the glycosidic bonds are broken to yield simple pentoses and hexoses. The difficulty of breaking these bonds relies on the fact that all of these glycosidic bonds are not equally accessible and reactive (Abatzoglou et al., 1998; Lindberg et al., 1975). Mechanistic and kinetic interactions within the matrix further add to the complexity of the molecule (Abatzoglou et al., 1998).

In order to characterize a complex polysaccharide, it is most advisable to perform a stepwise degradation with acids of increasing strengths. To obtain information about the nature of and the glycosidic attachment of sugars, mild acid hydrolysis methods are recommended as these methods would gently remove the sugars without causing any major damage to the chemical structure (Adams, 1965b). As a result, this gentle degradation would produce molecules which are less complicated and their structural studies can be performed more easily.

Acid hydrolysis of weak linkages

Furanoside linkages have been reported to be more vulnerable to acid hydrolysis than the pyranoside bond. The exception to this would be the 3-6 anhydrohexosides (Adams, 1965a). Glycofuranosides containing de-oxy sugars are also susceptible to acid hydrolysis compared to glycopyranosides containing de-oxy sugars (e.g. fucose). The N-glycosides of de-oxy sugars are less stable to acid hydrolysis than the O-glycosides of the same sugars (Adams, 1965a). The methods for hydrolyzing the glycosidic bonds in O-2-deoxyglycosides and N-2-deoxyglycosides are discussed by Adams elsewhere (Adams, 1965a). The weak bonds are hydrolysed by dilute concentrations of acid (HCl) at about 100°C over a short period of time.

Acid hydrolysis of glycolipids

To date, the limitations of carrying out acid hydrolysis of glycolipids in an aqueous environment is not well documented. There has been no systematic investigation into this method that would give a series of structurally well-defined ceramide hexose products.

The methods of hydrolysing the sugars in glycoconjugates vary depending on the type of glycoconjugate. Many different acids of varying strengths have been used to cleave the glycosidic bonds. These include sulphuric acid (H_2SO_4), hydrochloric acid (HCl), trifluoroacetic acid (CF_3COOH) or a mixture of acids (Biermann, 1988). The use of H_2SO_4 has not been widely popular while HCl has been widely used by most researchers. The acid concentrations ranged from 0.5 M (Molar) to 6 M at temperatures of 100°C over periods of time ranging from 1 – 12 hours. It was observed that different hexoses were cleaved off the oligosaccharide chain at different times. This suggested that the presence of certain functional groups and the arrangement of these groups in space (axial or equatorial) together with the type of anomeric bond (α or β) contributed towards the different rates of cleavage for different monosaccharides (BeMiller et al., 1971; Biermann, 1988). Of particular interest was the work carried out by Kannan et al. They were able to isolate and hydrolyse the hexoses of the neutral glycolipids (of human brain origin) with 1M HCl over 6 hours at 100°C and found that the glucose-ceramide bond was less susceptible to acid hydrolysis (aqueous environment) than the galactose-ceramide bond (Kannan et al., 1974). They found that it took twice the amount of time to cleave the glucose-ceramide bond at the same acid concentration and temperature.

Other researchers have often used a mixture of equal amounts of two acids. The underlying principle of this method would be that one of the more dilute acids would be able to cleave glycosidic bonds of neutral sugars while a stronger acid concentration would be able to liberate the amino sugars such as GalNAc and GlcNAc (Biermann, 1988).

The acid hydrolysis process has been extensively used by researchers despite the drawbacks mentioned above. Sweeley's group hydrolysed the glycosidic bonds in human brain glycolipids and gangliosides by the methanolysis method in order to identify the individual monosaccharide units in these glycoconjugates (Sweeley et al., 1964). The acid hydrolysis process has undergone many refinements over time and the methods are often engineered to suit the type of sugar residues in the glycoconjugate being analysed. For example, Makino et al were able to develop a relatively sensitive method for analysing the oligosaccharide sequence in a N-linked sugar chain (glycoprotein) by employing both partial acid hydrolysis and enzymatic methods (Makino et al., 1998).

Leverly et al outlined a simple protocol for derivatising and analysing glycolipids by Capillary GC. In their investigation, they initially methylated the glycolipid samples with methyl iodide (CH_3I), dried the permethylated glycolipid and then subjected this sample to acetolysis (0.5 M H_2SO_4 in 90% acetic acid (CH_3COOH) at 80°C (Leverly et al., 1987).

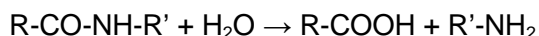
The nature of glycolipids in acidic environment needs to be established prior to the degradation experiments. An ideal degradation experiment involving cleavage of glycosidic bonds in glycolipids will be able to address the following issues;

- § The hydrolysis conditions will only cleave glycosidic bonds of interest,
- § The hydrolysis conditions will not further degrade or at worst minimally degrade the other sugar residues exposed after the scission of bonds,
- § The hydrolysis conditions will remove sugars from the non reducing end sequentially without affecting or modifying the ceramide unit of the glycolipid

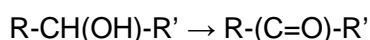
Stability of sphingolipids/ceramides

The degradation mechanisms involving lipids are well understood. The degradative effects of acid on sphingolipids, however, are not well documented. Nonetheless, there are three distinct sites on a ceramide molecule that could make the latter vulnerable to degradation. These include the amide linkage that links the long chain base to the fatty acid component, the presence of hydroxy groups and the presence of double bonds in unsaturated sphingolipids.

The amide bond is quite stable and can be hydrolysed under extreme acidic or basic conditions as follows, where R' is the long chain (amine) base and R is the fatty acid (Solomons, 1994).



In addition, the hydroxyl group can be oxidized to carbonyl groups thereby converting the long chain base from a secondary alcohol to a ketone (Solomons, 1994).



The alkene bonds can also undergo cleavage by oxidation reactions where oxygen is added to the double bonded carbon to form a hydroperoxide molecule which further decomposes to a hydroxyl and an alkoxyl radical (de Man, 1999). These double bonds can be also oxidatively cleaved to generate aldehydes or carboxylic acids. This concept is discussed in detail elsewhere (Mylvaganam et al., 2000).

Acid hydrolysis of blood group glycoconjugates

Isolation and identification of active blood group substances

Many researchers have utilised the acid hydrolysis processes for the structural analysis of a variety of glycoconjugates in a wide range of studies. They carried out both qualitative

and quantitative analysis of monosaccharides isolated from the oligosaccharide chain after the cleavage of the latter from the protein or ceramide entity. These oligosaccharide chains were cleaved mainly by acid hydrolysis methods.

The many studies reported on the ABH blood group active substances were largely based on glycoproteins and were primarily set out to determine the sugar sequences and identity of the residues making up the oligosaccharide chain. The first step involved cleaving oligosaccharide sequences in the form of di- or trisaccharides from the glycoprotein (or glycolipid) by either acid or alkali. These sequences were then subjected to a range of hydrolysis methods including methylation, periodate oxidation, methanolysis and as well as enzymatic degradation in order to cleave individual glycosidic bonds thereby identifying the individual monosaccharide residues and their linkage patterns by MS or other physical methods.

During the course of these various investigations, much was learnt about the nature of glycosidic bonds and some of these included

- § fucose residues are extremely labile to acidic conditions but are stable under alkaline conditions
- § the 1-3 glycosidic bond in glycoconjugates, especially in glycoproteins, is alkali-labile
- § the Glc-ceramide bond is not readily hydrolysed by acid except with extremely acidic solutions and high temperatures
- § glycosidic bonds linking GlcNAc and GalNAc residues are not readily hydrolysed by acid

Morgan and Watkins have described in detail, the evolution of research regarding the Lewis and ABO specificity since the late 1930s (Morgan et al., 2000). As stated earlier, a large portion of early research was based on the identifying sugars and sugar sequences that were responsible for A, B and H blood group activity. It has also been reported that fucose was not identified as a blood-group related monosaccharide until 1963 as this residue was readily lost upon acid hydrolysis methods used to isolate the oligosaccharide sequence of the blood group substances (McKibbin, 1978).

A near comprehensive list of researchers who have employed acid hydrolysis methods in their investigations of blood group determinants is shown in Table 5.

Some examples of work carried out on the blood group active substances are described as follows;

In the early days, Hakamori's team working on isolating and characterising glycolipids from human red blood cells utilised acid hydrolysis processes to identify the monosaccharides units present in the glycan chain of glycolipids. They employed both sulphuric acid (H_2SO_4) and hydrochloric acid (HCl) to determine the hexose and hexosamine constituents that make up the blood group sugars in the glycolipids (Hakomori et al., 1961). They were also one of the first few groups to claim that glycolipids also bear the blood group antigens and like glycoproteins, they exhibit antigenic properties.

An early study on the H substance by Rege's group degraded the blood group H substance obtained from the human ovarian cyst fluid by alkali to obtain two different fragments of di- and trisaccharides containing the fucose residues. Alkaline degradation, unlike acid hydrolysis, was found to preserve the fucose residues in the oligosaccharide chain of glycoconjugates. These fragments were then subjected to further degradation by acid hydrolysis in order to determine the linkages between the L-fucose and the two other blood group sugars (Gal, GlcNAc) making up the H substance. Consequently, they were able to demonstrate that the fucose is joined to a non reducing Gal residue by an α 1-2 glycosidic linkage in both types 1 and 2 H blood group substance (Rege et al., 1964).

Rege's group was also able to obtain trisaccharide sequences by partial acidic degradation of A, B, H and Le^a substances and reported that the hydrolysate of the A substance contains an A-active trisaccharide with a terminal non reducing GalNAc residue while the hydrolysate of the B substance contains a B-active trisaccharide with a terminal non reducing Gal residue (Rege et al., 1963). They also reported that the ABH and Le^a substances have a common inner Gal-GlcNAc moiety but were not able to demonstrate the structural differences between H and Le^a . At this time, it was speculated that the fucose residues had a vital role in Le^a and H specificities (Rege et al., 1963).

Table 5: Blood group substances isolated by researchers (in chronological order). These substances had their blood group oligosaccharide chains structurally characterized and analysed by one or a range of hydrolysis methods listed below. Acid/partial acid hydrolysis was the most common methods used. Blue font represents glycoproteins while red font represents glycolipids.

Substance	Purpose					Method							Reference
	Identify/analyse sugars	Sugar sequence	Linkage analysis	Release of active oligosaccharides	Removal of Fucose	Origin	Acid hydrolysis	Partial acid hydrolysis	Methanolysis	Acetolysis/formolysis	Periodate oxidation	Enzymatic cleavage/other	
A,B & O	ü				ü	Saliva and hog stomach	ü						(Kabat et al., 1948)
B	ü				ü	Human saliva and horse stomach						ü	(Baer et al., 1950)
A	ü			ü		Ovarian cyst fluid	ü						(Cote et al., 1956)
A, B & H	ü					Hog mucins	ü					ü	(Schiffman et al., 1958)
B	ü	ü	ü	ü		Ovarian cyst fluid		ü			ü		(Schiffman et al., 1960)
A & B	ü					Human RBCs	ü	ü			ü		(Hakomori et al., 1961)
A	ü	ü		ü		Mucopolysaccharide	ü	ü			ü	ü	(Cheese et al., 1961)
B	ü		ü	ü		Ovarian cyst fluid		ü			ü		(Painter et al., 1962)
A	ü	ü		ü		Ovarian cyst fluid		ü			ü		(Schiffman et al., 1962)
A, B,H,Le ^a	ü	ü		ü		Ovarian cyst fluid		ü			ü		(Painter et al., 1963)
Globoside	ü	ü	ü			Human RBCs		ü	ü				(Yamakawa et al., 1963)
A, B, H, Le ^a	ü	ü	ü	ü		Not stated		ü		ü	ü	ü	(Rege et al., 1963)
H	ü	ü		ü	ü	Ovarian cyst fluid		ü			ü	ü	(Rege et al., 1964)
A,B & H	ü	ü		ü		Ovarian cyst fluid					ü	ü	(Schiffman et al., 1964)
A & B		ü	ü	ü		Ovarian cyst fluid		ü			ü	ü	(Painter et al., 1965)
A,B & H	ü			ü		Ovarian cyst fluid		ü				ü	(Kabat et al., 1965)

Table 5 continued:

Substance	Purpose					Method							Reference
	Identify/analyse sugars	Sugar sequence	Linkage analysis	Release of active oligosaccharides	Removal of Fucose	Origin	Acid hydrolysis	Partial acid hydrolysis	Methanolysis	Acetolysis/formolysis	Periodate oxidation	Enzymatic cleavage/other	
Le ^a	ü	ü	ü	ü		Ovarian cyst fluid	ü	ü			ü	ü	(Marr et al., 1968)
H		ü	ü	ü		Ovarian cyst fluid	ü		ü		ü	ü	(Aston et al., 1968)
Le ^a , Le ^b , Le ^c	ü			ü		Human adenocarcinoma				ü		ü	(Hakomori et al., 1970)
Novel glycolipid	ü	ü		ü		Human adenocarcinoma	ü	ü		ü	ü	ü	(Yang et al., 1971)
Mucins	ü					Epithelial mucins	ü						(Griggs et al., 1971)
A	ü	ü				Human RBCs		ü		ü	ü	ü	(Hakomori et al., 1972)
B & H	ü	ü	ü			Human RBCs		ü	ü		ü	ü	(Koscielak et al., 1973)
H	ü	ü	ü	ü	ü	Human RBCs		ü	ü	ü		ü	(Stellner et al., 1973)
H	ü					Human RBCs	ü		ü		ü	ü	(Watanabe et al., 1975)
A, H	ü	ü	ü			Human RBCs		ü		ü	ü	ü	(Koscielak et al., 1976)
OLe ^b	ü					Human plasma				ü			(Hanfland, 1978)
Novel glycolipid	ü	ü	ü			Meconium				ü			(Karlsson et al., 1979)
Le ^a , Le ^b , H	ü	ü	ü			Human plasma				ü		ü	(Hanfland et al., 1981)
Le ^c	ü					Human RBCs		ü				ü	(Hanfland et al., 1982)
OLe(a-b-)	ü		ü			Human plasma						ü	(Hanfland et al., 1986)
Glycolipids			ü			Not stated		ü	ü	ü		ü	(Leverly et al., 1987)
Le ^b			ü			Human colonic adenocarcinoma cell				ü		ü	(Stroud et al., 1992)
Glycolipids & glycoproteins	ü					Not stated	ü	ü	ü			ü	(Yasuno et al., 1999)

A similar study undertaken by the same group set out to isolate blood group A, B, H and Le^a substances from the human ovarian cyst fluid and then degraded these structures with polystyrenesulphonic acid. The products (disaccharides) obtained had their sugar-content analysed and identified. The results showed that each of the hydrolysates contained the common monosaccharides, L-Fuc, D-Gal, D-GalNAc, D-GlcNAc. The Fuc residues were not obtained in significant amounts as they reported that this non reducing residue is cleaved from the parent chain during the early stages of the acid hydrolysis process before the Gal, GalNAc and GlcNAc are removed (Painter et al., 1963). Painter also

demonstrated that the active B substance in human ovarian cyst was partly due to the non reducing terminal disaccharide sequence Gal α 1-3Gal-. This disaccharide was obtained by hydrolysis of the glycoconjugates with mineral acids. He also proposed that the B structure is more complex than this simple disaccharide as the presence of other sugars in the complete sequence of oligosaccharides together with additional sugar residues branching off them may add to the complexity or full specificity of the B active substance (Painter et al., 1962). This group later recommended that the (defucosylated) active B trisaccharide had either the oligosaccharide sequence Gal α 1-3Gal β 1-3GlcNAc- (type 1) or Gal α 1-3Gal β 1-4GlcNAc- (type 2) as they were both able to inhibit the agglutination of the human B cells by human anti-B serum.

In a separate study, glycolipids (erythrocytes) consisting of fucose residues were isolated and then subjected to partial acid hydrolysis, periodate oxidation and methylation methods. to determine the types of monosaccharides and sequence in which they were present in the glycan chain The anomeric linkages in the oligosaccharide sequence were also described by the way of enzymatic degradation and immunological activities (Koscielak et al., 1973).

The Lewis blood group erythrocyte antigens were first purified and biochemically analysed by Hanfland (Hanfland, 1978). It was already known at the time that the Lewis antigens were acquired by the red blood cells from the plasma. He was able to isolate and characterize two Le^a and three Le^b active glycolipids obtained from human OLe^b plasma. The acetolysis method was used to cleave the sugars off the glycolipids followed by neutralisation, reduction and acetylation procedures. The sugars obtained were then finally subjected to GC for identification.

Catalysis by metal ions

Catalysts

'A catalyst is a substance which increases the rate at which a chemical reaction approaches equilibrium without itself becoming permanently involved in the reaction' (Bond, 1972). In the absence of a catalyst, the reaction would still occur but would only be able to proceed very slowly. A catalyst can only increase the rate of a reaction which is already thermodynamically feasible; it cannot initiate one which is thermodynamically unfeasible. In addition, different types of catalysts can cause the same reaction to yield different products; this is because different catalysts 'bring reactant molecules to different levels of activation energy which are thermodynamically feasible as well' (Bond, 1972).

These different products would have been formed in the absence of the catalysts in the same temperature range and the non-existence of some of the products in a homogenous reaction maybe entirely due to their instability at high temperatures. A catalyst may become temporarily involved in a reaction. It may form intermediate species with the reactant molecules which makes transformation into the product much easier than that of the reactant itself (Bond, 1972).

Metal catalysts normally consist of a transition-metal surrounded by a group of ligands that determine the catalytic behaviour of the centre metal ion (Crabtree, 2000). Metal induced catalysis is especially valuable for reactions that involve the removal or addition of one or more molecules of hydrogen from a molecule; in other words, metal ions can readily dissociate and as well as catalyse the formation of molecules (Bond, 1972). As the concept of catalysis developed, more chemically strong and unreactive bonds were attacked. Examples of such strong bonds are the carbon-hydrogen bonds in alkanes. This bond type is less reactive than the H-H bond in molecular hydrogen and resists cleavage by oxidative addition. Such molecules undergo catalytic dehydrogenation or reduction which converts them to more chemically reactive alkenes (Bond, 1972).

Oxidative addition involving the carbon-carbon bond is the most difficult to cleave (Crabtree, 2000). This bond is very strong and is at the same time hidden deep within the molecule due to the overlap of the *sp* hybridised orbitals.

In dissociative adsorption, the molecule is disrupted on adsorption. In associative adsorption, unsaturated molecules open up their double bonds to form new covalent bonds (Bond, 1972).

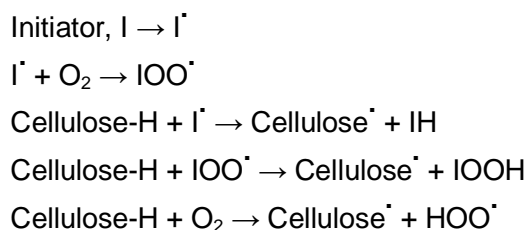
Transition metal ions form reactive oxygen species such as hydroxyl and superoxide radicals that result in extensive degradation through oxidative-reductive depolymerisation (Yalpani, 1988). Alginic acids, carrageenans, dextran, pectin, xanthan gum and hyaluronic acid have all been shown to be depolymerised by the presence of metal ions. Furthermore, millimolar amounts of Fe^{2+} and Cu^+ ions in the presence of oxygen and reducing agents such as L-ascorbic acid, thiols, hydroquinones cause oxidative-reductive depolymerisation reactions. The role of the reducing agent is to reduce the liberated oxidized groups thereby stabilizing them and as well as to regenerate the metal ions (Yalpani, 1988).

Metallic cations in cellulose degradation

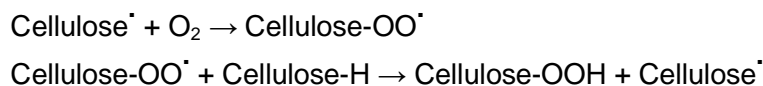
Metal cations have been found to play important roles in cellulose degradation. Transition metals, especially, are known to degrade paper. These metal ions catalyse the oxidation of cellulose in alkaline medium. Trace quantities of transition metals, iron, copper and manganese are usually adsorbed onto the cellulose during paper manufacturing processes as cellulose has a high affinity for such species (Shahani et al., 1986).

Generally a free radical mechanism of autoxidation of cellulose arises from the interaction of cellulose with atmospheric oxygen and occurs in 3 distinct steps and is the most commonly reported pathway as shown below. From (Shahani et al., 1986)

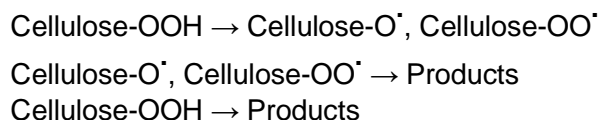
Initiation:



Propagation:

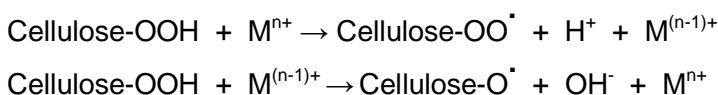


Product formation:



The hydrogen is usually removed from carbon 1 of the glucose and an oxygen molecule would react with this carbon to form a peroxy radical thereby cleaving the β 1-4 glycosidic bond. This cleavage also forms an alkoxy radical in the adjacent glucose unit (Shahani et al., 1986).

The transition metal ions can further increase the reaction rate of the above by catalysing the homolytic decomposition of the cellulose-peroxide molecules as shown below;



The absorption of atmospheric oxygen even in the smallest quantities can initiate the process outlined above. Metal ions present as salts that form acidic solutions also contribute towards paper degradation through acid hydrolysis process. Both alum (aluminium potassium sulphate) and $\text{Fe}_2(\text{SO}_4)_3$ are known for inducing acid hydrolysis of paper while copper cations were found to degrade paper in neutralized humid conditions (Shahani et al., 1986).

The autoxidative breakdown of cellulose has been found to be inhibited by magnesium compounds that are believed to stabilize the cellulose peroxide molecules (Shahani et al., 1986).

Bicchieri et al have also proposed that metal ions degrade cellulose by the sequence of events described above (Bicchieri et al., 1996). Bicchieri *et al* suggested that the saturation sites of papers treated with copper ions proceeds much quicker than that compared with untreated paper and those treated with iron ions. This observation suggested that there are differences in the interaction between metallic cations and cellulose. They recommended that Fe^{3+} catalyses the cleavage of the β 1-4 glycosidic bonds thereby increasing the hydrolysis rate while Cu^{2+} catalyses the opening of the glycopyranosic ring without having any effect on the rate of hydrolysis. These reactions were carried out in a low-acid medium (Bicchieri et al., 1996).

It is thought, that metal ions may also be involved in cleaving glycosidic bonds in the oligosaccharide chains of glycolipids by similar mechanisms described above.

Of further interest to glycolipids is that trace metals such as copper and iron have also been found to catalyse the oxidation of fatty acids in lipids (de Man, 1999).

Objective

The objective of the current project was to establish degradation concepts of neutral glycolipids and in doing so carry out systematic degradation of blood group active glycolipids by employing potential catalytic conditions and acid hydrolysis methods designed to modify blood group glycoconjugates into the uncommon blood group substances such the H-type 1, A-type 1, Le^a and the type 1 precursor, Le^c . The ability to control the degradation process to favour the production of a particular blood group product was also investigated.

2 Results Section A - Effects of metallic salts and acids on the globoside molecule

Part I

Effects of 0.02 M metallic solutions on globoside at 85°C

The degradative effects of metallic compounds on glycolipids have not been widely investigated. A previous project based on testing the stability of glycolipids led to the observation that incubating the globoside (G-4-4) glycolipid with certain metallic-compound solutions over a period of time at high temperatures resulted in the breakdown of G-4-4 (Gilliver, 2002). In addition, the breakdown of this glycolipid seemed to occur in an orderly fashion with the terminal sugar being removed first followed by the sub-terminal residue and so on and so forth. Hence it is suspected that the cleavage (or hydrolysis) of glycosidic bonds in the oligosaccharide unit of the glycoconjugate could be catalysed by metal ions.

The cleavage of glycosidic linkages by metal ion catalysts in cellulose degradation is discussed in Part VIII. The similarity between the reported investigations and the current project was the cleavage of the glycosidic bonds. The major difference between the two studies was long term polysaccharide degradation versus short term glycolipid degradation.

Globoside (G-4-4) is a type-4 glycolipid consisting of four sugar residues, three of which are bound by strong beta (β) glycosidic bonds to the sphingolipid chain. In greater detail G-4-4 has the following structure; $\text{GalNAc}\beta 1-3\text{Gal}\alpha 1-4\text{Gal}\beta 1-4\text{Glc}\beta 1-1\text{Cer}$. It is thought that the sequential removal of sugar residues catalysed by metallic salts could prove to be an economical process in obtaining pre-cursor molecules of the carbohydrate-based blood group biosynthetic pathways.

This section of the project will look at the various factors and variables affecting the degradation of the G-4-4 glycolipid and will try to establish a few degradation concepts of glycolipids before degrading the more valuable Lewis blood group glycolipids. The precursors for the blood group structures are difficult to obtain and are most likely acquired by either enzymatic cleavage processes or from the rare individuals lacking the common blood group glycosyltransferases.

Developing alternative pathways where these molecules can be obtained readily by inexpensive and rapid processes would prove to be useful and may further highlight risks and modifications which can be made to existing glycolipid extraction and handling processes.

The experiments in this section were carried out at 85°C as this temperature was shown to be ideal for degradation of glycolipids as seen in Part VII. The general method is outlined in Protocol 1 (General Method). Anisaldehyde staining was the main method used to identify new structures formed upon hydrolysis of the glycan chain of the G-4-4 molecule.

The G-4-4 molecule has a relative migration (*rm*) of 7.0 ± 0.5 units on a TLC plate as measured on a scale developed in-house. A 3-sugar glycolipid has an *rm* of 9.0 ± 0.5 while the 2- and 1-sugar glycolipid structures migrate further up on the TLC plate, off the *rm* scale.

The terms 'degradation' and 'hydrolysis' have been used interchangeably in this section. Degradation of the G-4-4 molecule refers to the hydrolysis of the glycan chain of this glycolipid unless otherwise specified.

The first set of experiments was carried out to assess the effects of various metal ions on the G-4-4 molecule upon incubating them together at 85°C over 48 hours. The experiments tested small amounts of glycolipids and metallic compounds merely to observe any hydrolysis patterns in the glycolipids. It is appreciated that some salts/compounds used were insoluble or partly/sparingly soluble while others hydrolysed to give solutions of varying acidity. The main objective of this section was to do a survey of those compounds that were able to cause some form of degradation to the G-4-4 structure.

In Figure 9, plate I shows the degradative effects of incubating G-4-4 with 0.02 M solutions of some metallic chlorides. The migration of the degradation products on the TLC plate are marked and compared with that of the controls.

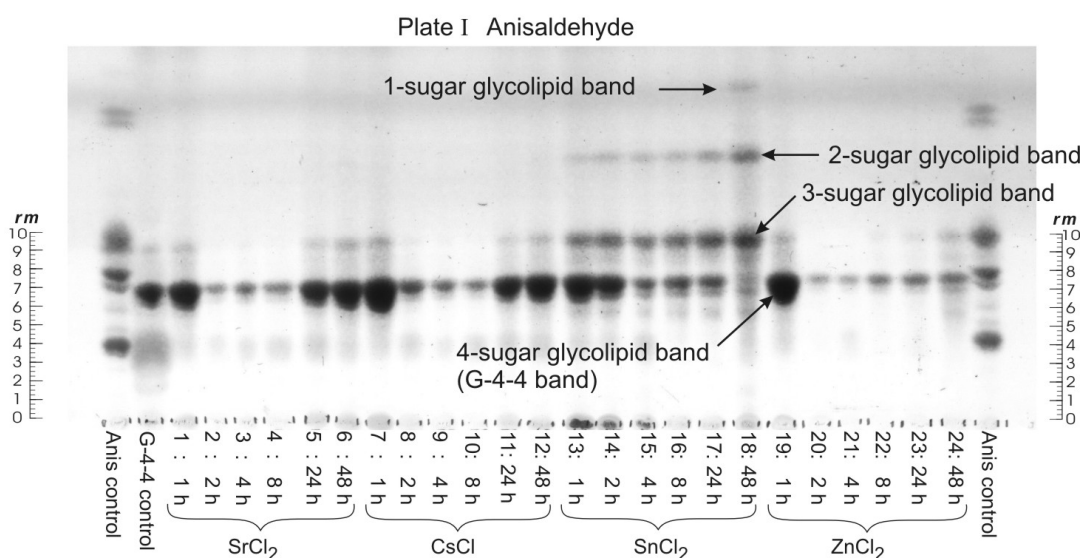


Figure 9: Effects of a range of 0.02 M metallic chlorides on G-4-4. Incubation was carried out at 85°C over a period of 48 hours. The concentration of G-4-4 was 0.02 M. Arrows in plate I indicate the migratory positions of the G-4-4, 3-, 2-, 1-sugar glycolipids.

A control was also set up to investigate the effects of temperature alone on G-4-4 over 24 hours and another G-4-4 sample set at room temperature for 24 hours as outlined in the general method section in Protocol 1. At 85°C, there was no visible degradation of the G-4-4 sample. The darker 3-sugar glycolipid bands in lanes 1, 4, 5, 9 and 10 (Figure 10, plate I) is entirely due to a high loading concentration and is purely an artifact of anisaldehyde staining. The darker 4-sugar G-4-4 bands at $rm\ 7.0 \pm 0.5$ is also due to a heavier sample loading on these lanes compared to that of lanes 2 and 3 (Figure 10, plate I).

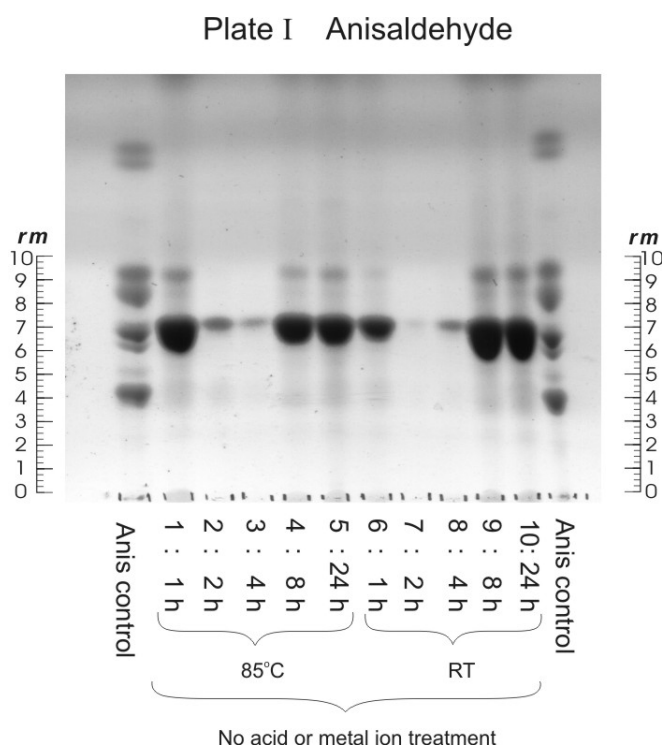


Figure 10: Effects of temperature on native G-4-4 sample. The effects of temperature were measured at 85°C and 20°C. RT = room temperature

The same holds true for G-4-4 sample set at room temperature. This was an expected result. There was no visible degradation of G-4-4.

Summary

Degradation of G-4-4 was assessed by measuring new or existing band intensities at various positions on the *rm* scale by anisaldehyde staining which is a semi-quantitative assay. The method for scoring degradation is outlined in Protocol 11. It is important to note that a difference in band intensity should not be confused with the differences in loading volumes.

Table 6 gives a summary of the degradation patterns observed when a solution of globoside was incubated with solutions of various compounds.

Table 6: Degradative effects caused by incubating 0.02 M metallic salts with G-4-4. Incubations were carried out at 85°C over 48 hours. (Blue font denotes nil degradation, green font denotes mild degradation and red font denotes considerable to extensive degradation).

Anions	Groups of the Periodic Table					Other salts	
	I	II	Transition metals	III	IV	Non metallic salts	Complex or double salts
Chloride	Na ⁺ K ⁺ Cs ⁺ Li ⁺	Mg ²⁺ Ca ²⁺ Sr ²⁺ Ba ²⁺	Fe ²⁺ Ni ²⁺ Cr ³⁺ Fe ³⁺ Cd ²⁺ Zn ²⁺ Mn ²⁺ Co ²⁺ Cu ²⁺	Al ³⁺	Sn ²⁺ Sn ⁴⁺ Pb ²⁺	NH ₄ ⁺	NH ₄ (Cu ⁺) NH ₄ (Fe ³⁺)
Sulphate	Na ⁺ Li ⁺	Mg ²⁺ Ca ²⁺	Fe ³⁺ Cd ²⁺ Zn ²⁺ Ni ²⁺ Ag ⁺ Cr ²⁺	Al ³⁺			K(Cr) K(Al)
Nitrate	Na ⁺ K ⁺	Mg ²⁺ Ba ²⁺	Fe ³⁺ Cr ³⁺ Fe ²⁺ Ag ⁺ Ni ²⁺ Zn ²⁺ Cd ²⁺ Cu ²⁺	Al ³⁺	Pb ²⁺		
Ammonium sulphate			Fe ³⁺ Fe ²⁺ Ni ²⁺ Co ²⁺	Al ³⁺			
Ammonium oxalate			Fe ³⁺				
Oxide			Fe ³⁺ Cr ³⁺ Cr ⁶⁺				

Generally, compounds containing metals belonging to groups I and II of the periodic table did not cause G-4-4 to degrade. They form neutral solutions and included chlorides, sulphates and nitrates of Na⁺, K⁺, Ca²⁺, Mg²⁺, Ba²⁺. Al₂O₃ (group III metal) and a complex salt of this metal (aluminum ammonium sulphate) were not able to induce degradation of G-4-4 and neither did PbCl₂ chloride and Pb(NO₃)₂ (Group IV).

Salts from Group III of the Periodic table that were able to cause mild degradation were AlCl₃ and Al(NO₃)₃ while Al₂(SO₄)₃ caused significant degradation.

Significant degradation of the G-4-4 molecule was also observed with SnCl₂ and SnCl₄ (Group IV).

Most of the transition metal salts were not able to cause G-4-4 to degrade except some salts of Fe and Cr. Significant degradation was noticed with FeCl₂, ferric ammonium sulphate, Fe₂(SO₄)₃, Fe(NO₃)₃ and Cr(NO₃)₃.

Further transition metal salts that caused mild degradation of G-4-4 were NiCl_2 , CrCl_3 and FeCl_3 and $\text{Fe}(\text{NO}_3)_2$. The complex salts, copper (I) ammonium chloride, ferric ammonium chloride and ferric ammonium sulphate caused some degradation of G-4-4 molecule together with the double salt chromium potassium sulphate.

On the other hand, complex salts such as ferric ammonium oxalate and double salts such as nickel and cobalt ammonium sulphates were unable to induce degradation.

The non metallic salt, NH_4Cl , was also able to cause mild degradation of the G-4-4 molecule.

Overall, the results indicated that the salts of the transition metals Fe^{2+} , Fe^{3+} and Cr^{3+} and Sn^{2+} , Sn^{4+} (from Group IV) caused degradation of G-4-4. These salts were also found to form very acidic solutions when dissolved in water. Thus, from these observations, it is evident that the acidity created by the dissolution of salts may have a role in the degradation of the G-4-4 glycolipid. This concept will be investigated further.

Those salts that did not induce degradation were those that did not form acidic solutions upon dissolution e.g. metals of groups I and II do not form acidic salts and were not observed to induce G-4-4 degradation.

Other salts that formed slightly acidic solutions were able to mildly degrade the G-4-4 sample. Complex and double salts, as seen in Table 6, were also able to induce mild degradation and would have most likely formed mildly acidic solutions upon dissolution.

The metal ions acting as catalysts in hydrolysing the glycosidic bonds in the glycan chain of the G-4-4 structure is also suspected and will be further investigated in the following sections.

Part II

Effects of varying metal ion concentrations on G-4-4

The degradative effects of varying metal ion concentration on a fixed concentration of globoside (G-4-4) at 85°C were investigated in this section. The method for this experiment is outlined in Protocol 1, v 1 with an incubation period of 48 hours. Five compounds which included copper (II) nitrate ($\text{Cu}(\text{NO}_3)_2$), chromium (III) oxide (Cr_2O_3), aluminium chloride (AlCl_3), iron (III) chloride (FeCl_3) and tin (II) chloride (SnCl_2) were chosen to investigate this. From earlier results, $\text{Cu}(\text{NO}_3)_2$ and Cr_2O_3 were not able to induce degradation of G-4-4, while AlCl_3 was able to mildly degrade the glycolipid sample. FeCl_3 and SnCl_2 were able to significantly degrade G-4-4. This section was set out to explore if lowering the ion concentrations would have any degradative effects on G-4-4. It is suspected that less degradation would be observed with decreasing the concentrations of compounds that caused degradation i.e. FeCl_3 and SnCl_2 . These salts form acidic solutions and the acidity of their solutions would increase by lowering their concentrations. Further, those compounds that caused mild or nil degradation and formed neutral or mild acidic solutions upon dissolution would not be expected to show degrade G-4-4 at lower concentrations.

This experiment was designed to explore if metal ions or the acidity of the solutions catalyse the degradation process of G-4-4. Minute quantities of metal ions are required for catalysing reactions such as degradation or synthesis processes. If metal ions that do not form acidic solutions can induce degradation, then the metallic ions may be acting as catalysts. However, if metal ions that form acidic solutions are able to degrade glycolipid in low pH (higher M^+ concentration) but are not able to induce degradation at higher pH (lower M^+ concentration), then low pH (acidity) is most likely causing G-4-4 to degrade.

Cr_2O_3 and $\text{Cu}(\text{NO}_3)_2$ versus G-4-4

Varying volumes of 0.02 M Cr_2O_3 and $\text{Cu}(\text{NO}_3)_2$ (560 μl –4 μl) solutions were suspended with 280 μl of 0.02 M G-4-4 solution. The pH of each solution mixture containing Cr_2O_3 was recorded and was found to be in the range of 6.0 -7.0 (as Cr_2O_3 is an insoluble compound) while those with $\text{Cu}(\text{NO}_3)_2$ had a pH range of 3.0 - 4.5. These solution mixtures were then incubated in an 85°C water-bath for periods of time ranging from 1-48 hours and the treated G-4-4 samples were isolated and stained with anisaldehyde according to Protocol 12.

Undegraded native G-4-4 and TLC controls are present on each plate for comparison purposes. The native G-4-4 sample migrates to a position of $rm\ 7.0 \pm 0.5$ and is a mixture of 3- and 4-sugar glycolipids. The 3-sugar glycolipid has a migration of $rm\ 9.0 \pm 0.5$.

There was no evidence of G-4-4 degradation caused by incubation with varying volumes of 0.02 M Cr_2O_3 or $Cu(NO_3)_2$ as there were no additional bands present apart from the original 4- and 3-sugar glycolipid bands at $rm\ 7.0 \pm 0.5$ and $rm\ 9.0 \pm 0.5$ respectively (results not shown).

$AlCl_3$ versus G-4-4

Globoside (G-4-4) again, was not affected by varying concentrations of Al^{3+} ions over 48 hours. This is in contrast to the observation seen in Part I. This could have been due to concentration differences of $AlCl_3$ solution between the two experiments. The pH of each of the solution mixtures was in the range 3.0 – 4.5. The addition of G-4-4 may have increased the pH of the solution thereby resulting in nil degradation of G-4-4 by $AlCl_3$ (results not shown). The globoside (G-4-4) was not available in reasonable quantities to repeat the experiment.

$FeCl_3$ and $SnCl_2$ versus G-4-4

Treatment of G-4-4 with varying volumes of 0.02 M $FeCl_3$, in contrast, caused significant degradation of the G-4-4 molecule. The pH of the solution mixtures ranged from 1.0 (for 560 μ l solutions) to 6.0-7.0 (for 4 μ l of 0.02 M $FeCl_3$).

Significant degradation of G-4-4 was noticed as early as 24 hours when it was incubated with 560 μ l of 0.02 M $FeCl_3$ solutions shown by a dark staining of the 3-sugar glycolipid structure at an rm of 9.0 ± 0.5 and as well as the appearance of a weak band in the 2-sugar glycolipid region off the rm scale and towards the top of the TLC plate (Figure 11, plate I, lanes 4-6). Another band in a 1-sugar glycolipid region appeared at 48 hours of incubation (lane 6). This band migrated slightly further up the TLC than the standard 1-sugar glycolipid and this could be due to degradation of the ceramide tail or to the sugar residue resulting in a changed polarity and migration of the glycolipid. The pH of the solutions were in the range of 1.0 - 2.0.

Incubation of G-4-4 with 280 μ l of 0.02 M $FeCl_3$ had a milder degradation effect on the G-4-4 glycolipid. The 3-sugar glycolipid bands had darkened over time and an additional 2-sugar glycolipid band appeared after 24 hours of incubation (Figure 11, plate I, lanes 11-12).

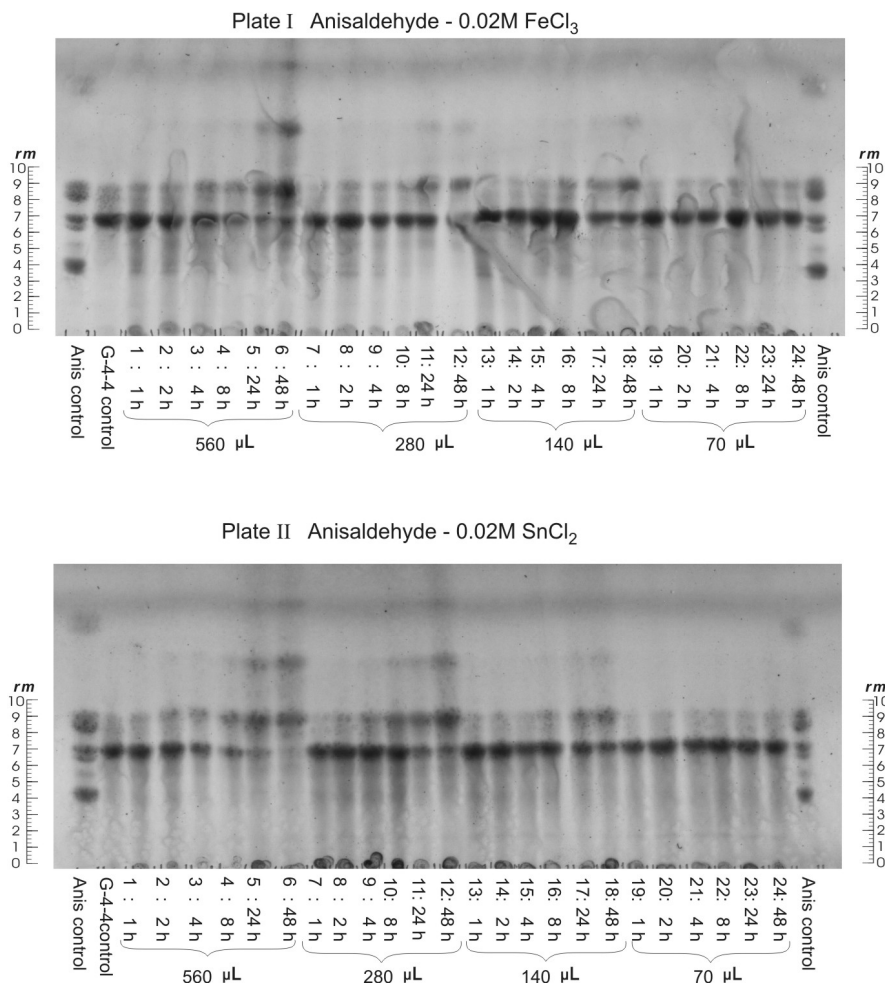


Figure 11: Effects of varying volumes of 0.02 M FeCl₃ and SnCl₂ solutions on G-4-4. Incubation was carried out at 85°C over a period of 48 hours. The concentration of G-4-4 was 0.02 M.

140 µl of 0.02 M FeCl₃ was able to induce degradation only after 48 hours of incubation evident by the gradual darkening of the 3-sugar glycolipid (at $rm\ 9.0 \pm 0.5$) and the appearance of the 2-sugar structures (off scale) (Figure 11, plate I, lane 18). The pH with 140 µl of 0.02 M FeCl₃ was found to be 3.0.

In contrast, no degradation bands were noticeable for G-4-4 suspended in 70 µl, 35 µl, 18 µl, 9 µl and 4 µl of 0.02 M FeCl₃ (results not shown) where the pH ranged from 4.0-7.0.

Plate II illustrates the effects of various concentrations of 0.02 M SnCl₂ on G-4-4 sample. The SnCl₂ solution was able to induce the strongest degradation out of all the 5 salts tested in this section. The pH of the differing concentrations mixtures ranged from 2.0 -6.0.

560 μl of 0.02 M SnCl_2 when suspended with G-4-4 at 85°C caused significant degradation after 8 hours of incubation as seen with the gradual darkening of the 3-sugar band ($m 9.0 \pm 0.5$) and appearance of additional 1- and 2-sugar glycolipid bands (Figure 11, plate II, lanes 4-6). The pH at this particular concentration was 2.0.

280 μl of 0.02 M SnCl_2 was also able to cause G-4-4 to degrade in a similar fashion; significant degradation was noticed as early as 8 hours (Figure 11, plate II, lanes 10-12) evident by the appearance of bands in the migratory regions of 1- and 2-sugar glycolipids. The pH was recorded as 2.0.

140 μl of 0.02 M SnCl_2 showed some degradation as well, starting at 24 hours (Figure 11, plate II, lanes 17 and 18). This degradation is evident by darker 3-sugar bands and faint 2-sugar bands at 24 and 48 hours (Figure 11, plate II, lanes 17 and 18). The pH at this particular concentration was 2.0.

70 μl , 35 μl , 18 μl , 9 μl and 4 μl of 0.02 M SnCl_2 solution did not seem to cause G-4-4 to degrade when suspended with the latter at 85°C (results not shown). The pH values ranged from 5.0 (for 70 μl 0.02 M SnCl_2) to 6.0 (for 4 μl 0.02 M SnCl_2).

With these observations, it can be said that increasing concentrations of both FeCl_3 and SnCl_2 when suspended with 0.02 M G-4-4 solution resulted in G-4-4 degradation. Lower volumes of these two salt solutions were not able to induce G-4-4 break-down. Hence it could be said that degradation of G-4-4 is most likely due to a low pH rather than the catalytic effect of metal ions. This was investigated further in the next set of experiments.

Summary

The results are summarised in Table 7 and Table 8.

The results suggested that the acidity of the degrading medium was directly linked to globoside (G-4-4) degradation (or hydrolysis of the glycosidic bonds in the glycan chain). This is well represented by the degradation patterns obtained with the treatment of G-4-4 with varying volumes of 0.02 M FeCl_3 and SnCl_2 i.e. solutions with a high pH did not induce G-4-4 degradation while those with a lower pH (less than 3.0) were able to degrade G-4-4.

Larger volumes of both SnCl_2 and FeCl_3 solutions were able to induce G-4-4 degradation as the pH of the salt and glycolipid mixture was 3.0 or below. Degradation did not take place when the pH of the solution was greater than 3.0.

Table 7: The degradative effects of incubating varying volumes of 0.02 M FeCl_3 with G-4-4. Incubations were carried out at 85°C over 48 hours. The pH of the globoside and varying volumes of 0.02 M FeCl_3 was measured and recorded prior to the incubation period as shown in the final row.

Hours of incubation (h)	Volume of 0.02 M FeCl_3 salt solution (μl)							
	560	280	140	70	35	18	9	4
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
8	±	±	-	-	-	-	-	-
24	++	++	-	-	-	-	-	-
48	+++	++	++	-	-	-	-	-
pH	1.5	2.0	3.0	4.0	6-7.0	6-7.0	6-7.0	6-7.0

Key (see Protocol 11)

Colour code for degradation

1. +++++ = total degradation, no sugar bands = extensive degradation
2. ++++ = no globoside band, presence of 3-, 2- and 1-sugar bands = considerable degradation
3. +++ = reduced globoside band, presence of 3-, 2- and 1-sugar bands = significant degradation
4. ++ = reduced globoside band, presence of 3-, 2- sugar bands = mild degradation
5. + = reduced globoside band and relatively darker 3-sugar band = mild degradation
6. ± = minor reduction in globoside band = minimum degradation
7. - = no change in the globoside band = nil degradation

Table 8 : The degradative effects of incubating varying volumes of 0.02 M SnCl_2 with G-4-4. Incubations were carried out at 85°C over 48 hours. The pH of the globoside and varying volumes of 0.02 M SnCl_2 was measured and recorded prior to the incubation period as shown in the final row.

Hours of incubation (h)	Volume of 0.02 M SnCl_2 salt solution (μl)							
	560	280	140	70	35	18	9	4
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
8	++	++	-	-	-	-	-	-
24	+++	++	++	-	-	-	-	-
48	+++	+++	++	-	-	-	-	-
pH	2.0	2.0	2.0	5-6.0	5-6.0	5-6.0	5-6.0	5-6.0

Scoring system as per Protocol 11.

It was interesting to note that 0.02 M AlCl_3 was capable of inducing degradation of G-4-4 in Part I. However, this degradative action of AlCl_3 was not observed in this section. One of the reasons for obtaining these results may be due to a higher pH of the degrading medium. (The pH values for these earlier experiments were not recorded as the acidity of

the metal ions causing the degradation was not suspected at this stage). Further, it could be an indication that the Al^{3+} ions are not involved in catalysing the degradation of the glycolipid.

The compounds that did not induce degradation of G-4-4 were Cr_2O_3 , $\text{Cu}(\text{NO}_3)_2$ and AlCl_3 . $\text{Cu}(\text{NO}_3)_2$ formed neutral or near neutral solution while Cr_2O_3 is an insoluble compound.

Part III

The implications of low pH on a fixed G-4-4 and salt concentrations

The previous section strongly implied that pH alone may be responsible for globoside (G-4-4) degradation. In order to further investigate this, a small amount of concentrated nitric acid (1.75 M HNO_3) was added to a range of 0.02 M salt solutions and 0.02 M G-4-4 solution and the mixture was incubated at 85°C over a period of 48 hours. Concentrated HNO_3 was chosen as it is a strong acid and the NO_3^- ion is a very poor base/nucleophile which should not have any secondary effects on the substrate.

A total of 36 different compounds (0.02 M solutions) were incubated where 280 μl of 0.02 M G-4-4 was suspended with 140 μl of each 0.02 M salt solution and then each solution mixture was spiked with 3 μl of 1.75 M HNO_3 . This mixture was incubated at 85°C over a 48-hour period. The pH of this solution mixture was measured using a Whatman's pH paper 1-4 range. The method for this experiment is outlined in Protocol 1, v 2.

The main objective of the current experiment was to further investigate if the acidity and not metal ions was responsible for catalysing the degradation of G-4-4. The secondary objective was to investigate if both metal ions and a low pH have a combined degradative effect on the G-4-4 structure.

It is suspected, that upon spiking with acid, those salts that formed neutral solutions and gave nil degradation when incubated with G-4-4 at 85°C will be able to induce degradation, salts that formed acidic solutions will be able to further degrade the G-4-4 structure while those salts that could slightly degrade G-4-4 would be able to significantly degrade the glycolipid. In other words, adding acid to the reaction medium would enhance the degradation of G-4-4.

Low pH versus G-4-4 and 0.02 M metallic salt and oxide solutions

Eight different 0.02 Molar solutions of iron compounds were acidified and tested for their roles in G-4-4 degradation (listed in Table 9). The results are shown in Figure 12 (plates I, II and III). Fe_2O_3 , being an insoluble oxide, was not able to degrade G-4-4 on its own (Part I). However, at low pH the insoluble oxides have a tendency to dissolve over time. Fe_2O_3 was included in this experiment for comparison purposes.

All acidified solutions had a pH of approximately 2.0. It was shown in Part II that a pH value below 3.0 was ideal for G-4-4 degradation. Undegraded globoside (G-4-4) is present on each plate for comparison purposes.

Table 9: List of iron compounds.

ferric chloride	FeCl_3	ferrous sulphate	FeSO_4
ferric nitrate	$\text{Fe}(\text{NO}_3)_3$	ferric ammonium sulphate	$\text{FeNH}_4(\text{SO}_4)_2$
ferric ammonium chloride	FeNH_4Cl_4	ferric sulphate	$\text{Fe}_2(\text{SO}_4)_3$
ferric oxide	Fe_2O_3	ferrous chloride	FeCl_2

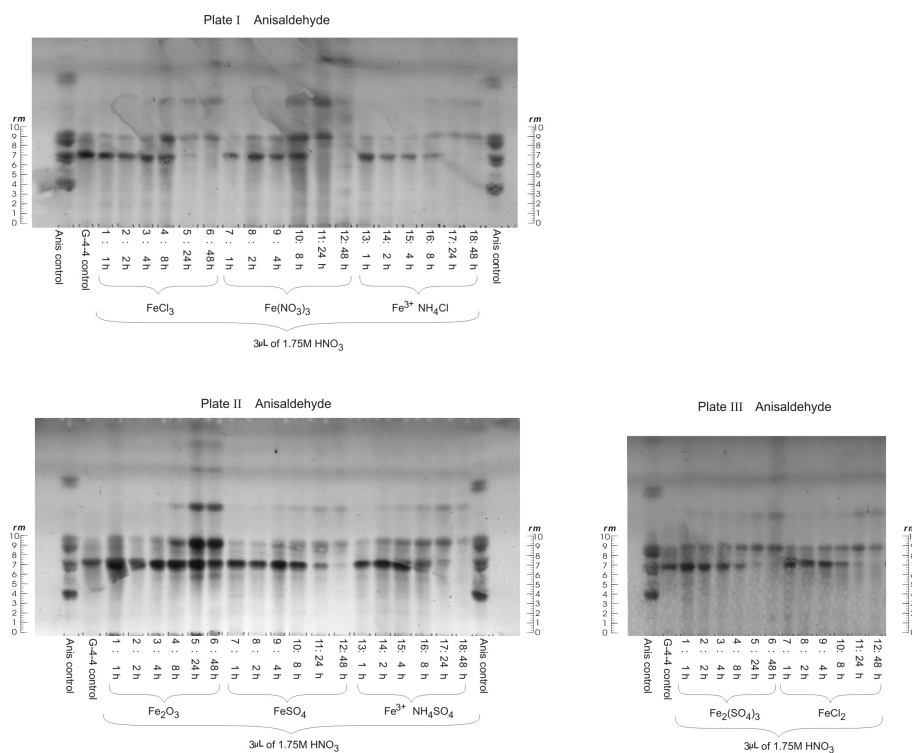


Figure 12: Effects of acidified solutions on G-4-4.

Incubation was carried out at 85°C over a period of 48 hours. The concentration of G-4-4 was 0.02 M.

The data in Table 10 shows the effects an acidified medium has on the breakdown of the G-4-4 glycolipid. Degradation, in general, was first noticeable from 8 hours of incubation and onwards and was most significant at 24 and 48 hours of incubation. The band present towards the top of the plate, (with a migration position similar to the 1-sugar glycolipid), is most likely a 1-sugar glycolipid with a slightly degraded sugar ring or ceramide tail as it migrated slightly further up the TLC plate than a standard 1-sugar glycolipid would. Any degradation in the glycan or ceramide entity would change the overall polarity of the glycolipid causing the latter to migrate differently on the TLC plate.

Table 10: Effects of acidified iron compounds on G-4-4 at 85°C over 48 hours.

Salt	Time intervals (hours)					
	1	2	4	8	24	48
FeCl ₃	-	-	+	++	+++	++++
Fe(NO ₃) ₃	-	-	-	+	++++	++++
Fe ³⁺ NH ₄ Cl	-	-	-	+	++++	++++
Fe ₂ O ₃	-	-	-	+	+++	+++
FeSO ₄	-	-	+	+	+++	++++
Fe ³⁺ NH ₄ SO ₄	-	+	+	+	+++	++++
Fe ₂ (SO ₄) ₃	-	-	-	+	++++	++++
FeCl ₂	-	-	-	+	+++	++++

Scoring system as per Protocol 11.

The results in Figure 13 show the degradative effects of more acidified compounds on G-4-4. These compounds are listed in Table 11.

Table 11: List of compounds.

chromium sulphate	Cr ₂ (SO ₄) ₃	chromium (III) oxide	Cr ₂ O ₃
tin (IV) chloride	SnCl ₄	copper (II) nitrate	Cu(NO ₃) ₂
tin (II) chloride	SnCl ₂	aluminium oxide	Al ₂ O ₃
chromium oxide	CrO ₃	tin (IV) oxide	SnO ₂
aluminium chloride	AlCl ₃	tin (II) sulphate	SnSO ₄

Degradation of G-4-4, in general, was first noticeable at 4 hours of incubation and was most significant at 24 and 48 hours of incubation as shown in Table 12. The tin compounds, SnO₂ and SnSO₄, form extremely acidic and corrosive solutions upon dissolution and were able to degrade G-4-4 in the very first hour of incubation. Acidifying these salts would have further dropped the pH of these solutions making them extremely acidic to be able to initiate G-4-4 degradation over a very short period. The acidified compounds, SnCl₄ and Cr₂O₃, were also able to degrade G-4-4 over a short incubation period i.e. 2 hours.

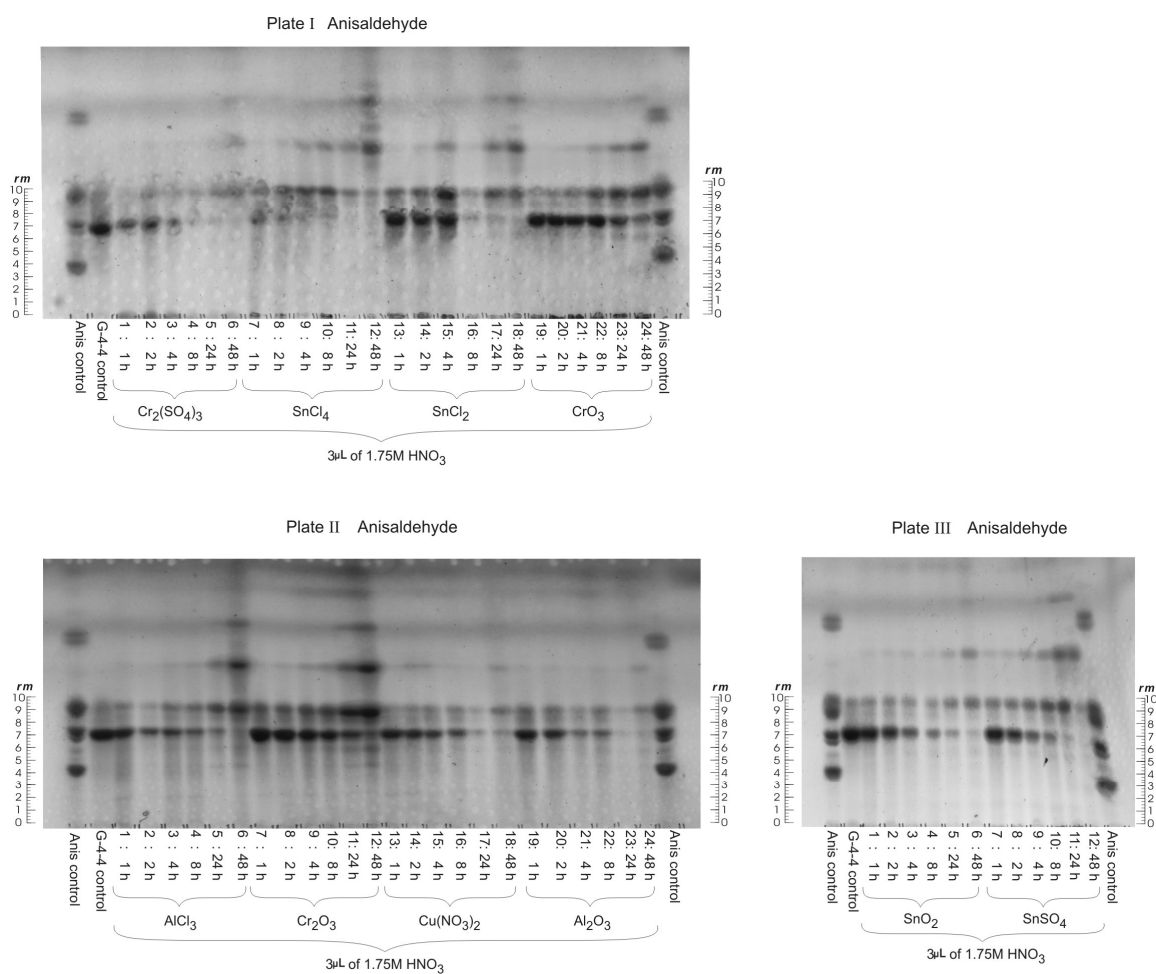


Figure 13: Effects of acidified metallic solutions on G-4-4. Incubation was carried out at 85°C over a period of 48 hours. The concentration of G-4-4 was 0.02 M.

Table 12: Effects of acidified metallic solutions on G-4-4 at 85°C over 48 hours.

Salt	Time intervals (hours)					
	1	2	4	8	24	48
Cr ₂ (SO ₄) ₃	-	-	+	++++	++++	++++
SnCl ₄	-	+++	+++	+++	++++	++++
SnCl ₂	-	-	+	+++	++++	++++
CrO ₃	-	-	-	++	+++	+++
AlCl ₃	-	-	+	+	++	++++
Cr ₂ O ₃	-	+	+	+	+++	++++
Cu(NO ₃) ₂	-	-	-	-	-	+++
Al ₂ O ₃	-	-	-	-	++	+++
SnO ₂	++	++	++	+++	+++	+++
SnSO ₄	++	++	++	++	++++	++++

Scoring system as per Protocol 11.

The results in Figure 14 show the degradative effects of more acidified compounds on the G-4-4 molecule. These compounds are listed in Table 13.

Table 13: List of compounds.

chromium potassium sulphate	CrK(SO ₄) ₂	aluminium ammonium sulphate	AlNH ₄ (SO ₄) ₂
aluminium nitrate	Al(NO ₃) ₃	chromium (III) chloride	CrCl ₃
aluminium potassium sulphate	AlK(SO ₄) ₂	aluminium sulphate	Al ₂ (SO ₄) ₃
ammonium ferrous sulphate	(FeNH ₄) ₂ (SO ₄) ₂	potassium nitrate	KNO ₃
chromium nitrate	Cr(NO ₃) ₃		

Acidified KNO₃ had a mild degradative effect on the G-4-4 molecule. This was an anomaly in the results as G-4-4 was expected to breakdown at the low pH created by adding a small amount of concentrated HNO₃. The G-4-4 band was not consumed in the reaction as shown by the distinctive band present at *rm* 7.0 ± 0.5.

The control for this experiment was incubating an equivalent amount of 0.02 M G-4-4 solution with concentrated (1.75 M) HNO₃ solution at 85°C over 48 hours. The result showed that G-4-4 degraded from as early as the 1st hour of incubation. This is shown by the presence of a weak 2-sugar band which gradually darkened over time i.e. 48 hours, together with the 3-sugar glycolipid band (Figure 14, plate III, lanes 7-12). At the same time, the G-4-4 band gradually weakened. At 48 hours, the 3-sugar glycolipid band was the most prominent followed by the 2-sugar glycolipid band and the G-4-4 band was the

weakest (Figure 14, plate III, lane 12). The 1-sugar glycolipid band was first noticeable and also prominent at 48 hours suggesting that the medium for degradation was reasonably potent. Degradation of G-4-4 took place in the absence of metal ions.

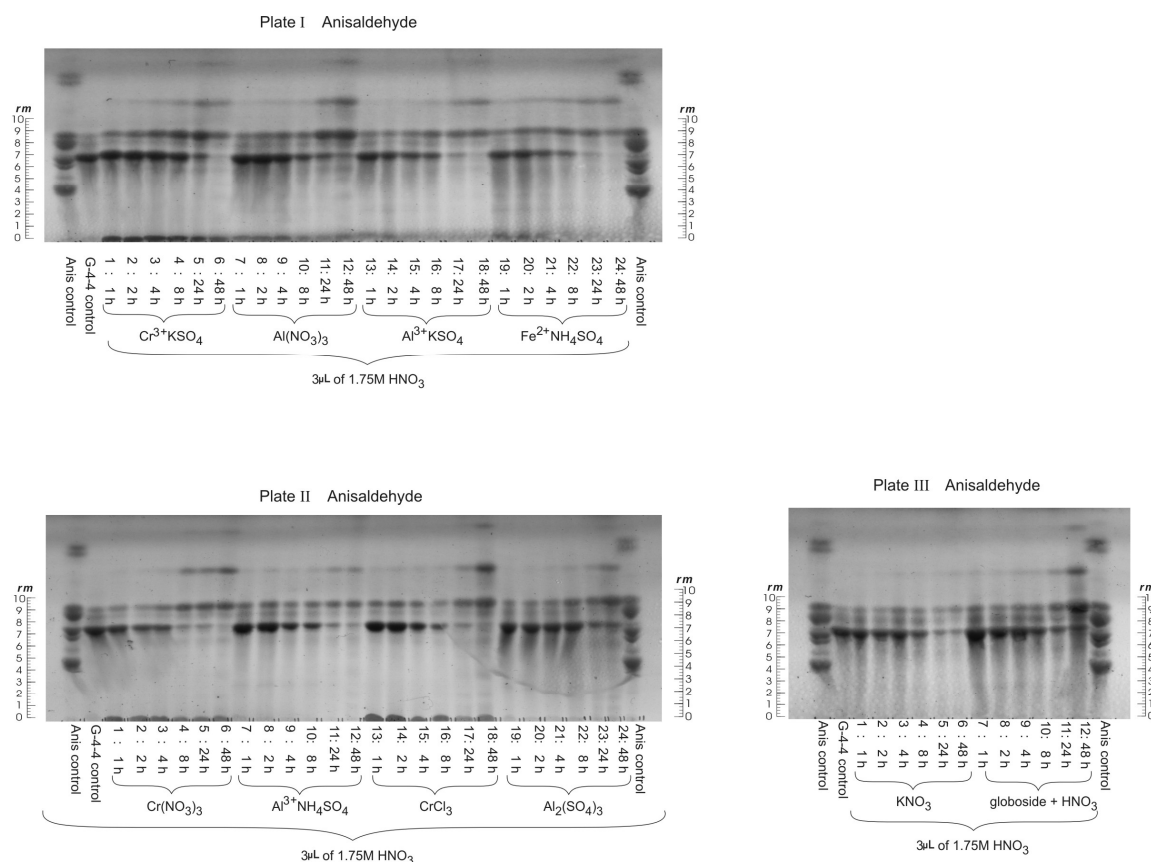


Figure 14: Effects of acidified metallic solutions and acid on G-4-4. Incubation was carried out at 85°C over a period of 48 hours. The concentration of G-4-4 was 0.02 M.

The data in Table 14 shows that G-4-4 degradation was noticeable from 2-4 hours of incubation at 85°C. As expected, degradation was most significant at 24 and 48 hours of incubation.

Table 14: Effects of acidified metallic solutions on G-4-4 at 85°C over 48 hours.

Salt	Time intervals (hours)					
	1	2	4	8	24	48
CrK(SO ₄) ₂	-	+	++	++	+++	+++
Al(NO ₃) ₃	-	+	+	+	++	+++
AlK(SO ₄) ₂	-	-	-	+	++	++++
(FeNH ₄) ₂ (SO ₄) ₃	-	+	+	+	+++	++++
Cr(NO ₃) ₃	-	-	-	+++	+++	++++
AlNH ₄ (SO ₄) ₂	-	+	+	++	+++	+++
CrCl ₃	-	+	+	++	+++	+++
Al ₂ (SO ₄) ₃	-	-	+	++	+++	+++
KNO ₃	-	±	±	±	±	±

Scoring system as per Protocol 11.

The anisaldehyde-stained plates in Figure 15 show the effects of more acidified solutions of metallic compounds on G-4-4 at 85°C for periods of time ranging from 1-48 hours. These compounds are listed in Table 15.

Table 15: List of compounds.

zinc nitrate	Zn(NO ₃) ₂	cadmium chloride	CdCl ₂
lead chloride	PbCl ₂	sodium chloride	NaCl
sodium nitrate	NaNO ₃	potassium chloride	KCl
nickel sulphate	NiSO ₄	copper (II) chloride	CuCl ₂
manganese chloride	MnCl ₂		

The data in Table 16 shows that G-4-4 breakdown was first noticeable at the first hour of incubation at 85°C and there was significant degradation by the eighth hour. All the salts tested were able to induce G-4-4 degradation upon being acidified with HNO₃.

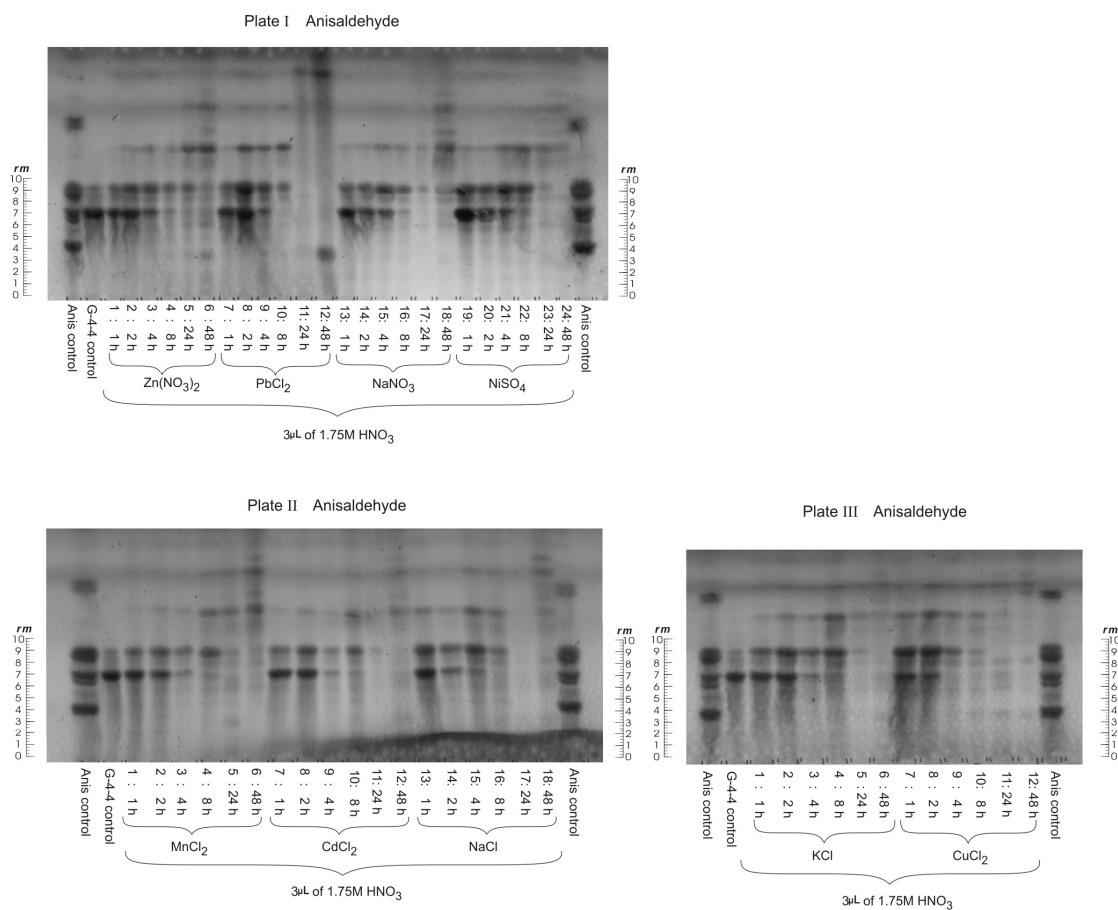


Figure 15: Effects of acidified metallic solutions on G-4-4. Incubation was carried out at 85°C over a period of 48 hours. The concentration of G-4-4 was 0.02 M.

Table 16: Effects of acidified metallic solutions on G-4-4 upon incubation at 85°C over 48 hours.

Salt	Time intervals (hours)					
	1	2	4	8	24	48
Zn(NO ₃) ₂	-	++	++	+++	++++	++++
PbCl ₂	-	+++	+++	++++	+++++	+++++
NaNO ₃	++	++	++	++	++++	++++
NiSO ₄	+++	+++	+++	+++	+++++	+++++
MnCl ₂	±	++	+++	++++	+++++	++++
CdCl ₂	++	++	++	+++	+++++	+++++
NaCl	++	++	+++	++++	+++++	+++++
KCl	++	++	+++	+++	+++++	+++++
CuCl ₂	+++	+++	++++	+++++	+++++	+++++

Scoring system as per Protocol 11.

Summary

The data in Table 17 showed that lowering the acidity of the medium resulted in G-4-4 degradation. Salts that did not induce degradation on their own, such as NaNO₃, Fe₂O₃ and NaCl were able to degrade the G-4-4 glycolipid once acidified with 1.75 M HNO₃. Salts that naturally formed acidic solutions upon dissolution, such as Fe and Sn salts were seen to further degrade the G-4-4 glycolipid more extensively when spiked with 1.75M HNO₃. The results are summarised in the Table 17 which shows the degradative effects of adding acid to a range of G-4-4 and salt solution mixtures.

Transition metal ions are known to act as catalysts in many chemical reactions. Hence, it is suspected that some of the salts used in the treatment of G-4-4 may be involved in catalysing the breakdown of G-4-4. However minute amounts of catalysts, usually less than milligram quantities are often required for reactions and from the results shown in Table 17, it is more likely that the acidic component was largely responsible for the degradation of G-4-4 as explained further below.

Transition metals such as Fe and Cr form acidic chlorides and sulphates and were able to degrade G-4-4 on their own. The addition of acid caused further extensive degradation of G-4-4. In some situations the G-4-4 glycolipid was so extensively degraded that the products were not visible with the anisaldehyde staining.

Table 17: Effects of acidified and non-acidified compounds on G-4-4 at 85°C over 48 hours. The pH after the addition of acid is also shown. Globoside is significantly degraded by acid alone and this result (+++ score) is the background score shown in black font in the 'degradation with acid' column. Any additional degradation is shown by red font which is more than likely due to the (acidic or catalytic effect due to metal ions of the) salt solution.

(Blue font denotes nil degradation, green font denotes mild degradation and red font denotes considerable to extensive degradation in the 'degradation without acid' column).

0.02 M Salt solution	Degradation without acid		Degradation with acid	
	pH		pH	
Globoside	5-6.0	-	2-3.0	+++
Iron Salts				
FeCl ₃		+++	2.0	++++
Fe(NO ₃) ₃		++++	2.0	++++
Fe ³⁺ NH ₄ Cl		++	2.0	++++
Fe ₂ O ₃		-	2.0	+++
FeSO ₄		++	2.0	++++
Fe ³⁺ NH ₄ SO ₄		+++	2.0	++++
Fe ₂ (SO ₄) ₃		+++	2.0	++++
FeCl ₂		++	2.0	++++
Fe ²⁺ NH ₄ SO ₄		-	2.0	++++
Tin salts				
SnCl ₄		++++	2.0	++++
SnCl ₂		++++	2.0	++++
SnO ₂		-	2.0	+++
SnSO ₄		++	2.0	++++
Chromium salts				
Cr ₂ O ₃		-	2.0	++++
CrO ₃		-	2.0	+++
Cr(NO ₃) ₃		++	2.0	++++
Cr ₂ (SO ₄) ₃		++	2.0	++++
(K)CrSO ₄		±	2.0	+++
CrCl ₃		+++	2.0	+++
Aluminium salts				
Al(NO ₃) ₃		±	2.0	+++
AlKSO ₄		-	2.0	++++
Al ₂ O ₃		-	2.0	+++
Al ³⁺ NH ₄ SO ₄		-	2.0	+++
Al ₂ (SO ₄) ₃		+++	2.0	+++
AlCl ₃		+++++	2.0	++++
*Other Transition M⁺				
Cu(NO ₃) ₂		±	2.0	+++
Zn(NO ₃) ₂		-	2.0	++++
NiSO ₄		-	2.0	+++++
MnCl ₂		-	2.0	++++
CdCl ₂		±	2.0	+++++
CuCl ₂		-	2.0	+++++
*Group I and IV M⁺ salts				
PbCl ₂		±	2.0	+++++
KCl		-	2.0	+++++
NaCl		-	2.0	+++++
KNO ₃		±	2.0	+++
NaNO ₃		-	2.0	++++

Scoring system as per Protocol 11 and is only applicable to the 'degradation without acid' column. M⁺ denotes metal ions.

Transition metals that did not form acidic solutions and were not capable of degrading G-4-4 were found to degrade this glycolipid upon acidification with HNO_3 . The catalytic role of transition metals was not observed in the results obtained. The same applied to compounds of Groups I and IV metals.

The compounds of Group IV metal, Sn, were also able to significantly degrade G-4-4 upon acidification. The chlorides of this salt form acidic solutions and they have the ability to readily degrade G-4-4 on their own.

Treatment of G-4-4 with acid only resulted in significant degradation. This was sufficient evidence to suggest that the breakdown of the glycolipid was mainly caused by the acid.

Part IV

The implications of varying pH on a fixed G-4-4 and salt concentrations

In the previous section, the results indicated that the acidification of the degrading medium resulted in G-4-4 degradation. However, there is a possibility of the metal ions in solution catalysing the degradation reaction. In this section of the project, the effects of varying HNO_3 concentrations on a fixed concentration of globoside (G-4-4) and 0.02 M salt solution were investigated. The objective of these experiments would be to determine if there is any degradation when the volume of acid added is minimal and while there are no changes in the concentration of metal ions. Any degradation observed while the pH of the medium is greater than 3 may imply catalysis by the metal ions.

The general method involved suspending 280 μl of 0.02 M G-4-4 solution with 280 μl of 0.02 M salt solution to which varying volumes of 0.1 M nitric acid was added. These solutions were then placed in an 85°C water bath for periods of time ranging from 1 to 48 hours. The pH of each solution was recorded using Whatman's pH paper. The control for this experiment was G-4-4 solution with no acidic or salt treatment set at room temperature. It was already known that acid would degrade G-4-4 over time at 85°C (Part VI) and it is also known that certain salt solutions are capable of degrading G-4-4 over time at 85°C (Part I). The method is outlined in Protocol 1, v 3.

The five compounds chosen were chromium trioxide (Cr_2O_3), copper(II) nitrate ($\text{Cu}(\text{NO}_3)_2$), aluminium chloride (AlCl_3), ferric chloride (FeCl_3) and tin (II) chloride (SnCl_2). These salts were the same as those chosen in Part II.

Varying $[\text{HNO}_3]$ versus fixed G-4-4 and salt concentrations

Chromium (III) oxide, Cr_2O_3

The anisaldehyde-stain plate in Figure 16, (plate I) shows the degradative effects of incubating varying volumes of 0.1 M HNO_3 with a fixed volume of 0.02 M Cr_2O_3 suspended solution on the G-4-4 glycolipid. (Note that Cr_2O_3 is an insoluble compound and may have heterogenous catalytic effects on the hydrolysis of the G-4-4 molecule).

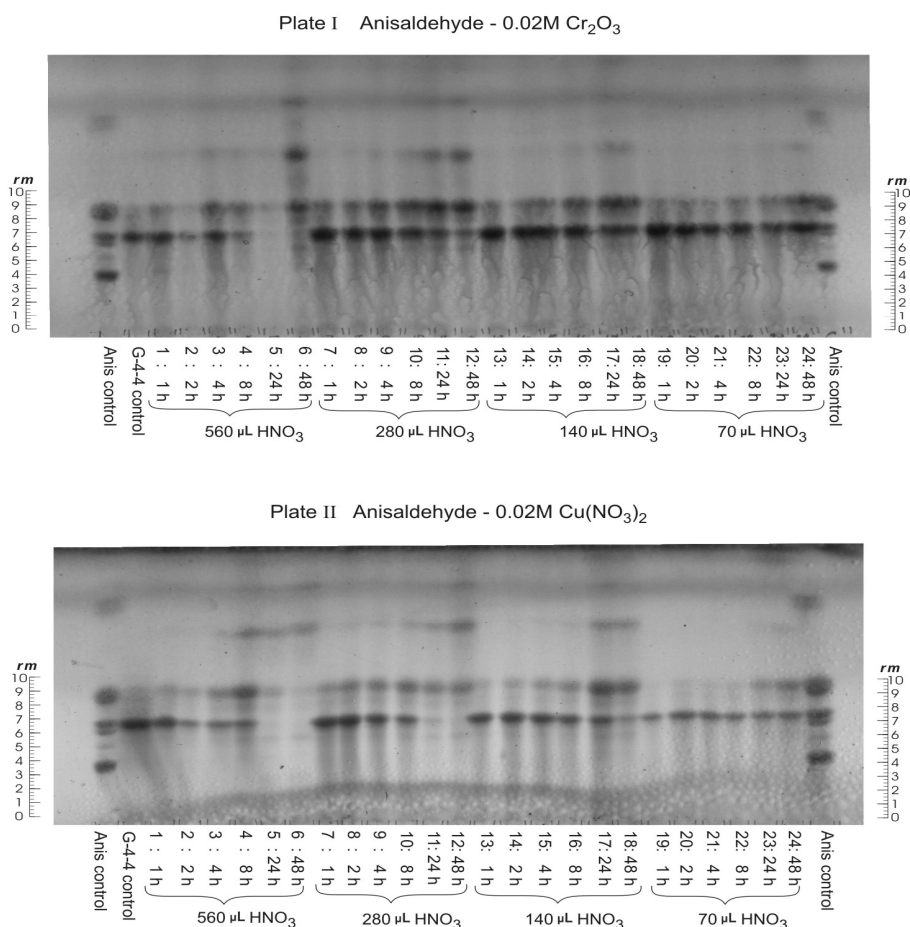


Figure 16: Effects of variable volumes of 0.10 M HNO₃ on G-4-4 and salt solutions. The salts used were Cr₂O₃ and Cu(NO₃)₂. Variable acid volumes were added to a fixed concentration of G-4-4 and salt solutions and the entire mixture was incubated at 85°C over 48 hours.

As expected, the most significant degradation of G-4-4 was observed at the lowest pH (1.0) i.e. when G-4-4 was suspended with 560 µl of 0.1 M HNO₃ and 280 µl of 0.02 M Cr₂O₃. In addition, degradation was noticed as early as the 4th hour of incubation seen by the presence of an additional band in the 2-sugar glycolipid region (Figure 16, plate I, lane 3). This band gradually darkened over time with a very prominent band at 48 hours (Figure 16, plate I, lane 6). The 3-sugar band at $rm\ 9.0 \pm 0.5$ also darkened over time suggesting the formation of this product from the G-4-4 degradation. Multiple bands present in lane 6, 48 hours indicated extensive fragmentation of the G-4-4 molecule. The prominent bands occurred in the 1-, 2- and 3- sugar glycolipid regions. The G-4-4 band at $rm\ 7.0 \pm 0.5$ was absent at 48 hours indicating that it was entirely consumed in the degradation process. (It is important to note that the 1-sugar glycolipid generated upon

G-4-4 hydrolysis migrated slightly further than the standard 1-sugar glycolipid and this may be due to the sugar or ceramide tails being slightly degraded by the acid thereby changing the polarity and hence the migration of the glycolipid on the TLC plate).

Suspending G-4-4 with 280 μl of 0.1 M HNO_3 and at a pH of 1.5, also had similar degradation effects on the former except degradation was apparent from as early as 8 hours of incubation at 85°C evident from the presence of a very faint 2-sugar glycolipid bands (Figure 16, plate I, lane 10). Furthermore, these bands in the 2-sugar region darkened over time and became prominent by the 24th hour of incubation. A band in the 1-sugar region also became visible by 24 hours (Figure 16, plate I, lane 11). At the same time, the G-4-4 band at $R_f 7.0 \pm 0.5$, became weaker with increased incubation times and had become significantly weak by 48 hours at 85°C . The 1-, 2- and 3-sugar glycolipid bands were prominent at 24 and 48 hours (Figure 16, plate I, lanes 11 and 12).

With 140 μl of 0.1 M HNO_3 and at pH 2.0, G-4-4 underwent a relatively mild degradation. Degradation was first noticed at 24 hours of incubation at 85°C . This was indicated by the presence of a very weak band in the 2-sugar glycolipid region on the TLC plate (Figure 16, plate I, lane 17). This band became slightly darker at 48 hours accompanied by the simultaneous weakening of G-4-4 band (Figure 16, plate I, lane 18).

70 μl of 0.1 M HNO_3 solution had even less degradative effects on the G-4-4 molecule. Degradation was only slightly noticeable by the presence of a very weak band in the 2-sugar glycolipid region at 48 hours of incubation (Figure 16, plate I, lane 24). There was virtually no degradation with this volume of HNO_3 .

Furthermore, 35 μl , 18 μl , 9 μl and 4 μl of 0.1 M HNO_3 had no degradative effects on the G-4-4 molecule. The pH for these solution mixtures ranged from 5.0-6.0 (results not shown).

Further, the control set up, i.e. 0.02 M suspended solution of Cr_2O_3 without acid was not able to induce degradation of G-4-4 over 48 hours at 85°C incubation (Part I).

Copper (II) nitrate, $\text{Cu}(\text{NO}_3)_2$

The anisaldehyde stain in Figure 16, plate II, shows the degradative effects of incubating varying volumes of 0.1 M HNO_3 with fixed volumes of 0.02 M $\text{Cu}(\text{NO}_3)_2$ salt solution and 0.02 M G-4-4 solution at 85°C over periods of time ranging from 1 - 48 hours.

Again, degradation of G-4-4 was most significant at the lowest pH (1.0) when it was suspended with 560 μl of 0.1 M HNO_3 solution. An additional band in the 2-sugar glycolipid region appeared at about 4 hours of incubation at 85°C which darkened over the 48-hour

incubation period (Figure 16, plate II, lane 3). The 3-sugar band also became slightly more prominent over time. In addition, the 1-sugar glycolipid band became just noticeable at 8 hours. (This band migrated slightly further than the standard 1-sugar glycolipid on the TLC plate due to reasons described earlier). The G-4-4 band had disappeared at 24 hours of incubation; the 2- and 3-sugar glycolipid bands had also weakened at this time (Figure 16, plate II, lane 24). However the 1-sugar glycolipid band remained consistent from 8 - 48 hours (Figure 16, plate II, lanes 4-6).

Suspending G-4-4 with 280 μ l of 0.1 M HNO_3 also caused G-4-4 to degrade in a similar fashion. The pH of the degrading medium was between 1.0 and 1.5 and degradation was noticeable as early as the 8th hour of incubation by the presence of a very weak band in the 2-sugar glycolipid region (Figure 16, plate II, lane 10). This band gradually darkened over time and was the strongest at 48 hours of incubation (Figure 16, plate II, lane 12). The 3-sugar glycolipid bands were prominent from the 1st hour of incubation. This band was very weak in the G-4-4 control lane. A darker band indicates an approximate measure of quantity with anisaldehyde staining. Therefore, the relatively dark 3-sugar glycolipid bands at R_f 9.0 ± 0.5 indicated product (3-sugar glycolipids) being formed. These bands were present from the 1st hour of incubation. The G-4-4 band gradually weakened as the 2- and 3-sugar glycolipid bands darkened over the 48-hour incubation period (Figure 16, plate II, lanes 7-12). At 24 hours, the G-4-4 band had almost completely disappeared. The 2- and 3-sugar glycolipid bands were the only prominent bands present at this time. Another band became visible in the 1-sugar glycolipid region at 48 hours (Figure 16, plate II, lane 12).

The effects of suspending 140 μ l of 0.1 M HNO_3 with G-4-4 had a relatively mild degradation effect on the latter. The pH of the degrading medium was 2.0. Degradation was visible at 24 hours of incubation at 85°C by the presence of a prominent band in the 2-sugar glycolipid region (Figure 16, plate II, lane 17).

70 μ l of 0.1 M HNO_3 on G-4-4 also had a very mild degradative effect on the latter (G-4-4). The pH of the degrading medium was 3.0. Degradation was only just noticeable at around 24 hours of incubation. This is evident by the presence of a very weak band in the 2-sugar glycolipid region on the TLC plate.

35 μ l, 18 μ l, 9 μ l and 4 μ l of 0.1 M HNO_3 suspended with $\text{Cu}(\text{NO}_3)_2$ had a pH 3.0, 3.0, 3.5 - 4.0 and 3.5 - 4.0 respectively and had virtually no degradative effects on G-4-4 molecule. However, the 3-sugar glycolipid bands were relatively darker at 24 and 48 hours of incubation with each of the volumes of 0.1 M HNO_3 (results not shown).

$\text{Cu}(\text{NO}_3)_2$ on its own was not able to induce G-4-4 degradation (Part I).

Aluminium chloride, AlCl_3

Figure 17, plates I and II show the effects of incubating varying volumes of 0.1 M HNO_3 with fixed volumes of 0.02 M AlCl_3 salt solution and 0.02 M G-4-4 solution at 85°C over periods of time ranging from 1-48 hours.

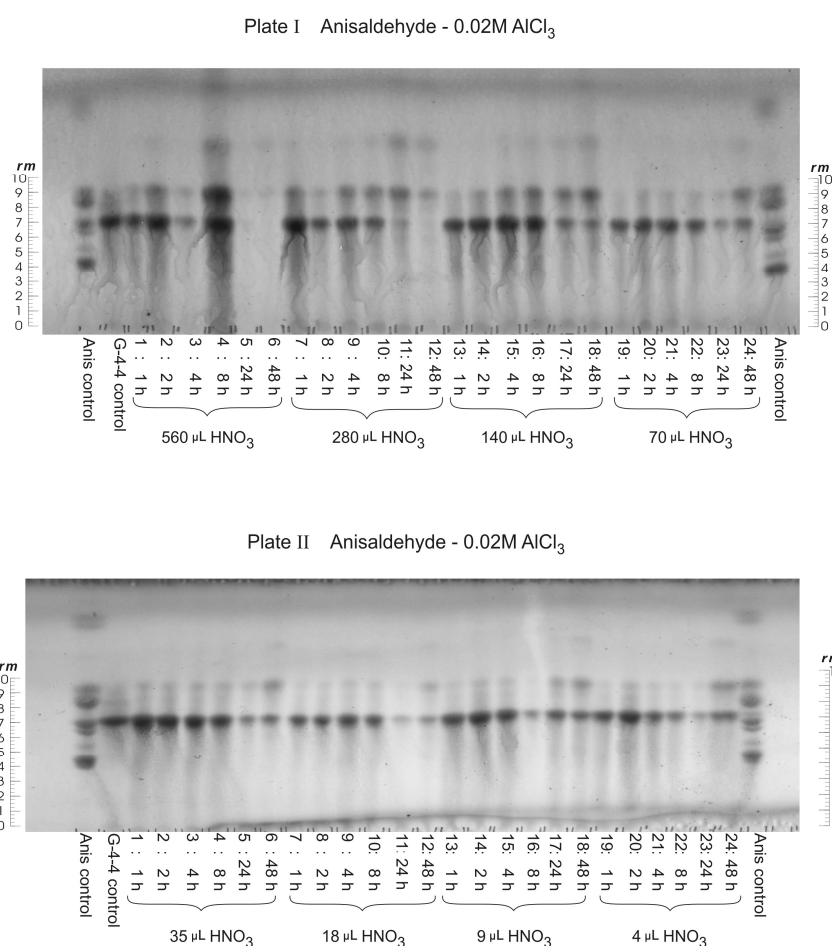


Figure 17: Effects of variable volumes of 0.10 M HNO_3 on G-4-4 and salt solutions. The salt used was AlCl_3 . Variable acid volumes were added to a fixed concentration of G-4-4 and salt solutions and the entire mixture was incubated at 85°C over 48 hours.

G-4-4 was extensively degraded when suspended with 560 μL of 0.1 M HNO_3 . The pH of the degrading medium was 1.0 and degradation was clearly noticeable from about 8 hours of incubation by the presence of a prominent band in the 3-sugar glycolipid region on the TLC plate together with a very weak band in the 2-sugar glycolipid region (Figure 17,

plate I, lane 4). A band in the 1-sugar glycolipid region was also present in this lane. (This band migrated slightly further than the standard 1-sugar glycolipid on the TLC plate due to reasons described earlier). At 24 and 48 hours the G-4-4 band at $rm\ 7.0 \pm 0.5$ had completely disappeared and extremely weak bands in the region of 2- and 3-sugar glycolipids were visible (Figure 17, plate I, lanes 5 and 6). This suggests that the rate of reaction after 8 hours of incubation at 85°C was very high. The very weak bands on plate I, lane 3 is due to a low volume of sample loading.

G-4-4 also degraded when it was suspended with 280 μ l of 0.1 M HNO_3 . The pH of the degrading medium was 1.5 and degradation was first noticed at around 8 hours of incubation at 85°C as indicated by the presence of an additional 2-sugar glycolipid band (Figure 17, plate I, lane 10). This band together with the 3-sugar glycolipid band, gradually darkened over the 48 hour incubation period (Figure 17, plate I, lanes 9-12). At the same time, the G-4-4 band had significantly weakened at 24 hours of incubation and had almost disappeared at 48 hours of incubation (Figure 17, plate I, lanes 11 and 12). Only the 2- and 3-sugar glycolipid bands were prominent at this time.

Degradation of G-4-4 with 140 μ l of 0.1 M HNO_3 was less severe. The pH of the degrading medium was recorded as 2.0 and degradation was noticed as early as 24 hours (Figure 17, plate I, lane 17). The 2-sugar glycolipid band had slightly darkened over the 48-hour incubation period (Figure 17, plate I, lanes 15-18). At the same time, the 3-sugar glycolipid band at $rm\ 9.0 \pm 0.5$ had become relatively darker over this incubation period. The G-4-4 band had significantly weakened by the 24th hour of incubation but did not disappear completely as seen with higher volumes of 0.1 M HNO_3 (Figure 17, plate I, lane 17).

The degradative effects of 70 μ l of 0.1 M HNO_3 on G-4-4 molecule was very mild. The pH of the degrading medium was recorded as 2.5 and bands in the 2- sugar regions were not observed at any time interval but the 3-sugar glycolipid band was relatively more prominent at 48 hours (Figure 17, plate I, lane 24).

Furthermore, 35 μ l, 18 μ l, 9 μ l and 4 μ l of 0.1 M HNO_3 had very minor degradation effects on the G-4-4 molecule. The pH of the degrading media was recorded as 2.5 - 3.0, 3.5 - 4.0, 3.5 - 4.0 and 3.5 - 4.0 respectively for each of the volumes of 0.1 M HNO_3 stated above. Very weak bands appeared in the 2-sugar glycolipid position at 48 hour incubation time for each volume of the 35 μ l, 18 μ l, 9 μ l and 4 μ l of 0.1 M HNO_3 added to the G-4-4 molecule (Figure 17, plate II, lanes 6, 18, 24). The 3-sugar bands also appear to be relatively dark at 48 hours with each volume of HNO_3 stated above suggesting that the

4-sugar G-4-4 molecule degrades first into a 3-sugar glycolipid molecule and then into a 2-sugar glycolipid structure.

AlCl_3 on its own was also able to induce significant G-4-4 degradation (Part I).

Ferric chloride, FeCl_3

The anisaldehyde stained plates in Figure 18, (plates I and II) show the effects of varying volumes of 0.1 M HNO_3 and a fixed volume of 0.02 M FeCl_3 salt solution on 0.02 M G-4-4 solution at 85°C over periods of time ranging from 1-48 hours.

Significant degradation of G-4-4 was observed at the lowest pH (1.0) i.e. when G-4-4 was incubated with 560 μl of 0.1 M HNO_3 . Degradation was noticed as early as the 1st hour of incubation evident by the presence of a weak band in the 2-sugar glycolipid region and a darker 3-sugar glycolipid band at R_f 9.0 ± 0.5 compared to that present in native G-4-4 molecule (Figure 18, plate I, lane 1 and G-4-4 control). These bands darkened over time and at over the same period, the G-4-4 band became weaker (Figure 18, plate I, lanes 1-4). The G-4-4 band had completely disappeared by 24 hours with only the 2- and 3- sugar glycolipid bands being prominent (Figure 18, plate I, lanes 5 & 6). The 1-sugar glycolipid band was not present at any time interval.

Incubation with 280 μl of 0.1 M HNO_3 also had severe degradative effects on the G-4-4 molecule. The pH of the degrading medium was recorded as 1.5 and G-4-4 breakdown was noticed as early as the 1st hour if incubation shown by the presence of an additional (but very weak) band in the 2-sugar glycolipid region on the TLC plate (Figure 18, plate I, lane 7). This band became more prominent over time while the G-4-4 band became weaker (Figure 18, plate I, lane 11). There was also a weak band visible in the region of 1-sugar glycolipid which had a slightly higher TLC mobility. At 48 hours, the G-4-4 band had virtually disappeared and the 1- and 2-sugar glycolipid band were the dominating structures present at this time (Figure 18, plate I, lane 12).

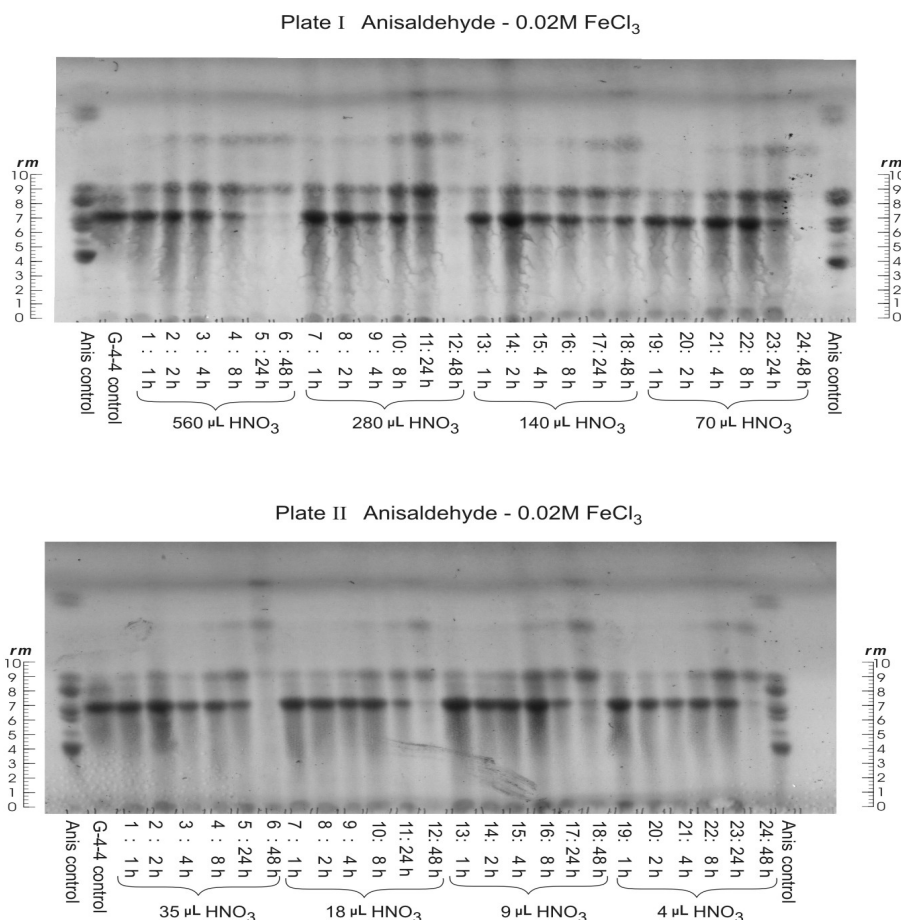


Figure 18: Effects of variable volumes of 0.10 M HNO₃ on G-4-4 and salt solutions. The salt used was FeCl₃. Variable acid volumes were added to a fixed concentration of G-4-4 and salt solutions and the entire mixture was incubated at 85°C over 48 hours.

Incubation with 140 µl of 0.1 M HNO₃ was also able to degrade the G-4-4 molecule fairly strongly. The pH of the degrading medium was 2.0. Degradation was noticed after 8 hours of incubation by the presence of an additional band in the 2-sugar glycolipid region which became more prominent over time (Figure 18, plate I, lane 16). At 48 hours, the 1-, 2-, 3- and 4-sugar glycolipid structures were present (Figure 18, plate I, lane 18). It is important to note that the G-4-4 molecule was not entirely consumed in the degradation process and that the 3-sugar glycolipid bands gradually darkened over the incubation period i.e. 48 hours.

The effects of 70 µl of 0.1 M HNO₃ on the G-4-4 molecule was observed only after 4 hours of incubation where an additional band in the 2-sugar glycolipid region became visible (Figure 18, plate I, lane 21). This band became more prominent over time. The pH of the

degrading medium was 2.0. At 24 hours of incubation, the G-4-4 band had significantly diminished with the 3-sugar glycolipid structure being the most prominent. There was also a weak band in the region of the 1-sugar glycolipid structure at 24 hours (Figure 18, plate I, lane 23). The G-4-4 band had completely disappeared at 48 hours of incubation time with only weak bands in the 2- and 1-sugar glycolipid regions on the TLC plate (Figure 18, plate I, lane 24).

Incubating G-4-4 with 35 μl of 0.1 M HNO_3 had a mild degradation effect on the former. The pH of the degrading medium was 2.0 and degradation was noticeable from 24 hours of incubation at 85°C. A very weak band in the 2-sugar glycolipid region appeared at this time which, together with the 3-sugar band, gradually darkened over the 48-hour incubation period (Figure 18, plate II, lanes 5-6). At 48 hours, both the G-4-4 and the 3-sugar glycolipid band had completely disappeared (Figure 18, plate II, lane 6). The only bands visible during this time were in the 2- and 1-sugar glycolipid regions.

Likewise 18 μl of 0.1 M HNO_3 had a similar degradation effect on the G-4-4 molecule. The pH of the degrading medium was 2.5. (Figure 18, plate II, lane 10). The 2- and 3-sugar glycolipid bands were the most prominent at 48 hours together (Figure 18, plate II, lane 12). The G-4-4 band had almost disappeared at 48 hours.

Incubation of G-4-4 with 9 μl and 4 μl of 0.1 M HNO_3 had identical degradative effects as discussed with 18 μl of 0.1 M HNO_3 above (Figure 18, plate II, lanes 13-24).

0.02 M FeCl_3 solution was able to significantly degrade the G-4-4 glycolipid in the absence of acid as this salt forms acidic solutions upon dissolution (see Figure 11).

Tin (II) chloride, SnCl_2

Figure 19, plates I and II show the effects of incubating varying volumes of 0.1 M HNO_3 and a fixed volume of 0.02 M SnCl_2 salt solution on 0.02 M G-4-4 solution at 85°C over periods of time ranging from 1-48 hours.

As expected, degradation of G-4-4 was noticed upon incubation with 560 μl of 0.1 M HNO_3 . The pH of the degrading medium was 1.0 and additional bands in the 2- and 1-sugar glycolipid regions were observed only after 24 hours of incubation (Figure 19, plate I, lanes 5-6). Both the G-4-4 and the 3-sugar glycolipid band had significantly weakened from the 2nd hour of incubation (Figure 19, plate I, lane 2). This could be due to the rapid degradation of the G-4-4 molecule into products that cannot be chemically stained by anisaldehyde. There is also a possibility that a small volume of sample was loaded on the TLC plate.

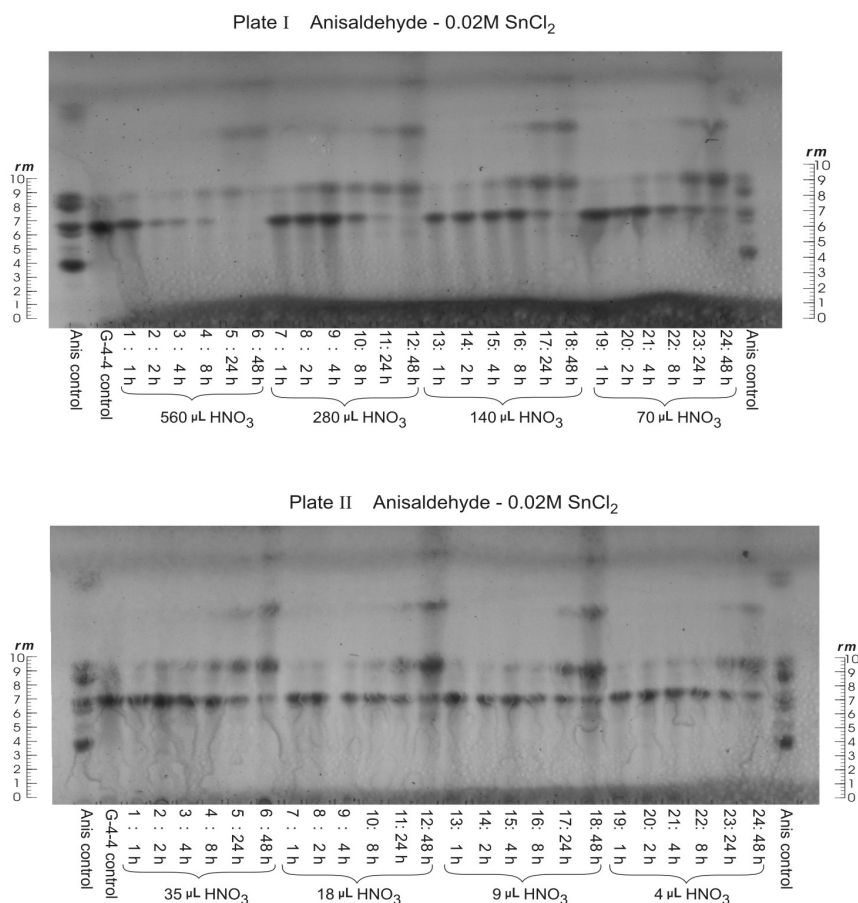


Figure 19: Effects of variable volumes of 0.10 M HNO₃ on G-4-4 and salt solutions. The salt used was SnCl₂. Variable acid volumes were added to a fixed concentration of G-4-4 and the salt solutions and the entire mixture was incubated at 85°C over 48 hours.

The degradative effects of 280 µl of 0.1 M HNO₃ on the G-4-4 molecule were also significant. The pH of this degrading medium was 1.0 and degradation was noticeable from the 2nd hour of incubation shown by the presence of a very weak band in the 2-sugar glycolipid region (Figure 19, plate I, lane 8). The 3-sugar glycolipid band at $rm\ 9.0 \pm 0.5$ gradually darkened over the 48 hours of incubation accompanied by the simultaneous weakening of the G-4-4 band suggesting breakdown of the latter (Figure 19, plate I, lanes 8-12). The 2-sugar glycolipid band became a solid band at 24 hours of incubation and the 1-sugar glycolipid band also appeared at around 24 and 48 hours. At 48 hours, the G-4-4 band had disappeared with the 3- and 2- and 1-sugar bands being prominent (Figure 19, plate I, lane 12).

Treatment of G-4-4 with 140 µl of 0.1 M HNO₃ caused the former to degrade after about 8 hours of incubation. The pH of the degrading medium was 1.5. The appearance of an additional 2-sugar glycolipid band was visible at 24 hours of incubation

(Figure 19, plate I, lane 17). The G-4-4 band at this time had notably weakened and had virtually disappeared at 48 hours (Figure 19, plate I, lane 18). At 48 hours, the 1-, 2- and 3-sugar glycolipid bands were the most prominent (Figure 19, plate I, lane 18).

The pH of the degrading medium containing 70 μ l of 0.1 M HNO_3 was 2.0 and this had a less severe degradative effect on the G-4-4 molecule. The 3-sugar glycolipid band became prominent from 4 hours of incubation and an additional band in the 2-sugar glycolipid region was observed at 24 hours (Figure 19, plate I, lanes 21 and 23). At the same time, the G-4-4 band at m/z 9.0 ± 0.5 had considerably weakened (Figure 19, plate I, lane 23). The G-4-4 band at 48 hours had significantly weakened and was present with 1-, 2- and 3-sugar bands; the most prominent band being the 3-sugar glycolipid (Figure 19, plate I, lane 24). The band in the 2-sugar glycolipid region appeared to be skewed. This could be due to salt, acid or water molecules present in the sample loaded onto the plate (Figure 19, plate I, lane 24).

35 μ l, 18 μ l, 9 μ l and 4 μ l of 0.1 M HNO_3 had identical degradative effects on G-4-4. The pH of each of the degrading medium was 2.0 and degradation was most prominent at 48 hours where the G-4-4, 1-, 2- and 3- sugar glycolipid bands were observed (Figure 19, plate II, lanes 1-24).

0.02 M SnCl_2 solution was also able to significantly degrade G-4-4 on its own (see Figure 9, plate I, lanes 13 - 18). This salt forms strong acid solutions.

The results are summarised in Table 18.

Summary

The method tested the degradative ability of salts that formed both acidic and neutral solutions upon dissolution. It is clearly evident from Table 18 that degradation took place at pH below 3.0. This table illustrates that salts that formed near-neutral solutions such as Cr_2O_3 and $\text{Cu}(\text{NO}_3)_2$ that were not able to induce degradation of G-4-4 previously, were observed to do so upon acidification with HNO_3 . Degradation of G-4-4 was observed to be directly proportional to the amount of acid added i.e. G-4-4 degradation became more significant as the pH of the solutions were lowered. If the pH of solution mixtures were in the range, 3.0-6.0, G-4-4 degradation was not observed. As there was no sign of G-4-4 degradation at higher pH (≥ 4.0), it was clear that the metal ions were not contributing towards the degradation of the glycolipid.

Table 18: Degradative effects of salts and varying pH on G-4-4 at 85°C at 48 hours. The G-4-4 sample was suspended in 280 µl of various salt solutions with varying volumes of 0.1 M HNO₃. The pH of each of the degrading medium was also recorded prior to incubation and is shown in brackets.

Volume of 0.1 M HNO ₃ (µl)	Degradation scores and pH				
	Cr ₂ O ₃	Cu(NO ₃) ₃	AlCl ₃	FeCl ₃	SnCl ₂
560	++++ (1.0)	++++ (1.0)	+++++ (1.0)	++++ (1.0)	+++++ (1.0)
280	+++ (1.5)	++++ (1.0-1.5)	++++ (1.5)	++++ (1.5)	++++ (1.0)
140	++ (2.0)	+++ (2.0)	++ (2.0)	+++ (2.0)	++++ (1.5)
70	++ (2.5)	++ (3.0)	++ (2.5)	+++ (2.0)	+++ (2.0)
35	- (5.0-6.0)	- (3.0)	± (2.5-3.0)	++++ (2.0)	+++ (2.0)
18	- (5.0-6.0)	- (3.0)	± (3.5-4.0)	+++ (2.5)	+++ (2.0)
9	- (5.0-6.0)	- (3.5-4.0)	± (3.5-4.0)	+++ (2.5)	+++ (2.0)
4	- (5.0-6.0)	- (3.5-4.0)	± (3.5-4.0)	+++ (2.5)	+++ (2.0)
No acid	-	±	+++++	+++	++++

Scoring system as per Protocol 11.

Acidic salts such as AlCl₃, FeCl₃ and SnCl₂ were able to degrade G-4-4 more extensively when acidified with HNO₃.

A slight irregularity in the results was seen when G-4-4 was incubated lower volumes of acid and AlCl₃. Significant degradation of G-4-4 was expected but was not observed.

Further evidence to rule out of catalytic activity would be that each mixture contained a consistent quantity of metal ions. Those mixtures with a lower pH were able to induce G-4-4 degradation while those at a higher pH gave nil degradation. Hence, regardless of

the metal ion concentration of each mixture, G-4-4 was only able to degrade in solutions with a low pH (pH range = 1.0-3.0).

Part V

Implications of decreasing the pH and $[M^+]$ on G-4-4 at 85°C

This experiment was designed to investigate the simultaneous effects of both acidity and salt solutions (M^+) on the globoside (G-4-4) molecule over 24 hours at 85°C. This set of experiments would confirm if degradation of G-4-4 only occurs at pH less than and equal to 3.0 ($pH \leq 3.0$). The general method involved suspending 50 μ l of 0.02 M G-4-4 solution with 50 μ l of varying concentrations of nitric acid (HNO_3) and 50 μ l of varying concentrations of salt solutions. The pH of each of the differing mixtures was measured with Whatman's litmus paper. The mixtures were then capped and incubated at 85°C for 24 hours in a water bath. The method is outlined in detail in Protocol 1, v 4. Ferric chloride ($FeCl_3$) and tin (II) chloride ($SnCl_2$) solutions were used in this experiment. These salts form acidic solutions upon dissolution and were chosen in particular so that varying concentrations of these salts would give rise to a range of pH (acidic to near-neutral pH). This property was ideal to investigate the direct degradative effects pH would have on the G-4-4 molecule and at the same time determine if the metal ions (Fe^{3+} and Sn^{2+}) are acting as catalysts in the glycolipid breakdown process.

Varying $[FeCl_3]$ versus varying $[HNO_3]$

The G-4-4 sample was treated as follows; 50 μ l of 0.02 M G-4-4 solution was suspended with each of 50 μ l of 0.002 M, 0.004 M, 0.006 M, 0.008 M and 0.01 M of $FeCl_3$ solution and 50 μ l of 0.02 M, 0.04 M, 0.06 M, 0.08 M and 0.1 M HNO_3 solution. These mixtures were then incubated at 85°C over a period of 24 hours. The anisaldehyde stained plates in Figure 20 show the effects of individual HNO_3 and metal ion concentrations on G-4-4.

It is important to note that the G-4-4 molecule migrated to a higher *rm* position on the TLC plate. The reason for this is not known but may have been due to the batch of TLC plates used or due to the presence moisture in the TLC developing chamber. However, the untreated G-4-4 also migrated to this higher position relative to the TLC control indicating that the samples loaded were salt and water free.

0.02 M, 0.04 M and 0.06 M HNO_3 versus varying $[FeCl_3]$

The anisaldehyde staining of G-4-4 molecule treated with 0.02 M, 0.04 M and 0.06 M HNO_3 and varying concentrations of $FeCl_3$ showed that the G-4-4 molecule did not undergo any degradation (Figure 20, plate I, lanes 1-5). The distinctive band at *rm* 7.0 ± 0.5 remained intact together with a weak band at *rm* 9.0 ± 0.5 . The pH of each of the different solutions was approximately 3.5.

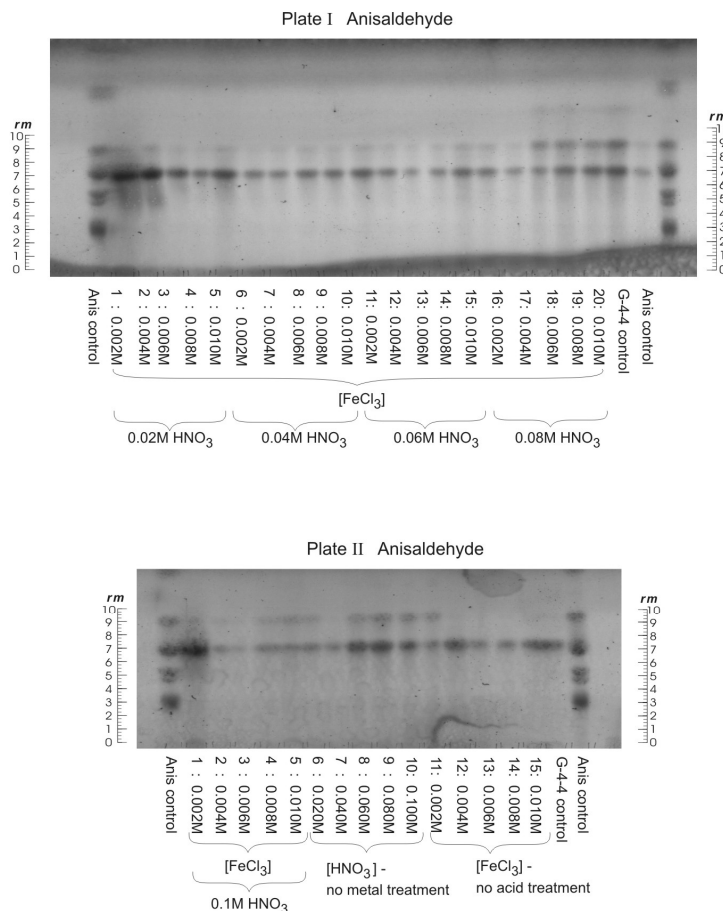


Figure 20: Effects of varying concentrations of FeCl_3 and HNO_3 on G-4-4.

The mixture was incubated at 85°C over 24 hours. The two sets of controls are also shown in plate II where the G-4-4 sample is treated with either varying concentrations of HNO_3 or FeCl_3 .

0.08 M HNO_3 versus varying $[\text{FeCl}_3]$

The pH of each of the solutions (FeCl_3 and G-4-4 mixture) was measured to be approximately 3.0. Degradation of the G-4-4 molecule was mild and was noticed with the full range of FeCl_3 concentrations used i.e. 0.004 M, 0.006 M, 0.008 M and 0.01 M except with 0.002 M FeCl_3 (Figure 20, plate I, lanes 16-20). The 3-sugar glycolipid band (at $rm\ 9.0 \pm 0.5$) at each concentration of FeCl_3 was prominent. The 3-sugar glycolipid bands were the darkest at 0.01 M FeCl_3 concentration and also had a relatively darker band present in the region of the 2-sugar glycolipid position on the TLC plate. In contrast to this result, 0.01 M FeCl_3 was not able to induce G-4-4 degradation when the acidic component was absent (Figure 20, plate II, lane 15). This observation suggested that the acidity and not metal ions caused the G-4-4 molecule to degrade.

0.10 M HNO₃ versus varying [FeCl₃]

Similarly, a mild degradation pattern was observed when G-4-4 molecule was suspended with a varying range of FeCl₃ concentrations. The pH of each of the degrading media was approximately 2.5. Weak bands in the 3-sugar glycolipid region (at *rm* 9.0 ± 0.5) appeared to be more prominent than that present in the untreated G-4-4 sample lane (Figure 20, plate II, lanes 1-5 and plate II, lane 'G-4-4 control'). This is a possible suggestion that the 4-sugar glycolipid molecule, G-4-4, was degrading into a 3-sugar glycolipid. However, there is no evidence of the 3-sugar glycolipid degrading into a 2- or 1-sugar glycolipid molecule as there were no bands present in the regions where these molecules would normally migrate.

G-4-4 versus varying [HNO₃]

This was the first set of controls with the variable salt concentrations absent. Mild degradation was observed when G-4-4 molecule was treated with varying HNO₃ concentrations and in the absence of FeCl₃ solution. The 3-sugar glycolipid band (at *rm* 9.0 ± 0.5) was prominent when G-4-4 was treated with 0.06 M, 0.08 M and 0.1 M nitric acid (Figure 20, plate II, lanes 8-10). The pH of the degrading media was 3.0, 2.0 and 2.0 respectively. However 0.02 M and 0.04 M HNO₃ were not able to induce degradation of G-4-4 and the pH of both media was 4.0.

G-4-4 versus varying [FeCl₃]

This was the second set of controls with nil acid treatment. There was no degradation observed when G-4-4 was treated with varying FeCl₃ concentrations in the absence of acid (Figure 20, plate II, lanes 11-15). The pH of each of the media was 4.0, 5.0, 4.0, 4.0 and 4.0 for 0.002 M, 0.004 M, 0.006 M, 0.008 M and 0.010 M of FeCl₃ respectively. There were no additional bands observed apart from the distinctive 4-sugar glycolipid band at *rm* 7.0 ± 0.5. However there is a slight irregularity shown by the presence of a 3-sugar glycolipid band in Figure 20, plate II, lane 11. This may have been due to a higher loading of sample on the respective lane or may have been due to a mild degradation process upon incubation.

Varying $[\text{SnCl}_2]$ versus varying $[\text{HNO}_3]$

50 μl of 0.02 M G-4-4 solution was suspended with each of 50 μl of 0.002 M, 0.004 M, 0.006 M, 0.008 M and 0.01 M of SnCl_2 solution and 50 μl of 0.02 M, 0.04 M, 0.06 M, 0.08 M and 0.1 M HNO_3 solution. The pH of each of the different mixtures is shown in the Table 20. The anisaldehyde stains are shown in Figure 21.

As with FeCl_3 , the G-4-4 structure migrated to a higher position than normal on the TLC plate (see above).

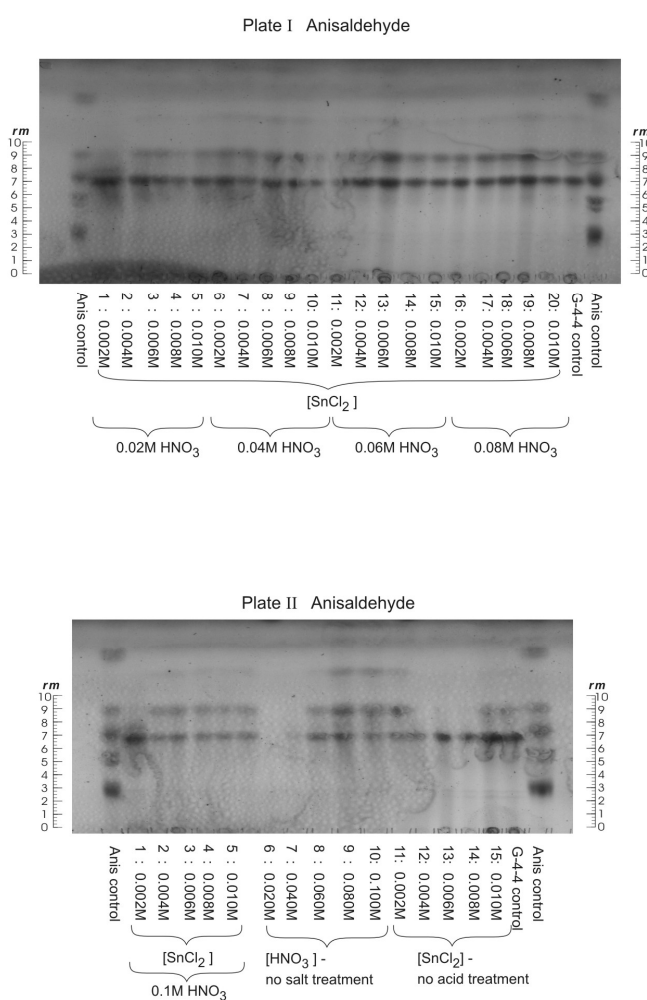


Figure 21: Effects of varying concentrations of SnCl_2 and HNO_3 on G-4-4.

The mixture was incubated at 85°C over 24 hours. The two sets of controls are also shown in plate II where the G-4-4 sample is treated with either varying concentrations of HNO_3 or SnCl_2 .

0.02 M HNO₃ versus varying [SnCl₂]

There was very mild degradation noticed when G-4-4 was suspended with 0.02 M HNO₃ and varying SnCl₂ concentrations (Figure 21, plate I, lanes 1-5). The G-4-4 band remained intact together with the 3-sugar glycolipid band (at *rm* 9.0 ± 0.5) present at all time intervals. The pH of each of the media was 4.0.

0.04 M, 0.06 M and 0.08 M HNO₃ versus varying [SnCl₂]

There was a very mild form of degradation observed when 0.04 M HNO₃ was suspended with varying concentrations of SnCl₂. The 3-sugar glycolipid bands, as expected were more intense than treatment with 0.02 M HNO₃. Upon closer observation, there was an extremely weak band present in the region of the 2-sugar glycolipid on the TLC plate (Figure 21, plate I, lanes 6-10). The G-4-4 band was slightly weak in Figure 21, plate I, lane 10. This could be due to a lower volume of sample loaded onto this lane and should not be confused with extensive degradation of the G-4-4 molecule. The G-4-4 and 3-sugar glycolipid bands remained consistent with the varying concentrations of SnCl₂ indicating that degradation took place in its mildest form. The pH of the degrading media was in the 3.5 – 4.0 range.

0.06 M HNO₃ was also able to mildly degrade G-4-4 when suspended with a range of SnCl₂ concentrations. There was an additional weak band present in the 2-sugar glycolipid range (off the *rm* scale) treated with each concentration of the salt solution (Figure 21, plate I, lanes 11-15). Figure 21, plate I, lane 13, has a very dark and prominent band in both the 4- and 3-sugar glycolipid positions and a relatively darker band in the 2-sugar glycolipid position compared to the other lanes due to a higher loading volume. The pH of the degrading media was in the 2.5 – 3.0 range.

0.08 M HNO₃ also degraded G-4-4 molecule in a similar fashion described with 0.06 M HNO₃. Degradation was mild with very weak bands present in the 2-sugar glycolipid region for each concentration of salt solution (Figure 21, plate II, lanes 16-20). The pH of the degrading media was in the 2.0 – 2.5 range.

0.10 M HNO₃ versus varying [SnCl₂]

Degradation of G-4-4 was slightly more extensive than that described with 0.06 M and 0.08 M HNO₃. The 2-sugar glycolipid bands appeared to be slightly darker and more visible (Figure 21, plate II, lanes 1-5). The 3-sugar glycolipid band at *rm* 9.0 ± 0.5 was also more intense than that described above. In addition, the G-4-4 band at *rm* 7.0 ± 0.5 appeared to be much more reduced again suggesting that the degradation reaction was more intense than that obtained from treating G-4-4 with lower concentrations of HNO₃.

G-4-4 versus varying [HNO₃]

Varying concentrations of HNO₃ in the absence of salt solution, was able to degrade G-4-4 molecule upon incubation at 85°C. This degradation was slightly more intense than any described above but still scored as a mild reaction. The pH of the degrading media ranged from 2.0 (for 0.10 M HNO₃) to 4.0 (for 0.02 M HNO₃). Degradation was visible by the presence of new bands in the 2-sugar glycolipid region where G-4-4 was treated with 0.06 M, 0.08 M and 0.10 M HNO₃ (Figure 21, plate II, lanes 8 -10). There were no bands visible in Figure 21, plate II, lanes 6 and 7 (0.02 M and 0.04 M HNO₃ respectively). Overall, G-4-4 was not expected to show any degradation with these two concentrations (0.02 M and 0.04 M) of HNO₃ because the pH of the degrading medium was in the 3.5 - 4.0 range.

G-4-4 versus varying [SnCl₂]

In contrast, varying concentrations of salt solutions in the absence of acid was only able to induce mild degradation upon incubation at 85°C as seen by the appearance of darker 3-sugar glycolipid bands in Figure 21, plate II, lanes 14 and 15. The pH of each of the degrading mixtures was approximately 4.0. There were no bands observed in the 2-sugar glycolipid region on the TLC plate.

Summary

The data in Table 19 and Table 20 suggested, once again, that a low pH is probably the most critical factor regarding G-4-4 degradation. Metal ions may not be involved in the short term degradation as the solutions containing varying volumes of SnCl₂ and FeCl₃ were not able to induce degradation at lower concentrations where the pH was higher than 3.0. On the other hand, both SnCl₂ and FeCl₃ were able to induce degradation at a concentration of 0.02 M as seen in Part I. In the current section, degradation ranged from (+) to (++) since the pH of the mixture incubated at 85°C was in the higher pH range. A higher pH resulted from the dilution of the varying concentrations of acid solutions with G-4-4 and salt solutions. This, in turn, resulted in the mild degradation of the G-4-4 glycolipid.

Table 19: Degradative effects of varying FeCl_3 and HNO_3 concentrations on G-4-4. Incubations were carried out at 85°C over 24 hours and the pH of each mixture was recorded prior to incubation and is shown in brackets.

		[FeCl_3] (Molar)					
		0.002	0.004	0.006	0.008	0.010	0.000
[H^+] (Molar)	0.02	- (3.5)	- (3.5)	- (3.5)	- (3.5)	- (3.5)	- (4.0)
	0.04	- (3.0)	- (3.0)	- (3.0)	- (3.0)	- (3.0)	- (4.0)
	0.06	- (3.0)	- (3.0)	- (3.0)	- (3.0)	- (3.0)	+ (3.0)
	0.08	- (3.0)	+ (3.0)	++ (3.0)	++ (3.0)	++ (3.0)	+ (2.0)
	0.10	+ (2.5)	+ (2.5)	+ (2.5)	+ (2.5)	+ (2.5)	+ (2.0)
	0.00	- (4.0)	- (5.0)	- (4.0)	- (4.0)	- (4.0)	- (4.0)
Scoring system as per Protocol 11.							

Degradation of G-4-4 was visible both in the absence and presence of metal ions but only when the pH of the degrading environment was below 3.0. In other words, the G-4-4 glycolipid generally underwent degradation at pH below 3.0 and the concentration of metal ions did not seem to have any contribution towards the degradation process. These results suggested that a low pH and not the metal ions are responsible for G-4-4 degradation i.e. metal ions do not catalyse the hydrolysis of glycosidic bonds in the oligosaccharide chain of G-4-4.

	[H ⁺] (Molar)	[SnCl ₂] (Molar)					
		0.002	0.004	0.006	0.008	0.010	0.000
	0.02	+	+	+	+	+	-
		(4.0)	(4.0)	(4.0)	3.5	4.0	4.0
	0.04	+	+	+	+	+	-
		(4.0)	(4.0)	(3.5)	3.5	3.5	3.5-4.0
	0.06	+	+	+	+	+	-
		(3.0)	(3.0)	(3.0)	2.5	3.0	2.5-3.0
	0.08	+	+	+	+	+	++
		(2.0-2.5)	(2.0-2.5)	(2.0-2.5)	(2.0-2.5)	(2.0-2.5)	(2.0-2.5)
	0.10	+	+	+	++	++	++
		(2.0)	(2.0)	(2.0)	(2.0)	(2.0)	(2.0)
	0.00	++	-	-	-	-	-
		(4.0)	(4.0)	(4.0)	(4.0)	(4.0)	(4.0)

Scoring system as per Protocol 11.

Part VI

The influence of different acids and pH on G-4-4 and ceramide at 85°C

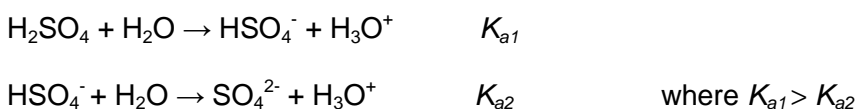
Various 0.10 M acids versus G-4-4 at 24 hours

This experiment was set up to investigate the effects of various 0.10 M acids on the G-4-4 molecule and to determine if the pattern of degradation is consistent at a pH of approximately 2.0. The method in Protocol 2, v 2, was followed and 50 µl of 0.02 M G-4-4 solution was suspended with 100 µl of various 0.10 M acids and incubated at 85°C for 24 hours. The pH of each of the solutions was measured using Whatman's pH paper. The acids tested were 0.10 M sulphuric acid (H₂SO₄), 0.10 M nitric acid (HNO₃), 0.10 M hydrochloric acid (HCl), 0.10 M phosphoric acid (H₃PO₄) and 0.10 M acetic acid (CH₃COOH). The control for this experiment was subjecting the G-4-4 sample at 85°C without any acid treatment.

The strength of an acid is measured by its dissociation constant K_a . Strong acids have larger K_a values i.e. reaction equilibrium is normally at the far right while weaker acids dissociate partially and have lower K_a values. Most organic acids such as acetic acid are weak acids while mineral acids such as nitric, sulphuric and hydrochloric acids are strong.

Further H₂SO₄ (a diprotic acid) has twice the acid concentration of HCl while H₃PO₄ (triprotic acid) has three times more protons but is less able to ionise.

The ionisation mechanisms of H₂SO₄ are as follows;



Similarly, a triprotic acid, (such as H₃PO₄), can undergo one, two and three dissociations and has three dissociation constants.

The anisaldehyde staining revealed that each of the acids had a different mode of degrading the G-4-4 molecule. HCl degraded the G-4-4 molecule into multiple fragments as shown by the multiple bands present in Figure 22, plate I, lane 2. The G-4-4 band at $rm\ 7.0 \pm 0.5$ had completely disappeared. This acid could have possibly attacked the lipid tail of the glycolipid.

Degradation of G-4-4 induced by H₃PO₄, CH₃COOH and HNO₃ were similar as the bands formed by the action of the acid migrated to the similar positions on the TLC plate (Figure 22, plate I, lanes 3, 4 and 6).

H_2SO_4 had consumed the entire G-4-4 molecule over the 24 hour incubation process at 85°C (Figure 22, plate I, lane 5).

The controls for this experiment were G-4-4 at room temperature and G-4-4 solution at 85°C over 24 hours. In both instances, there was no degradation of G-4-4 observed as there were no additional bands on the respective lanes and the single G-4-4 band had migrated to its standard position of $rm\ 7.0 \pm 0.5$ (Figure 22, plate I, lanes 1 and 7).

HNO_3 was chosen as the degrading acid for further experiments. The basis for choosing this particular acid was that it was readily available and was able to cleave the G-4-4 molecule in a 'clean' and ordered pattern. In addition HNO_3 being a simple molecule would form simple degradation products.

Plate I Anisaldehyde

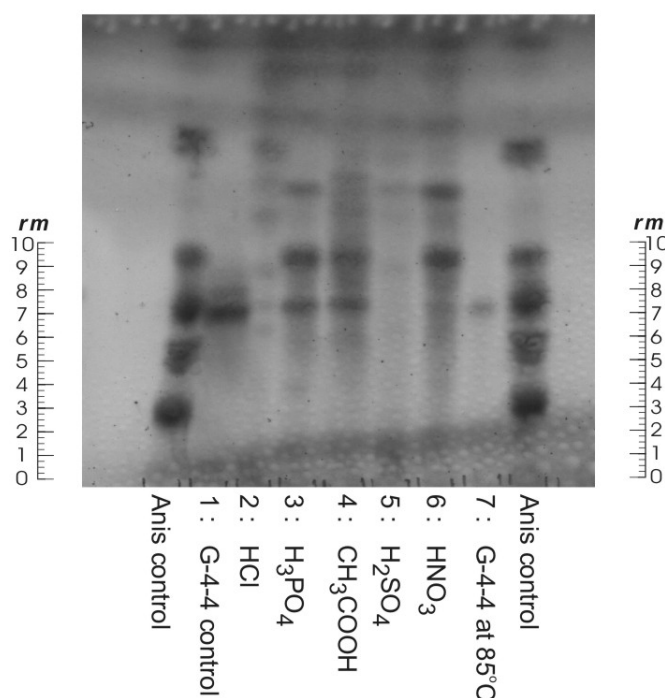


Figure 22: Degradative effects of various 0.10 M acids on G-4-4. G-4-4 was incubated with the various acids at 85°C over 24 hours. The control for this experiment was G-4-4 sample incubated at 85°C without any acidic treatment.

Summary

The results are summarised in Table 21.

From the results presented in Table 21, it can be seen that 0.1 M HCl and 0.1 M H₂SO₄ caused extensive degradation of the G-4-4 molecule over 24 hours. Both HNO₃ and H₃PO₄ also caused maximum degradation and the degraded structures appeared to be in the regions of the 3-, 2- and 1-sugar glycolipid structures on the TLC plate. Similarly, CH₃COOH was able to induce a similar degradative effect on G-4-4 but was excluded due to organic interferences in the degradation reactions of the G-4-4 molecule. H₃PO₄ was also excluded due to its dangerous nature and special requirements for handling and storage. HNO₃ was chosen on the basis of its ready availability and ability to cleave the G-4-4 structure sequentially thereby forming clean degradation patterns.

Table 21: Effects of various 0.10 M acids on G-4-4 at 85°C over 24 hours. The pH of each mixture was recorded prior to incubation.

Type of 0.10 Molar acid		Degradation score	pH
Sulphuric acid	H ₂ SO ₄	+++++	1.5
Nitric acid	HNO ₃	++++	1.5
Hydrochloric acid	HCl	+++++	2.0
Phosphoric acid	H ₃ PO ₄	++++	2.0
Acetic acid	CH ₃ COOH	++++	3.0
Control; G-4-4 solution		-	7.0
Scoring system as per Protocol 11. * nil treatment at 85°C			

0.10 Molar acids versus G-4-4

The effects of various acids of 0.10 M strength were investigated over periods of time ranging from 1 to 24 hours at 85°C. Degradative effects of each acid were examined at each time interval. Approximately 300 µl of 0.02 M G-4-4 solution was suspended with 600 µl of 0.1 M acid and incubated at 85°C according to Protocol 2, v 2. The control for this experiment was G-4-4 acidified as described above with the appropriate acids left at room temperature for a period of 24 hours. The standard control was untreated G-4-4 solution at room temperature.

There were no degradations observed with G-4-4 solutions suspended in acid and kept at room temperature over 24 hours. The G-4-4 band remained intact at $rm\ 7.0 \pm 0.5$ and no other additional bands were observed in these lanes (Figure 23, plate I, lanes 7, 14, 21, and 28). This result remained consistent with that of the standard control. Untreated G-4-4 molecule also did not degrade at room temperature (Figure 23, plate I, lane 29).

0.10 M Nitric acid - HNO_3

The G-4-4 molecule was able to degrade when suspended with 0.10 M HNO_3 at $85^\circ C$ over 24 hours. Degradation was noticed as early as the 4th hour of incubation evident by the presence of a relatively dark band in the 2-sugar glycolipid region (Figure 23, plate I, lane 3). This band darkened over the incubation period and was most prominent at 24 hours. The 3-sugar glycolipid band at $rm\ 9.0 \pm 0.5$ also darkened over the incubation period. Another band in the region of the 1-sugar glycolipid appeared at 24 hours (Figure 23, plate I, lane 6). This band was extremely weak. The G-4-4 band at $rm\ 7.0 \pm 0.5$ had virtually disappeared at the 8th hour of incubation (Figure 23, plate I, lane 4).

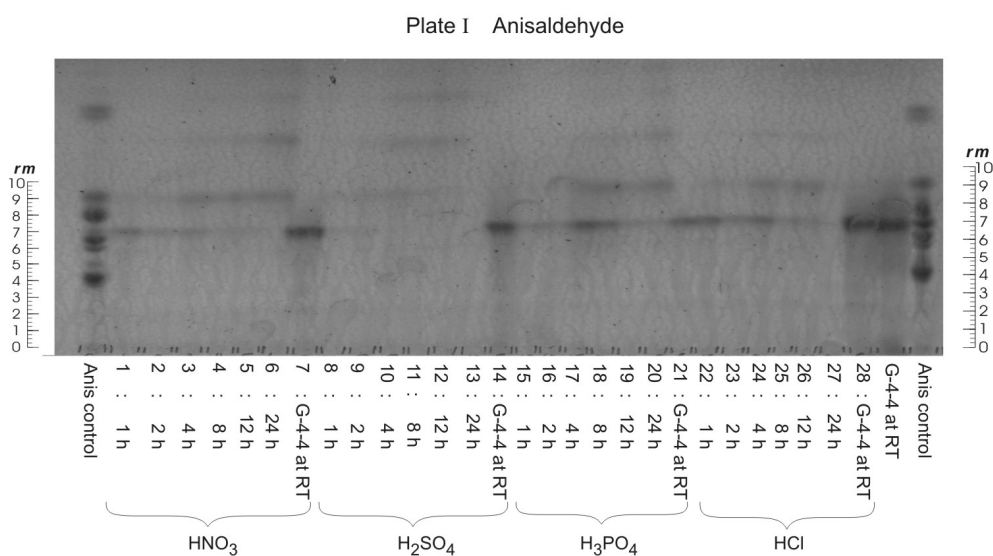


Figure 23: Effects of various 0.10 M acids on a fixed volume of G-4-4. The acids were incubated with G-4-4 over a period of 24 hours at both $85^\circ C$ and room temperature (RT).

0.10 M Sulphuric acid - H_2SO_4

H_2SO_4 of all the acids tested seemed to have the most severe degrading effects on the G-4-4 molecule. The latter was entirely consumed upon the 1st hour of incubation at 85°C as evident by the absence of the distinctive G-4-4 band at $rm\ 7.0 \pm 0.5$ (Figure 23, plate I, lane 8). The 3-sugar glycolipid band was present, although relatively weak from the 2nd hour of incubation (Figure 23, plate I, lane 9). Additional bands in the 1- and 2-sugar glycolipid regions appeared from the 4th hour of incubation and persisted until 24 hours (Figure 23, plate I, lanes 10-13). The band in the 1-sugar glycolipid region was extremely weak.

0.10 M Phosphoric acid - H_3PO_4

H_3PO_4 was able to degrade the G-4-4 molecule in a much more gentle fashion as the G-4-4 band at $rm\ 7.0 \pm 0.5$ persisted until the 24th hour of incubation. The band in the 3-sugar glycolipid region at $rm\ 9.0 \pm 0.5$ appeared to be relatively darker on the 4th hour of incubation (Figure 23, plate I, lane 17). Darkening of this band may relate to more of this molecule being formed during the degradation process as is seen with the results just described. The 2-sugar glycolipid band also appeared on the 4th hour of incubation. The 1-sugar glycolipid band was just visible at 24 hours (Figure 23, plate I, lane 20). However, the G-4-4 band did not completely disappear by 24 hours but had reasonably weakened at this time.

0.10 M Hydrochloric acid - HCl

G-4-4 was able to be degraded by HCl as expected. However, degradation was only noticeable from the 4th hour of incubation evident by the presence of an additional band in the 2-sugar glycolipid region (Figure 23, plate I, lane 24). The 3-sugar glycolipid bands were reasonably darker at 2 hours of incubation at 85°C and could possibly suggest that the terminal non reducing sugar was cleaving off the G-4-4 molecule to form the 3-sugar glycolipid molecule (Figure 23, plate I, lane 23). This resulted in darker 3-sugar glycolipid bands. The G-4-4 band at $rm\ 7.0 \pm 0.5$ had significantly weakened at 8 hours and had completely disappeared at 24 hours of incubation (Figure 23, plate I, lanes 25 -27).

Varying $[\text{HNO}_3]$ versus G-4-4

This section investigated the effects of various concentrations of nitric acid on the G-4-4 structure. The aim was to obtain the most appropriate concentration of nitric acid (HNO_3) that would be able to cleave the sugars off the G-4-4 glycolipid in an ordered manner over a period of 48 hours. This concentration of HNO_3 would then be employed in the

degradation of the more valuable Lewis blood group glycolipids in the next part of the project.

The effects of varying concentrations on G-4-4 molecule was tested by incubating 250 μ l of 0.02 M G-4-4 solution with 125 μ l of varying concentrations of HNO_3 solutions at 85°C over periods of time ranging from 1 hour to 48 hours according Protocol 2, v 1. The pH of each degrading mixture was measured using Whatman's pH paper.

From the anisaldehyde stains, degradation of the G-4-4 molecule was not observed with 0.01 M and 0.02 M HNO_3 . The G-4-4 band at $rm\ 7.0 \pm 0.5$ remained intact together with the secondary band (due to the 3-sugar glycolipid) at $rm\ 9.0 \pm 0.5$ (Figure 24, plate I, lanes 1-12). This was an expected result as the pH of the degrading medium was in the 5.0 – 7.0 range.

Mild degradation of G-4-4 was noticed with incubation with 0.04 M and 0.08 M HNO_3 . However, at 48 hours, the G-4-4 band had almost disappeared after 48 hours of incubation with 0.08 M HNO_3 . The 3- and 2-sugar glycolipid bands were most prominent at this time (Figure 24, plate I, lane 24). The pH of the degrading medium was in the 2.0 - 4.0 range.

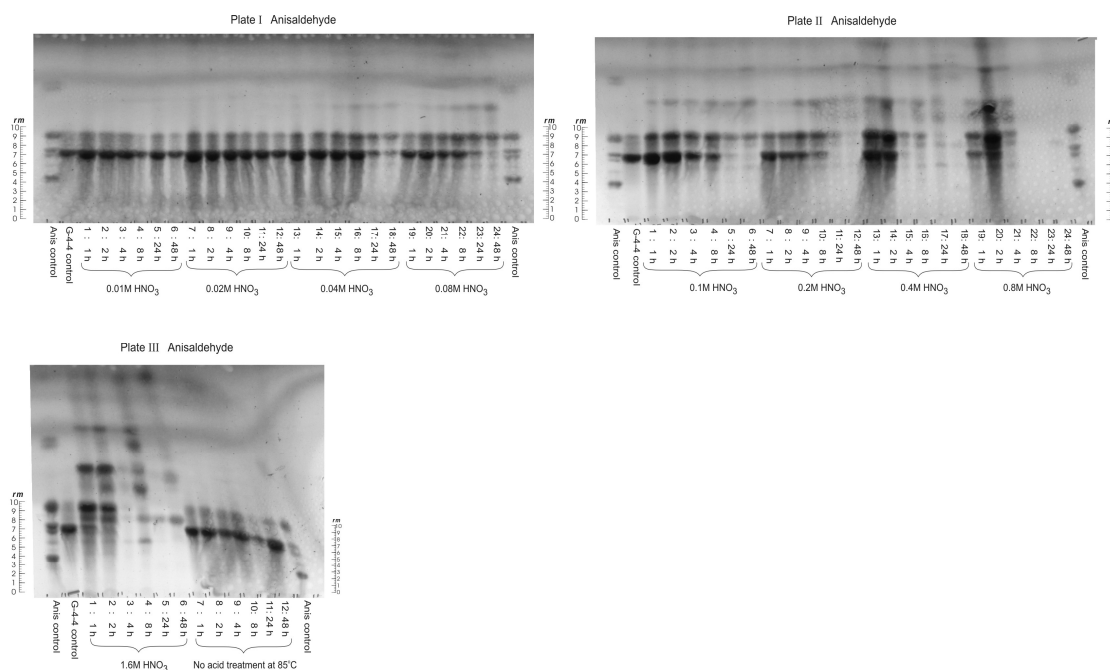


Figure 24: Effects of varying HNO_3 concentrations on G-4-4.

The G-4-4 acid mixtures were incubated at 85°C over 48 hours. The control for this experiment was G-4-4 at 85°C without any acid treatment.

0.10 M HNO_3 was able to degrade G-4-4 from as early as the 1st hour of incubation (Figure 24, plate II, lane 1). This was evident by the presence of a prominent band in the 2-sugar glycolipid region. The pH of the degrading medium was 2.0. Furthermore, this band increased in its intensity over the incubation period (Figure 24, plate II, lanes 1-6). The 3-sugar glycolipid band at $rm\ 9.0 \pm 0.5$ had also darkened over time and at 8 hours of incubation, the G-4-4 band at $rm\ 7.0 \pm 0.5$ had shown the first signs of weakening (Figure 24, plate II, lane 4). At 48 hours this band had completely disappeared with the presence of 1-, 2- and 3-sugar glycolipid bands (Figure 24, plate II, lane 6).

0.20 M HNO_3 also degraded the G-4-4 molecule in a similar fashion. Degradation was noticed as early as the 1st hour of incubation by the presence of a weak band in the 2-sugar glycolipid region (Figure 24, plate II, lane 7). The pH of the degrading medium was 1.0. This band intensified over the incubation period i.e. 48 hours and was prominent from 4 hours of incubation and onwards (Figure 24, plate II, lane 9). Concurrently, the G-4-4 band at $rm\ 7.0 \pm 0.5$ had completely disappeared at 24 hours of incubation with the 2- and 3-sugar glycolipid bands being prominent (Figure 24, plate II, lane 11). At 48 hours, both the G-4-4 band and the 3-sugar glycolipid band at $rm\ 7.0 \pm 0.5$ and $rm\ 9.0 \pm 0.5$ respectively had entirely disappeared. The 2-sugar glycolipid band was only very weakly represented at 48 hours. In addition, there were multiple bands observed at 48 hours.

0.4 M HNO_3 had extremely severe degradation effects on the G-4-4 molecule. The 2- and 1-sugar glycolipid bands were present from the 1st hour of incubation (Figure 24, plate II, lanes 13 and 14). The G-4-4 band at $rm\ 7.0 \pm 0.5$ had virtually disappeared at the 4th hour of incubation and multiple bands were also present at this time (Figure 24, plate II, lane 15). These multiple bands were present until 24 hours of incubation (Figure 24, plate II, lanes 15-17). The absence of bands at 48 hours indicated that the entire G-4-4 molecule (glycan + ceramide) was extensively degraded by the acid hydrolysis process (Figure 24, plate II, lane 18). The pH of the degrading medium was 1.0.

0.80 M HNO_3 also had a very severe degradation effect on the G-4-4 molecule. Extensive fragmentation was observed in the 1st and 2nd hour of incubation (Figure 24, plate II, lanes 19 and 20). The extremely dark band on lane 20, plate II is due to a higher loading volume of the sample on this particular lane. The 1-sugar glycolipid band was also present from the 2nd hour of incubation had disappeared by the 8th hour (lanes 20-22). The G-4-4 band at $rm\ 7.0 \pm 0.5$ had virtually disappeared by the 4th hour of incubation with only the 2- and 3-sugar glycolipids bands present together with other unidentifiable bands. The pH of the degrading medium was 1.0. There were no visible bands present from 8 hours and onwards (Figure 24, plate II, lanes 22-24).

Finally 1.6 M HNO_3 also showed extensive degradation of the G-4-4 molecule. Degradation was noticeable from the very 1st hour of incubation evident by the presence of additional bands in the region of 1- and 2-sugar glycolipid structures on the TLC plate and as well as a relatively darker 3-sugar glycolipid band at $rm\ 9.0 \pm 0.5$. This pattern was consistent at 2 hours of incubation. The pH of the degrading medium was 1.0. At these times, (1 and 2 hours) the G-4-4 band had significantly weakened (Figure 24, plate III, lanes 1 and 2). There were no prominent bands present at 4 hours. However this may have been due to a lower loading volume of sample onto this particular lane (Figure 24, plate III, lane 3). Multiple bands were observed at 8 hours of incubation (Figure 24, plate III, lane 4). At 24 and 48 hours, there was a prominent band present between the G-4-4 (4-sugar glycolipid) and the 3-sugar glycolipid bands at $rm\ 9.0 \pm 0.5$ (Figure 24, plate III, lanes 5 and 6). However, at 24 hours, this band existed as a single band but at 48 hours there were other bands present as well (Figure 24, plate III, lanes 5 and 6).

The control for this experiment was nil acid treatment of the G-4-4 molecule. 250 μl of 0.02 M G-4-4 solution was placed in a water bath for periods of time ranging from 1 to 48 hours. From the anisaldehyde stains, it can be said that heat alone was not able to degrade the G-4-4 molecule as the 4-sugar (G-4-4) band at $rm\ 7.0 \pm 0.5$ and the secondary 3-sugar band at $rm\ 9.0 \pm 0.5$ remained intact throughout the incubation process i.e. 48 hours (Figure 24, plate III, lanes 7-12).

Summary

The results are summarised in Table 22.

The results suggested that degradation of G-4-4 became more intense with increasing concentrations of HNO_3 . It is also apparent from the results that G-4-4 degradation is only evident at pH below 3.0. The pH of 0.1 M HNO_3 was 2.0 and incubation of G-4-4 at this concentration gave a visually clear and sequential cleavage of the sugar residues from the glycolipid over periods of time ranging from 1 to 48 hours. Hence, 0.10 M HNO_3 was selected as the concentration to be used for breaking down Lewis blood group glycolipids discussed later in the project.

Table 22: Degradative effects of varying concentrations of HNO_3 on G-4-4. Incubation was carried out at 85°C over 48 hours and the pH of each mixture was recorded prior to incubation.

[HNO_3] Molar	Degradation Score	pH
0.01	-	5.0-7.0
0.02	-	5.0
0.04	+	3.5-4.0
0.08	++	2.0
0.10	++++	2.0
0.20	+++++	1.0
0.40	+++++	1.0
0.80	+++++	1.0
1.60	+++++	1.0
Control; G-4-4 solution *	+	7.0

Scoring system as per Protocol 11.
* nil treatment at 85°C .

The removal of sugars or cleaving the glycosidic bonds between sugar residues by acid is termed (acid) hydrolysis. In the G-4-4 structure, $\text{GalNAc}\beta 1-3\text{Gal}\alpha 1-4\text{Gal}\beta 1-4\text{Glc}\beta 1-1\text{Cer}$, the terminal GalNAc sugar (residue) is removed first by hydrolysis of the $\beta 1-3$ glycosidic bond to form the 3-sugar glycolipid followed by the removal of the sub-terminal Gal residue by the hydrolysis of the $\alpha 1-4$ glycosidic linkage. This forms the 2-sugar glycolipid which is hydrolysed further in a similar manner into the 1-sugar structure.

Dilute and concentrated HNO_3 versus G-4-4 and ceramides

Implications of dilute and concentrated nitric acid versus hydroxy and non-hydroxy ceramides over 24 hours

The effects of temperature and dilute and concentrated nitric acid (HNO_3) on blood group ceramides were investigated and the results are shown in Figure 25, plate I. Blood group ceramides consist of a long chain or sphingolipid base linked to a fatty acid through an amide bond. The polar hydroxy group on the hydroxy ceramides makes them more susceptible to attack by radicals.

Undegraded ceramides migrate towards the top of the TLC plate due to their highly non polar nature and inability to react with the hydroxyl groups of the silica stationary phase. However, the hydroxy ceramide migrates to a slightly lower position than non hydroxy ceramide because the extra hydroxy group on the hydroxy ceramide interacts with the hydroxy groups of the silica plate thus slowing down its movement up the TLC plate. This is seen in Figure 25, plate I, and they served as experimental controls.

The hydroxy and non hydroxy ceramides, are not susceptible to breakdown upon heating at 85°C over 24 hours (Figure 25, plate I, lanes 3 and 4).

Similarly, suspending 50 µl of 5 mg/ml ceramides in 50 µl of 0.10 M HNO₃ and incubating the mixture at 85°C for 24 hours was also not able to cause the ceramides to degrade (Figure 25, plate I, lanes 5 and 6). The method is outlined in detail in the general method section in Protocol 3. This result indicated the robust nature of the ceramide and also suggested that this concentration of HNO₃ was ideal to use in glycolipid degradation experiments as the ceramide is literally unaffected by the acid.

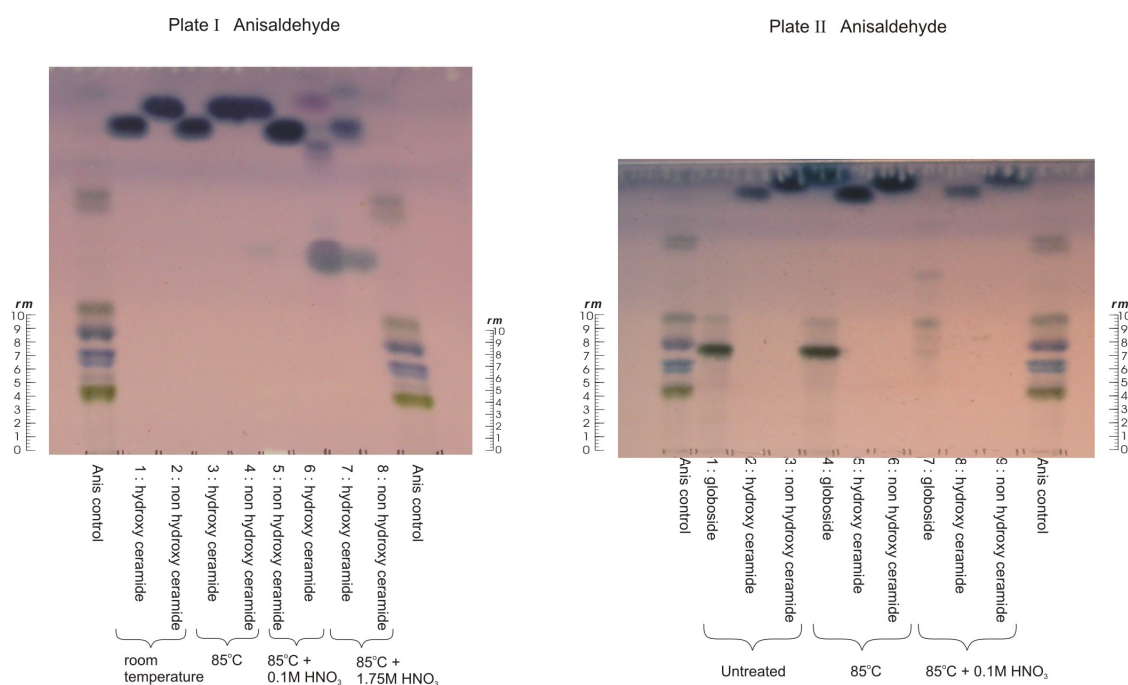


Figure 25: Effects of temperature and dilute/concentrated HNO₃ on ceramides and G-4-4. The ceramides tested included both hydroxyl and non-hydroxy types. The ceramide / glycolipid mixtures were incubated at 85°C over 24 hours.

In contrast, suspending 50 µl of each of the ceramides with 50 µl of 1.75 M HNO₃ caused them to degrade when incubated at 85°C for 24 hours. This was evident by the presence of intense blue bands in the middle section of the TLC plates and as well as other weaker bands present towards the top section of the TLC plate (Figure 25, plate I, lanes 7 and 8). These bands were absent when G-4-4 underwent degradation in all earlier work with the acid and metallic salt solutions indicating that the ceramide entity of the glycolipid (G-4-4) was not degraded by these elements. In other words, the acid cleaved off the sugars from the glycolipid molecule without affecting the sphingolipid tail. This removal of sugars

occurred in an ordered sequential manner with the terminal sugar most likely to be removed first followed by the sugar residue beside it. Once the sugars were removed, the acid then may most likely commence the breakdown of the ceramide tail (that appear as intense blue bands upon staining with anisaldehyde) but this was not seen when G-4-4 was treated with concentrated acid.

The TLC plate in Figure 25, plate II, included the G-4-4 structure for comparison with the ceramide structures. The experiment in this section was set up to test the robustness of the G-4-4 molecule in an acidic environment at 85°C and compare with that of the hydroxy and non hydroxy molecules treated in the exact same manner.

The method is outlined in Protocol 3, v 1.

The three samples tested migrated to their respective positions when left at room temperature (Figure 25, plate II, lanes 1, 2 and 3). These structures also seemed to be stable when subject to 85°C heat for 24 hours (Figure 25, plate II, lanes 4, 5 and 6).

The control experiment consisting of ceramides suspended with acid at room temperature was not included as it was known from previous experiments that nil degradation was expected.

However, G-4-4 degraded upon incubation with 0.1 M HNO₃ at 85°C over 24 hours as the distinctive G-4-4 band at *rm* 7.0 ± 0.5 was absent and another more faint band was present at *rm* 10.0 ± 0.5. (Figure 25, plate II, lane 7). Moreover, there were two weak but distinctive bands present in the 3-sugar glycolipid region and the 2-sugar glycolipid region which are the degradation products of the G-4-4 molecule. It is important to note that the blue bands observed with ceramide degradation in Figure 25, plate I, lanes 7 and 8 was absent when G-4-4 was degraded by acid. This observation strongly suggested that the lipid tail of the G-4-4 was not degraded by incubating with 0.10 M HNO₃ at 85°C over 24 hours.

The hydroxy and non hydroxy ceramides were not affected by incubation with 0.10 M HNO₃ at 85°C over 24 hours (Figure 25, plate II, lanes 8 and 9). This is an important aspect of the project as it confirms that the ceramide entity of glycolipids were not degraded when the former was treated with HNO₃ concentration lower than and equal to 0.1 M.

0.20 Molar and 0.50 Molar HNO₃ versus hydroxy and non-hydroxy ceramides over 24 hours

This experiment was carried out to determine the stability of the hydroxy and non hydroxy ceramides in different HNO₃ concentrations. The objective was to observe if the ceramides were unstable to acid at concentrations over 0.1 M HNO₃. The method is outlined in Protocol 3, v 2. Briefly, 50 µl of each of the ceramides was incubated with 50 µl of 0.20 M and 0.50 M HNO₃ at 85°C for 24 hours. Both hydroxy and non-hydroxy ceramides degraded at both concentrations of HNO₃ tested (Figure 26, plate I, lanes 5-8). Degradation was shown by the presence of blue bands similar to those seen in Figure 25. There was no degradation observed when ceramides were incubated with heat only (Figure 26, plate I, lanes 3 and 4).

There were no controls containing the ceramides/globoside (G-4-4) with acid set at room temperature as it was known from previous experiments that degradation will not occur. Refer to Figure 23, plate I, lanes 7, 14, 21 and 28. Also refer to Figure 27, plate I, lanes 1-5.

Plate I Anisaldehyde

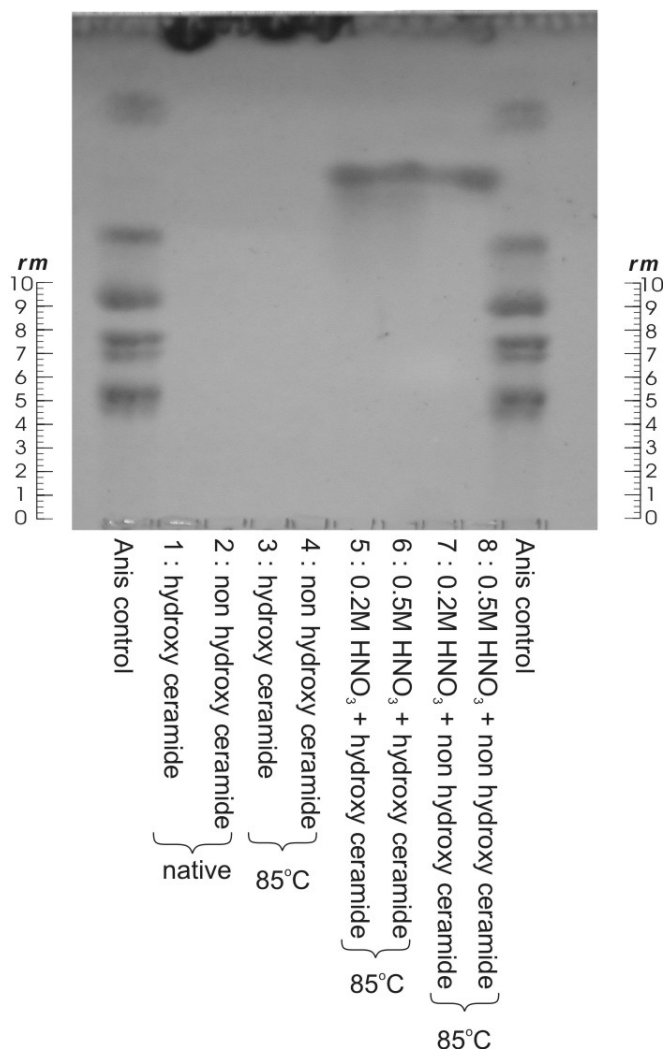


Figure 26: Effects of 0.2 M and 0.5 M HNO_3 on G-4-4.
The G-4-4 and acid mixtures were incubated at 85°C over 24 hours.

Summary

The results are summarised in Table 23.

Table 23: Effects of varying HNO_3 concentrations on ceramides and G-4-4. Both hydroxy and non-hydroxy ceramides were tested at 85°C over 24 hours.

Variables	Globoside (G-4-4)	Hydroxy ceramide	Non hydroxy ceramide
Room temperature- 20°C	-	û	û
Heat- 85°C	-	û	û
0.1 M HNO_3	++++	û	û
0.20 M HNO_3	+++++	ü	ü
0.50 M HNO_3	+++++	ü	ü
1.75 M HNO_3	+++++	ü	ü

Scoring system as per Protocol 11.
 Colour code for ceramide degradation
 1. ü = ceramide degradation
 2. û = no ceramide degradation

The data in Table 23 shows that the G-4-4 structure is labile to the acid conditions stated and degraded significantly upon incubation at 85°C . The hydroxy and non hydroxy ceramides are also degraded by 1.75, 0.5 and 0.2 M HNO_3 but are quite stable when treated with 0.1 M HNO_3 . This data was useful in the sense that it gave a safe cut-off concentration of HNO_3 for carrying out degradation reactions where the sphingolipid tail of the glycolipid will not be damaged i.e. future experiments involving the degradation of Lewis blood group glycolipids by acid will employ HNO_3 concentrations less than or equal to 0.1 M. The untreated ceramides and G-4-4 did not undergo any degradation when incubated at 85°C .

Another important conclusion that can be drawn from the results was that the sugar residues may make a glycolipid structure susceptible to acid. The polar hydroxy groups present on the sugar rings may make the glycolipid structure more vulnerable to attack compared to a ceramide structure which has minimal hydroxy groups. Both heat and acid are required for degradation. In this way, degradation or hydrolysis of glycosidic bonds proceed from the glycan end of a glycolipid (it can be said that the glycan chain 'protects' the ceramide entity from degradation).

Part VII

Validation of optimal temperature for G-4-4 degradation with 0.10 M HNO_3

Prior to Lewis blood group degradation work, the current experiment was set up to re-evaluate the optimal temperature for degradation. The temperature used in previous experiments was 85°C as this concept was developed in an earlier research project (Gilliver, 2002). 240 μl of 0.02 M globoside (G-4-4) solution was suspended with an appropriate amount of 0.10 M HNO_3 so that the pH of the glycolipid/acid mixture would be approximately 1.5 to 2.0. This mixture was then incubated at various temperatures ranging from 20°C, 40°C, 60°C and 85°C and over periods of time ranging from 2, 4, 8, 24 and 48 hours. The method is outlined in Protocol 2, v 4.

There was no degradation observed when G-4-4 was suspended with HNO_3 at 20°C and 40°C. This is evident by the absence of additional bands apart from the distinctive G-4-4 band at $rm\ 7.0 \pm 0.5$ and a weaker band at $rm\ 9.0 \pm 0.5$ which represents the 3-sugar glycolipid molecule (Figure 27, plate I, lanes 1-10).

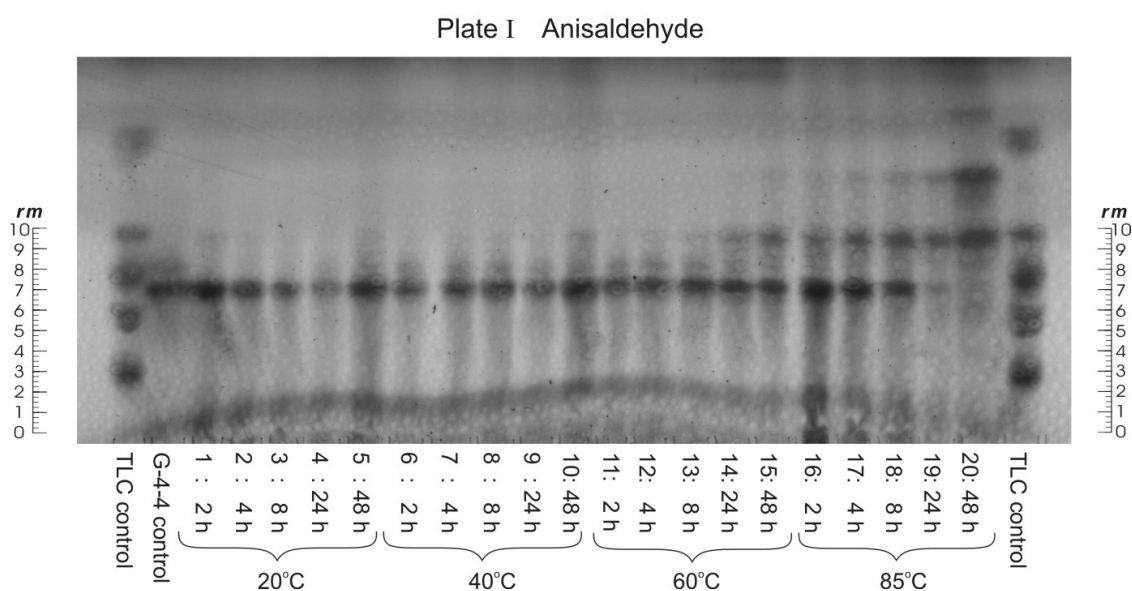


Figure 27: Effects of various temperatures upon treatment of G-4-4 with 0.10 M HNO_3 . Incubations were carried out over 48 hours.

However, degradation of G-4-4 molecule was observed when it was incubated at 60°C and 85°C as shown by the presence of additional bands in the 2- and 1-sugar glycolipid regions on the TLC plate (Figure 27, plate I, lanes 15-20).

Mild degradation at 60°C was noticeable at around 24 and 48 hours of incubation (Figure 27, plate I, lanes 14 and 15). The 3-sugar glycolipid band at $rm\ 9.0 \pm 0.5$ was significantly darker at these given times compared to the band in the G-4-4 control lane. In addition, there was a very weak band present in the region of the 2-sugar glycolipid molecule at 48 hours.

At 85°C, there was significant degradation of the G-4-4 molecule. Degradation was observable from as early as the 2nd hour of incubation (Figure 27, plate I, lane 16). There was an additional band in the 2-sugar glycolipid region on the TLC plate which darkened over time i.e. 48 hours. The G-4-4 band had virtually disappeared at around 24 hours of incubation (Figure 27, plate I, lane 19). At 48 hours, the 3- and 2-sugar glycolipid bands were the most prominent and there was also a reasonably significant band in the 1-sugar glycolipid region (Figure 27, plate I, lane 20). (The 1-sugar glycolipid band had a higher TLC mobility than the standard molecule as it is likely that the ceramide tail or the sugar residue may have undergone some chemical modification by acid hydrolysis. This would have resulted in a structure with a slightly different polarity and resulted in the structure migrating slightly differently on the TLC plate).

The results are summarised in Table 24.

Summary

Table 24: Degradative effects of 0.10 M HNO₃ on G-4-4 at varying temperatures. Incubations time was 48 hours.

Temperature (°C)	Degradation Score
20	-
40	-
60	++
85	++++
Scoring system as per Protocol 11	

The degradation results obtained by incubating G-4-4 with 0.10 M HNO_3 at varying temperatures over 48 hours shows that the most efficient degradation over 48 hours was achieved at 85°C with 'clean' bands appearing in the 3-,2- and 1-sugar glycolipid region over time. In contrast, the G-4-4 molecule was not readily degraded by 0.10 M HNO_3 at 20°C, 40°C and 60°C. The degradation patterns achieved at 85°C gave excellent results and this temperature was therefore estimated to be the practical upper limit for incubation. This experiment, hence, revalidated the use of 85°C as the optimal temperature for degradation experiments.

Part VIII

Reinvestigation of catalytic effect

0.005 M Fe³⁺ and 0.005 M Cu²⁺ vs. G-4-4 at 80°C over 28 days

The journal article by (Bicchierri et al., 1996) investigated the effects of acid hydrolysis and oxidation on cellulose. Cellulose is a polymer consisting of repeating units of glucose residues. These glucose residues are joined to each other by β 1-4 glycosidic bonds. They proposed that cellulose degradation is induced by the metallic cations, Fe³⁺ and Cu²⁺ by a free radical mechanism where the metal cations catalyse either the hydrolysis of glycosidic bonds in the glycan chain or oxidation of the gluco-pyranose rings.

This experiment involving the metal ions Fe³⁺ and Cu²⁺ was repeated with the G-4-4 molecule as outlined in Protocol 13. The ratio of the metal ion solution to G-4-4 was 1 ml/10 mg, the same ratio used by (Bicchierri et al., 1996). The pH of these solutions were recorded using Whatman's pH paper and the solutions were then placed in an 80°C heating block and incubated for varying periods of time ranging from 7 hours to 28 days (Figure 28 and Figure 29). Degradation of G-4-4 was noticed after 16 days of incubation with the presence of notable/darker 3-sugar glycolipid bands as seen in Figure 29. Upon closer examination, the 3-sugar glycolipid bands were prominent only when G-4-4 was treated with FeCl₃, CuCl₂ and Cu(NO₃)₃ and as well as Fe₂O₃ (an insoluble oxide) to some extent (Figure 29, plate II, lanes 2, 4, 6, 7, 9, 11, 14). Further, an additional band, though very weak, appeared in the 2-sugar glycolipid region after 16 and 28 days of incubation with the metal ions stated above (Figure 29, plate II, lanes 2, 4, 9, 11 and 14). The pH of each of the degrading media was between 6.0 and 6.5.

At the same time, degradation was not observed when G-4-4 was treated in the absence of metal ions (Figure 29, plate II, lanes 3, 5, 10, 12). This observation led to the conclusion that the metal ions are most likely involved in catalysing the cleavage of the glycosidic bonds. However, hydrolysis of glycosidic bonds by metal ions does not occur readily i.e. metal ions contribute towards the long term degradation of G-4-4.

Overall, the 4-sugar G-4-4 band at *rm* 7.0 \pm 0.5 remained consistent throughout the incubation period indicating that G-4-4 is a relatively robust molecule and underwent minimal degradation/hydrolysis under the influence of Fe³⁺ and Cu²⁺ ions to form the 3- and possibly the 2-sugar glycolipid structures.

Plate I Anisaldehyde

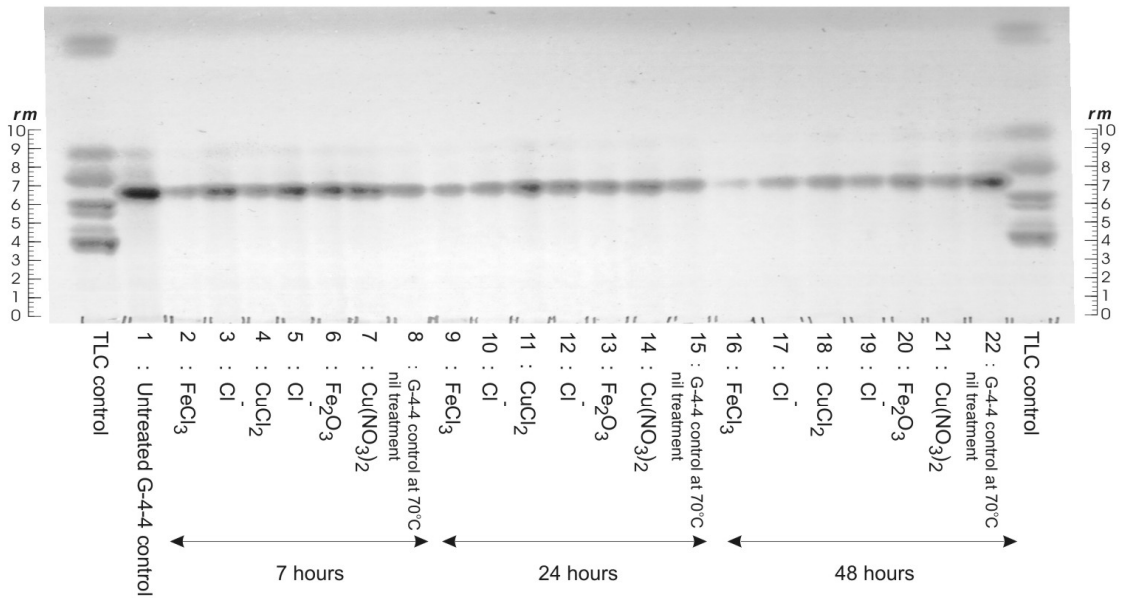


Plate II Anisaldehyde

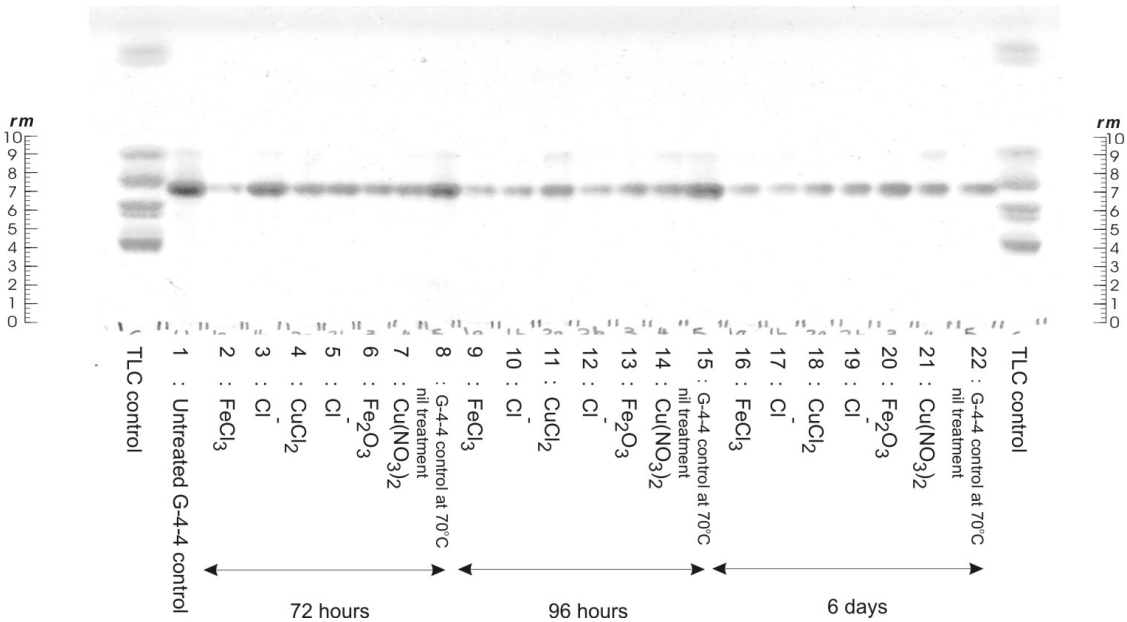


Figure 28: The influence of various salt solutions on G-4-4 over 6 days at 80°C.

Plate I Anisaldehyde

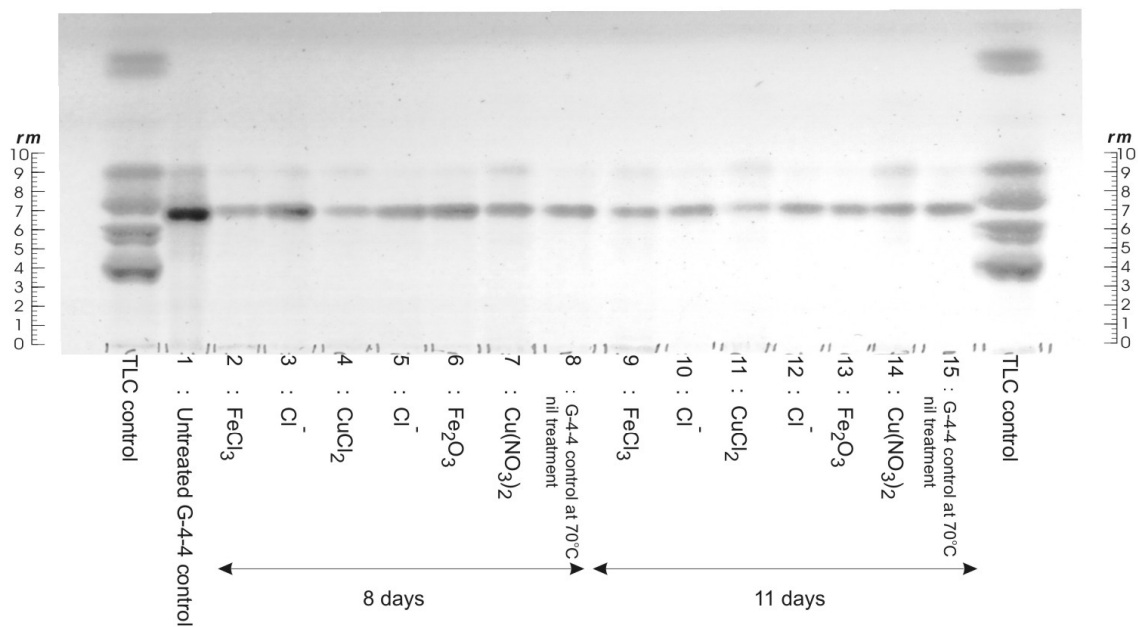


Plate II Anisaldehyde

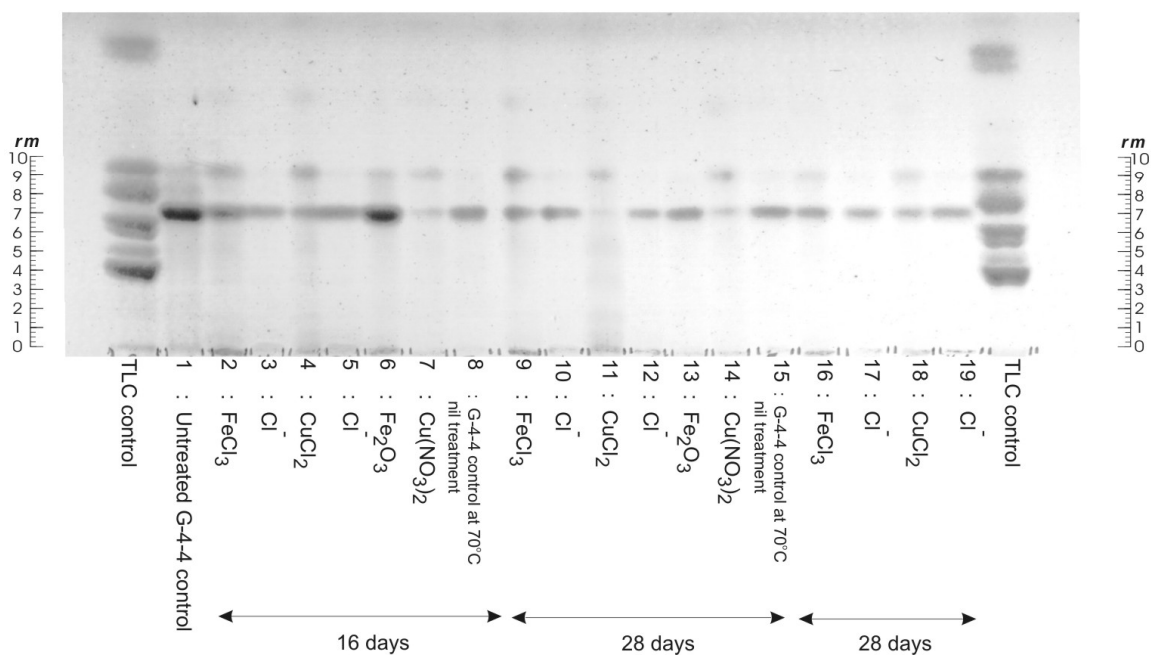


Figure 29: The influence of various salt solutions on G-4-4 between 8 days to 28 days at 80°C.

Summary

Metal cations may have long term degradative effects on glycolipids. The results suggested that Fe^{3+} and Cu^{2+} ions were able to induce mild degradation of the G-4-4 molecule over a long period of time. Degradation, however, was fairly mild with the 3-sugar band at $rm\ 9.0 \pm 0.5$ and another extremely weak band in the 2-sugar glycolipid region off the rm scale. Chloride salts of Fe^{3+} and Cu^{2+} were used.

The experiment conducted by (Bicchieri et al., 1996) was almost exactly reproduced with the G-4-4 replacing the cellulose structure. They used chloride salts of Fe^{3+} and Cu^{2+} . On a short-term basis, the metal ions mentioned above were not seen to have any involvement in the breakdown of the G-4-4 structure. However, on a long term basis, mild degradation of G-4-4 was observed. The metal ions seemed to be catalysing the hydrolysis of glycosidic bonds possibly by mechanisms proposed by Bicchieri and Shahani (Bicchieri et al., 1996; Shahani et al., 1986).

It is also possible that the degrading medium may have changed its pH, possibly increasing the acidity which contributed towards degradation. The chloride ions in solution may have contributed towards the acidity of the incubation mixture thereby inducing degradation. The metal ions also had some contribution towards the catalysis of the degradation reaction.

On the other hand, there could have been a combination effect of both a lower pH and the metal ions catalysing the degradation of the G-4-4 molecule. This is the case with paper degradation where transition metal ions are absorbed by cellulose during paper manufacture. These metal ions, in the presence of oxygen/moisture can catalyse the homolytic decomposition of the cellulose peroxide molecules as reported by Shahani (Shahani et al., 1986).

3 Results Section B - Lewis Blood Group Glycolipids

Acid Hydrolysis of Lewis b (Le^b) glycolipids

The degradative effects caused by acid hydrolysis on the Lewis blood group structures were investigated once the relationship between hydrolysis/degradation and pH was established with the more readily available globoside (G-4-4) molecule.

It is proposed that the Lewis structures such the Lewis b (Le^b), a six-sugar glycolipid with the structure $(\text{Gal}(\text{Fuc}\alpha 1-2)\beta 1-3\text{GlcNAc}(\text{Fuc}\alpha 1-4)\beta 1-3\text{Gal}\beta 1-4\text{Glc}\beta 1-1\text{Cer})$ can be broken down into other molecules such as Lewis a (Le^a), Lewis d (Le^d) or Lewis c (Le^c) structures by controlled acid hydrolysis processes similar to those employed for G-4-4 degradation that resulted in the sequential removal of sugars from the non reducing end of the glycan chains of glycolipids.

It is known that glycosidic bonds involving de-oxy sugars are readily cleaved in acidic conditions. Hence the Le^b was expected to lose either its outer fucose ($\alpha 1-2$ glycosidic bond) residue to form the Le^a structure or its inner fucosyl ($\alpha 1-4$ glycosidic bond) residue to form the Le^d structure. In addition, both fucose residues on the Le^b structure can be lost to form the Le^c moiety as shown in Figure 30.

According to the literature, the increasing order of stability of glycosidic bonds of aldohexoses in an acid medium are $\alpha\text{-D-(1-2)}$, $\alpha\text{-D-(1-3)}$, $\alpha\text{-D-(1-4)}$, $\beta\text{-D-(1-2)}$, $\beta\text{-D-(1-3)}$, $\beta\text{-D-(1-4)}$, $\alpha\text{-D-(1-6)}$ and $\beta\text{-D-(1-6)}$ (Shallenberger, 1982; Wolfrom et al., 1963). This property can be extrapolated to the glycosidic bonds involving de-oxy aldohexoses. Hence the conversion of Le^b into the Le^a structure should occur more readily than into the H-type 1 (Le^d) glycolipid in an acidic medium. In addition, the $\text{Fuc}\alpha 1-2$ bond can also be cleaved more readily due to its relatively more 'exposed' position on the Le^b molecule which makes it more susceptible to cleavage. On the other hand, the $\text{Fuc}\alpha 1-4$ bond could be less vulnerable to such cleavage mechanisms due to its internal and more 'protected' location within the glycolipid structure.

The Le^c glycolipid is not as readily available as other Lewis glycolipids and is only present in non-secretor individuals with the Le(a-b-) phenotype. These individuals make up less than 2% of the general Caucasian population (Table 3).

The hypothesised pathway for Le^b hydrolysis is shown in Figure 30.

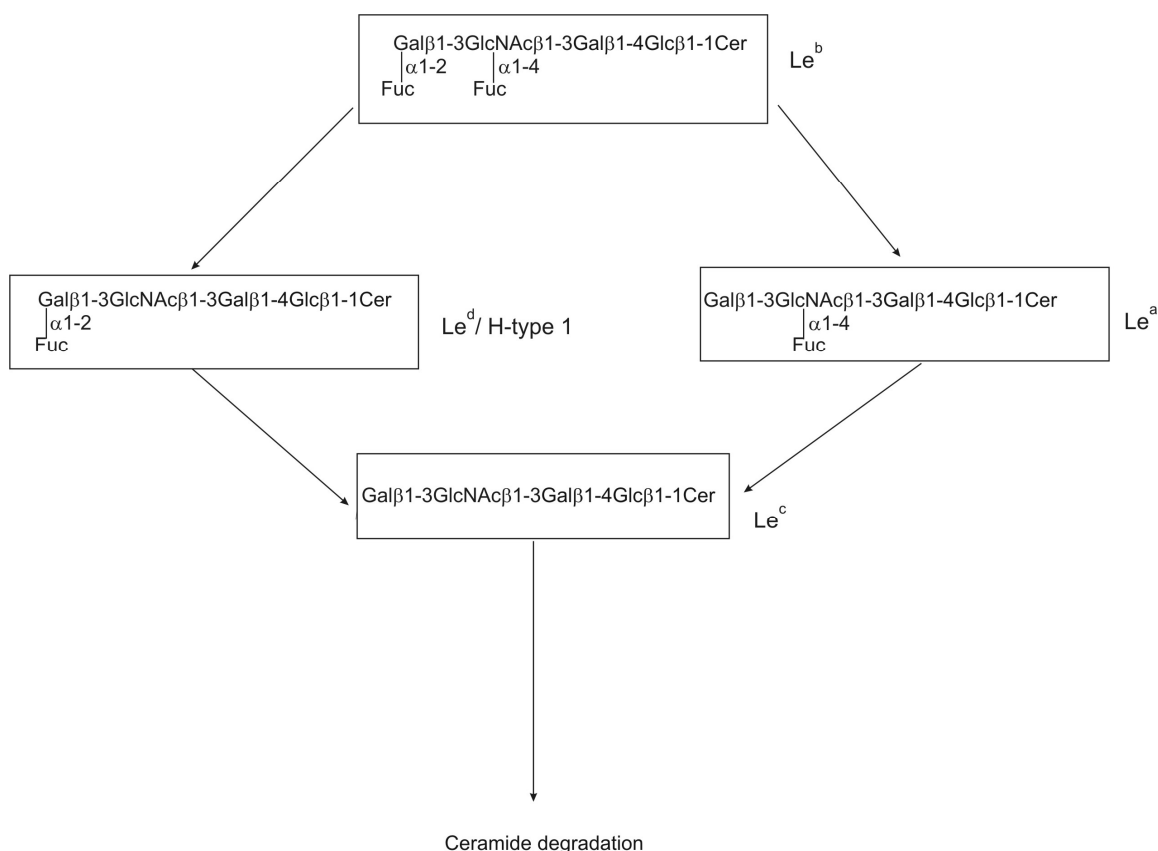


Figure 30: Flow diagram showing the possible degradation pathways of the Le^b molecule.

This section investigated the effects of acid hydrolysis and methanolysis on the Lewis b (Le^b) molecule with varying concentrations of HNO_3 over periods of time at $85^\circ C$. The main objective of the experiments was to observe the breakdown pathways. Anisaldehyde staining and immunostaining assays were the main techniques used to identify new and existing structures. Anisaldehyde stains provide quantitative data while immunostain assays generate more accurate information on the new and existing oligosaccharide sequences (antigenic determinants) formed from the acid hydrolysis of the Le^b glycolipids. The ability of the new antigens (formed upon hydrolysis of the Le^b molecule) to migrate to identical positions as the naturally occurring equivalent structures is further proof that unlike the glycan entity of a glycolipid, the ceramide moiety is not affected by the acid.

Any chemical modifications of the ceramide tail moiety by acid would be expected to change the overall polarity of the entire glycolipid molecule thereby causing differences in migratory positions on the TLC plate.

The migrations of some standard antigenic glycolipids were measured against a relative migration (*rm*) scale developed in-house and are as shown in Table 25.

Table 25: Relative migrations (*rm*) of some glycolipids. These were measured against a scale developed in-house.

Glycolipid	Number of sugar residues	Migration based the <i>rm</i> scale (± 0.5)
ALe ^b	7	3.5
Le ^b	6	4.25
A -6-1	6	5.0
Le ^a	5	5.25
Le ^d / H-type 1	5	5.5
Le ^c / type 1 precursor	4	6.75
Globoside	4	7.0
3-sugar glycolipid	3	9.0
2-sugar glycolipid	2	Off scale
1-sugar glycolipid	1	Off scale

Anisaldehyde staining was carried out as outlined in Protocol 12 while the procedure for immunostains assays is outlined in Protocol 14. The antibodies employed were; anti-Le^{ac}, anti-Le^{ab}, anti-Le^bH, anti-Le^b and anti-Le^d (anti-H type 1).

The terms 'degradation' and 'hydrolysis' are used interchangeably in this section and refers to the hydrolysis of the glycosidic bonds in the glycan chain of the Le^b molecule unless otherwise specified.

Le^b versus 0.10 M HNO₃

0.5 mg of Le^b sample was treated with 100 μ l of 0.10 M nitric acid (HNO₃) and incubated at 85°C for periods of time ranging from 1 hour to 48 hours as outlined in Protocol 7, v 1. The main objective of this experiment was to observe the degradative effects of 0.10 M HNO₃ on the Le^b molecule over time and to observe if new antigenic structures were formed over a 48 hour period. The results are shown in Figure 31.

The anisaldehyde stain in plate I showed that the Le^b sample underwent extensive degradation (indicated by multiple bands present) upon treatment with 0.10 M HNO₃ and the immunostains revealed that the precursor and as well as Le^a glycolipid molecules were generated during certain time intervals. Native Le^b migrates to an *rm* position of 4.25 ± 0.5 as a single band.

Plate II, Figure 31, is an immunostain of Le^a and Le^c controls against the anti-Le^{ac} reagent.

The native Le^b molecule gave an overall negative staining with the anti- Le^{ac} reagent (Figure 31, plate III, lane 8). Due to its extremely sensitive nature, the anti- Le^{ac} reagent was able to detect the very minute amounts of Le^a present in the untreated sample. This band was not visible in the anisaldehyde stain (Figure 31, plate I, lane 8) and as stated earlier, this form of staining provides quantitative data therefore Le^a was present only in negligible amounts.

In contrast, the various anti- Le^b reagents gave a strong positive result with the control Le^b sample (Figure 31, plate IV, V and VI, lane 8).

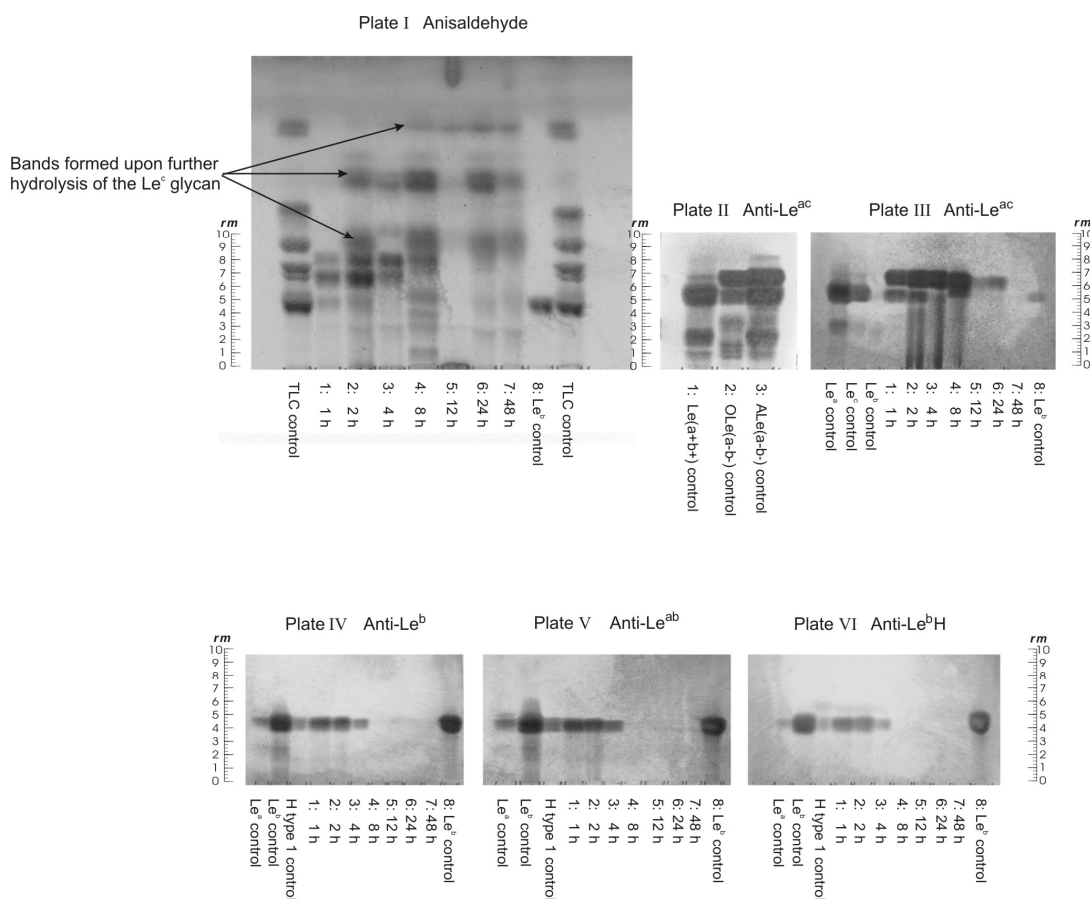


Figure 31: Effects of acid hydrolysis on Le^b upon treatment with 0.10 M HNO_3 . Incubation was carried out at 85°C over a period of 48 hours. Multiple bands shown in plate I are due to extensive degradation of the Le^c glycolipid. Plate I is an anisaldehyde stain and plates II – VI are immunostains with various antibodies. Note that the Le^c control had trace amounts of Le^a (plates II and III), and H-type 1 and Le^a controls contained Le^b molecules (plates IV, V and VI).

1 hour

The Le^b molecule had clearly degraded in the very 1st hour of incubation as shown in Figure 31, plate I, lane 1). The immunostain with the anti-Le^{ac} reagent showed the presence of both Le^a and Le^c structures at $rm\ 5.25 \pm 0.5$ and 6.75 ± 0.5 respectively (Figure 31, plate III, lane 1). These bands were not present in the original Le^b sample before degradation and hence were products of Le^b hydrolysis.

Plates VI, V and VI all showed positive staining of the Le^b band at $rm\ 4.25 \pm 0.5$ with the anti-Le^b, anti-Le^{ab} and anti-Le^{bH} reagents respectively (Figure 31, lane 1).

2 hours

At 2 hours, the band at $rm\ 6.75 \pm 0.5$ became more intense together with the band present at $rm\ 8.0 \pm 0.5$ in the anisaldehyde stain. An additional green diffuse band was present at $rm\ 9.0 \pm 0.5$ and another off the rm scale. These bands most likely represent the 3-sugar and 2-sugar glycolipids formed from further hydrolysis of the Le^c structure (Figure 31, plate I, lane 2).

The same degradation pattern with the anti-Le^{ac} reagent was observed as described in the 1st hour of degradation except that some streaking from the origin was observed. The streaking could be a significant indicator of the presence of Le^c as it is poorly soluble in solvents and gets partly deposited on the lanes as it migrates on the TLC plates. This pattern of streaking by Le^c glycolipids was also observed by S.Henry (S. Henry, personal communication).

Positive staining in the Le^b region ($rm\ 4.25 \pm 0.5$) was observed with the various anti-Le^b reagents tested as shown in Figure 31, plates VI, V and VI, lane 2.

4 hours

The anisaldehyde staining at the 4th hour of incubation showed two intense black bands in the region of $rm\ 6.75 \pm 0.5$ and $rm\ 8.0 \pm 0.5$. The diffuse green bands noticed at 2 hours of incubation were absent but there were other bands present off the rm scale.

Immunostaining with the anti-Le^{ac} reagent gave positive staining in the Le^c ($rm\ 6.75 \pm 0.5$) region together with distinctive streaking on the TLC plate (Figure 31, plate III, lane 3). The Le^a band at $rm\ 5.25 \pm 0.5$ was absent and this observation suggested the Fuc α 1-4 (from the Le^a glycolipid) was readily cleaved to form Le^c.

Again positive staining in the Le^b region, 4.25 ± 0.5 , was observed as shown in Figure 31, plates VI, V and VI. The anisaldehyde showed minimal staining for the 6-sugar glycolipid, Le^b, suggesting that a significant quantity of this glycolipid was degraded.

8 hours

The two main bands present at 8 hours of incubation were two diffuse green bands as seen at 2 hours together with a weak band at $rm\ 5.25 \pm 0.5$ (Figure 31, plate I, lane 4). These were most likely to be the 3- and 2-sugar glycolipids formed from further hydrolysis of the Le^c molecule. A thin band also appeared in the 1-sugar glycolipid region towards the top of the TLC plate.

In contrast there was negative staining observed in the Le^b regions when the degraded samples were tested against various anti- Le^b reagents (Figure 31, plates VI, V and VI, lane 4). At this stage, the entire Le^b sample had degraded.

12, 24 and 48 hours

There were no bands observed at 12 hours in the chemical (anisaldehyde) stain but there were large diffuse bands present at both 24 and 48 hours at $rm\ 9.0 \pm 0.5$ (Figure 31, plate I, lanes 5, 6 and 7). This migration is consistent with that of a 3-sugar glycolipid structure (observed with the hydrolysis of G-4-4 earlier) and could have formed from further hydrolysis of the Le^c (4-sugar glycolipid) structure. Other two bands present off the rm scale and migrating towards the top of the TLC plate could most likely represent the 2- and 1- sugar glycolipid structures, also formed from the further hydrolysis of the 3-sugar glycolipid. These bands are clearly marked in Figure 31.

It is important to note that these bands can not represent the free sugars formed upon hydrolysis as they were removed upon washing with water during the removal of salt impurities.

Immunostain with the anti- Le^{ac} and anti- Le^b reagents gave negative results at 12, 24 and 48 hours (Figure 31, plate III, IV, V and VI, lanes 5, 6 and 7). There were very weak Le^c bands present at 12 and 24 hours and could be termed negligible.

The results of Le^b hydrolysis with 0.1 M HNO_3 at 85°C is summarised in Table 26.

Summary

Table 26: Lewis antigens formed upon hydrolysis of Le^b molecule with 0.10 M HNO₃.

Incubation was carried out at 85°C over 48 hours. + and – indicate the presence or absence of specific structures respectively. (-) indicates that although immunostaining is present, it is not due to degradation of the Le^b sample. These symbols are non-quantitative.

Incubation time (hours)	Lewis Structures		
	Le ^b	Le ^{a*}	Le ^c
0	+	(-)	-
1	+	+	+
2	+	+	+
4	+	+	+
8	-	-	+
12	-	-	-
24	-	-	-
48	-	-	-

* Le^b had insignificant amounts of Le^a present and this was regarded as negative staining
This could not be separated by the HPLC separation programme.

The data obtained showed that the Le^b structure underwent extensive degradation and the cleavage of glycosidic bonds by acid seemed to proceed in an orderly fashion. The fucosyl bonds were removed initially, followed by the cleavage of sugar residues from the non reducing end of the remaining Le^b glycan as seen by bands in the region of 3-, 2- and 1-sugar-glycolipid migrations. In addition, the Le^a and Le^c structures seemed to be generated randomly thereby indicating that the fucose residues are bound by extremely acid-labile glycosidic bonds that readily cleave in low pH. The Le^d glycolipid was also a possible product of Le^b hydrolysis. However, a suitable anti-Le^d reagent was not available at the time to test the presence of this antigen. There was an accumulation of the Le^c structure up until 8 hours of incubation which then most likely further degraded into 3-, 2- and 1-sugar glycolipids.

Ceramide degradation was not observed as the distinct blue bands indicating this were absent.

Le^b versus 0.10 M HNO₃ and comparison of hydrolysis with globoside

This experiment was set up to observe the effects of 0.10 M nitric acid (HNO₃) on Le^b over a shorter time range (90 minutes) at 85°C and to compare the degradation patterns obtained with that of the globoside (G-4-4) molecule treated in a similar fashion. 100 µl of HNO₃ was used to degrade 0.5 mg of Le^b according to Protocol 7, v 2.

2 mg of G-4-4 was treated with 200 µl of HNO₃ according to Protocol 2, v 3.

The native Le^b molecule migrated to a position of $rm\ 4.25 \pm 0.5$ on the rm scale as a single band (Figure 32, plate I, lane 1).

From Figure 32, it can be seen that the Le^b structure was readily degraded by acid.

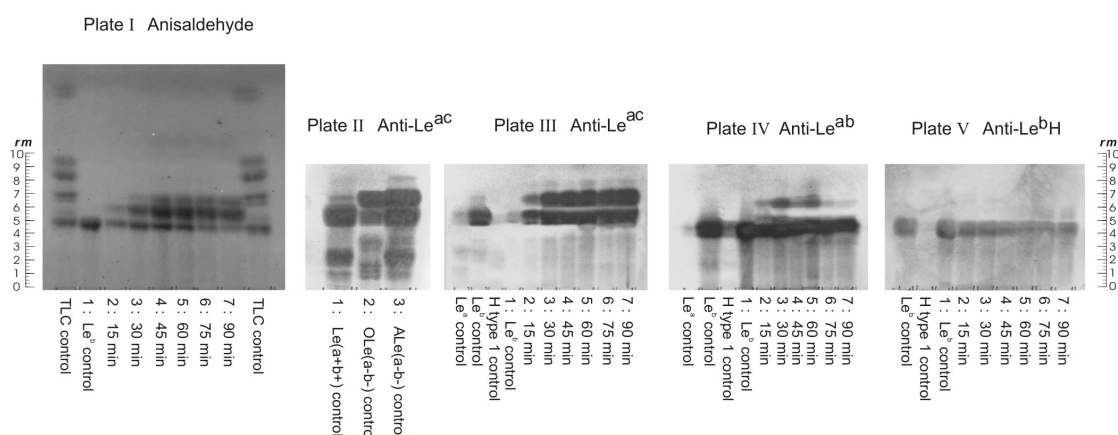


Figure 32: Effects of acid hydrolysis upon treatment of Le^b with 0.10 M HNO₃.

Incubation was carried out at 85°C over 90 minutes.

Plate I is an anisaldehyde stain and plates II – V are immunostains with various antibodies. Note that the Le^c control had trace amounts of Le^a (plates II and III), the Le^b control had some Le^c structures (plate III) and the H-type 1 and Le^a controls contained Le^b molecules (plates IV, and V).

The anisaldehyde stain showed that Le^b had begun degradation within the first 15 minutes of incubation. The Le^b band at $rm\ 4.25 \pm 0.5$ had weakened and an additional band, although weak, appeared at an rm position of 5.25 ± 0.5 which stained positive with the anti-Le^{ac} reagent confirming it was an Le^a band (Figure 32, plate I, lane 2 and plate III, lane 2). It is also important to note that the anisaldehyde staining of the degraded samples showed a clean and orderly cleavage of bonds and as well as the gradual formation of other Lewis structures. This pattern of degradation was not shown in plate I, Figure 31, where longer incubation times were used.

Plate II is an immunostain of Le^a and Le^c controls against the anti-Le^{ac} reagent (Figure 32)

Immunostaining with the anti-Le^{ac} reagent stained positive with both the Le^a and Le^c structures (which migrated to *rm* 5.25 ± 0.5 and 6.75 ± 0.5 respectively) from 15 - 90 minutes suggesting that these products were readily formed upon acid hydrolysis of Le^b (Figure 32, plate III, lanes 2-7).

The various anti-Le^b reagents namely anti-Le^{ab} and anti-Le^bH (specificities described earlier) also gave a positive staining in the Le^b region from 15 – 90 minutes of incubation (Figure 32, plates IV and V, lanes 2-7). This result suggested that the entire Le^b sample was not degraded / hydrolysed by the acid treatment over 90 minutes of incubation.

However, at 75 minutes of incubation, the Le^b band at *rm* 4.25 ± 0.5 had started to visibly diminish as seen in the anisaldehyde stain in Figure 32, plate I, lane 6. There were bands present in the *rm* region of 6.0 ± 0.5 with the anti-Le^{ab} reagent (Figure 32, plate IV). This was an anomaly in the staining pattern with this particular antibody. It could not be further determined what these structures were but are most likely to be the Le^a-like configurations since anti-Le^{ab} can only detect Le^a and Le^b structures (Figure 32, plates IV lanes 2-5).

There was an accumulation of the Le^c structure at 90 minutes suggesting that the Le^c structure could not be readily broken down by the acid. This observation suggested that the Le^c structure was quite stable to acid hydrolysis (0.1 M HNO₃) over an incubation period of 90 minutes (Figure 32, plates I and III).

Globoside versus 0.10 M HNO₃ over 90 minutes

Globoside (G-4-4) was set to incubate in similar conditions as the Le^b glycolipid. The results clearly demonstrated that the G-4-4 molecule was not degraded upon incubation with 0.10 M HNO₃ over 90 minutes (and 85°C) as the G-4-4 band at *rm* 7.0 ± 0.5 remained consistent throughout the incubation period together with the secondary 3-sugar glycolipid band at *rm* 9.0 ± 0.5 (Figure 33, plate I). The difference in their susceptibility to acid hydrolysis is most likely due to the chemical nature of the sugar residues and that of the glycosidic bonds that link them in the glycan chains in both G-4-4 and Le^b.

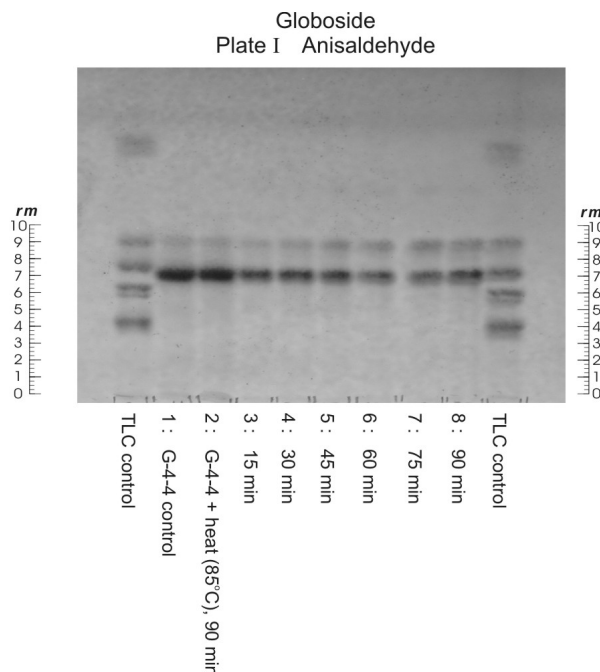


Figure 33: Nil degradative effects of 0.10 M HNO_3 on G-4-4 upon incubation at 85°C .
Incubation time was 90 minutes.

The results of Le^b and G-4-4 degradation upon incubation with 0.1 M HNO_3 over 90 minutes is summarised in Table 27.

Summary

The Le^a and Le^c structures were generated readily upon incubation of Le^b with acid. The Le^b glycolipid contains two fucose residues that occur as side groups. Being de-oxy sugars makes the glycosidic bonds linking them extremely labile to acid and are, as a result, easily cleaved at low pH. This behaviour of the fucose bonds was observed where the Le^a and Le^c structures were readily generated upon incubation with acid.

In addition, the Le^c structure was observed to be quite stable to low pH as further hydrolysis of this 4-sugar glycolipid was not observed over an incubation time of 90 minutes. Bands in the regions of 3-, 2- and 1-sugar glycolipid were absent.

An anti- Le^d reagent (anti-H type 1 reagent) was not available at this time to test the presence of the H-type 1 (Le^d) glycolipid. However, it is possible that Le^d was also a product of Le^b hydrolysis.

Table 27: Lewis antigens/glycolipids formed upon hydrolysis of Le^b and G-4-4. Incubation was carried out with 0.10 M HNO₃ at 85°C over 90 minutes. + and – indicate the presence or absence of specific structures respectively. (-) indicates that although immunostaining is present, it is not due to degradation of the Le^b sample. These symbols are non-quantitative.

Incubation (minutes)	Lewis Structures			Globoside (G-4-4)		
	Le ^b	Le ^{a*}	Le ^c	**3-sugar	2-sugar	1-sugar
0	+	(-)	-	-	-	-
15	+	+	+	-	-	-
30	+	+	+	-	-	-
45	+	+	+	-	-	-
60	+	+	+	-	-	-
75	+	+	+	-	-	-
90	+	+	+	-	-	-

* Le^b had insignificant amounts of Le^a present and this was regarded as negative staining. This could not be separated by the HPLC separation programme.

** G-4-4 sample also contained 3-sugar glycolipids as a natural contaminant. The band intensity of this glycolipid at *rm* 9.0 ± 0.5 did not change throughout the 90 minute incubation period indicating that additional 3-sugar structures were not generated; hence this regarded as negative staining.

On the other hand, the results suggested that G-4-4 is a more robust structure compared to the Le^b glycolipid. Three of the four sugars in a G-4-4 molecule are bound by β linkages (except the sub terminal Gal which is bound by an α glycosidic bond) which, according to literature, are relatively more stable to acidic conditions. This property, together with the absence of de-oxy sugars in G-4-4 makes glycosidic bonds in the latter more stable in low pH.

HPLC separation of degraded Le^b components

The components of the degraded Le^b sample were separated using High Performance Liquid Chromatography (HPLC) as outlined in Protocol 4. The main objective of this separation was to determine if the degraded components (in particular the Le^a and Le^c structures) of Le^b could be separated and purified by the HPLC programme for potential structural analyses.

A small quantity of pure Le^b sample was degraded over 90 minutes with 0.1 M HNO₃ and then neutralised and washed as outlined in Protocol 7.

Figure 34, plate I shows the anisaldehyde staining of the native and degraded Le^b molecule after 90 minutes. There was virtually no Le^b present after 90 minutes as shown by the absence of this band at $rm\ 4.25 \pm 0.5$ (Figure 34, plate I, lane 2).

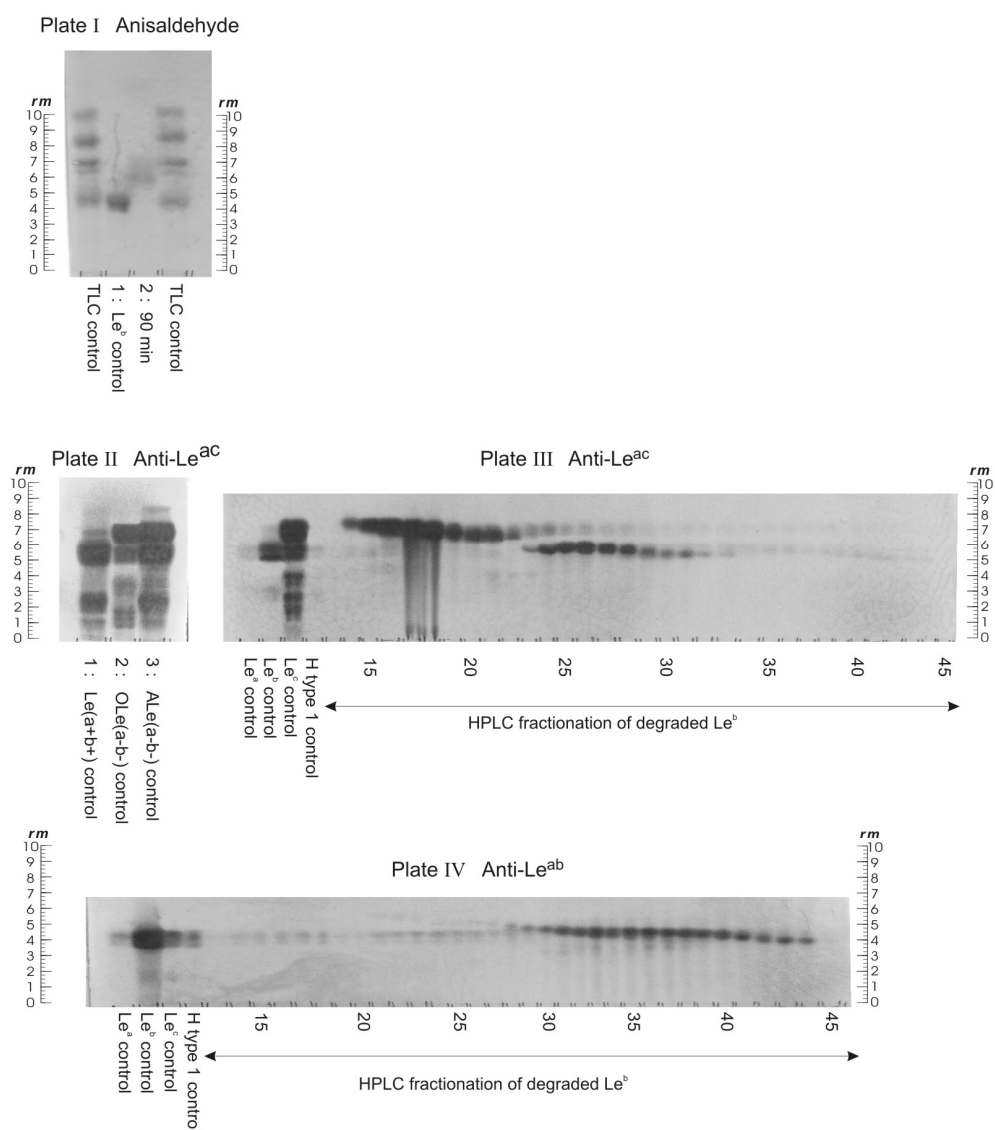


Figure 34: HPLC separation of the products of Le^b degradation with 0.1 M HNO_3 . Plate I is an anisaldehyde stain and plates II – IV are immunostains with various antibodies. Note that the Le^c control had trace amounts of Le^a (plates II and III) and the Le^a , Le^c and H-type 1 controls had trace amounts of Le^b structures (plate IV).

The Le^a and Le^c immunostain controls are shown in Figure 34, plate II against the anti-Le^{ac} reagent. Lane 1 shows a positive stain at $rm\ 5.25 \pm 0.5$ which is the Le^a band while lanes 2 and 3 contain the Le^c band at $rm\ 6.75 \pm 0.5$.

Figure 34, plate III shows immunostained fractions with anti-Le^{ac} antibody. The Le^c molecules were the first to elute through the column as seen from fractions 14 - 22 at $rm\ 6.75 \pm 0.5$. It is imperative to note that there was some streaking on the TLC plate observed in regions where the Le^c structures migrated to their standard position. This is almost certainly due to the known insoluble nature of purified Le^c structures. The degraded products migrated to identical positions as the native Le^a and Le^c glycolipids i.e. 5.25 ± 0.5 and 6.75 ± 0.5 respectively.

Le^a molecules eluted after Le^c as seen in fractions 23 - 32 at 5.25 ± 0.5 . The Le^c molecules, however, continued to co-elute with the Le^a structures but was present in extremely small amounts. These are represented by very weak bands present above the Le^a bands from fractions 25 - 29 on plate II (Figure 34). The original Le^b sample did not contain any Le^c structures and had extremely small amounts of Le^a as shown in the immunostain in Figure 32, plate III, lane 1. The Le^b immunostain control contains Le^a structures and this explains positive staining in the Le^a region in Figure 34, plate II. The Le^c immunostain control contains both Le^a and Le^c structures as shown by positive staining in their respective regions.

The fractions also stained with the anti-Le^{ab} reagent as shown in Figure 34, plate IV, fractions 29 - 45. This observation suggested that the Le^b sample subjected to acid hydrolysis was not completely degraded over the 90 minute incubation period. Fractions 43 - 45, however, were present in the exact region of Le^b migration ($rm\ 4.25 \pm 0.5$) but fractions 29 - 42 were present somewhat in between the Le^a and Le^b region i.e. region between $rm\ 4.25 \pm 0.5$ and $rm\ 5.25 \pm 0.5$. These structures are more likely to be Le^b molecules as anti-Le^{ab} reagent detects the former more strongly than Le^a structures. The Le^c, Le^a and H-type 1 immunostain controls contain very small amounts of Le^b which explains positive staining with each of these controls in the region of $rm\ 4.25 \pm 0.5$.

Summary

The acid hydrolysis of Le^b molecules with 0.10 M HNO₃ over 90 minutes yielded a mixture of the products Le^a, Le^c and possibly Le^d as well which could not be detected due to the unavailability of the anti-Le^d reagent at this stage. The HPLC can be programmed to separate and purify these components as shown in Figure 34.

The rare Le^c molecule can be prepared by the simple (mild) acid hydrolysis methods of the more readily available Lewis structures such as Le^b glycolipids. Le^c in particular is a very valuable molecule and occurs naturally in less than 2% of a given Caucasian population. Acid hydrolysis has proved to be an inexpensive means of obtaining this rare structure readily. To date, Le^c has not been prepared by this process.

Le^b versus varying $[\text{HNO}_3]$ over 90 minutes

The degradative effects of varying nitric acid (HNO_3) concentrations on Le^b were tested to observe if the degradation process can be controlled at 85°C over a 90 minute incubation period. The method is outlined in Protocol 7, v 3. The HNO_3 concentrations tested were 0.06 M, 0.04 M, 0.02 M and 0.005 M. The control for this experiment was Le^b sample without any treatment with acid or heat. It was known from earlier experiments with globoside (G-4-4) sample that degradation (with acid) does not occur at room temperature (Figure 27). For this reason and due to the limited availability of the Le^b sample, a control Le^b sample with acid at room temperature was not set up. Moreover, the degradative effects caused by 0.08 M HNO_3 were not investigated as they were expected to be very similar to that of 0.10 M HNO_3 .

The anisaldehyde stain in Figure 35, plate I gave rise to an expected pattern of degradation with varying HNO_3 concentrations. The most concentrated acid solution i.e. 0.06 M HNO_3 was able to degrade the Le^b molecule much more rapidly than 0.005 M HNO_3 . For example, the Le^b sample, when treated with 0.06 M HNO_3 had completely degraded at 90 minutes compared to nil degradation observed at the same time interval when Le^b was treated with 0.005 M HNO_3 (Figure 35, plate I, lanes 6 & 24).

0.06 M HNO_3

The most significant degradation of the Le^b molecule was observed with 0.06 M HNO_3 . Degradation commenced after 30 minutes of incubation as seen by the presence of an additional band in the region of Le^a migration at $rm\ 5.25 \pm 0.5$. This band together with the Le^b band at $rm\ 4.25 \pm 0.5$ persisted throughout the 90-minute incubation period (Figure 35, plate I, lanes 1-6).

The Le^a , Le^c and Le^d structures were present from 30 -90 minutes (Figure 35, plates III, and IV, lanes 2-6).

Anti- Le^{ab} also stained positive for Le^b throughout the 90 minute incubation indicating that the Le^b glycolipid was not completely consumed when incubated with 0.06 M HNO_3 (Figure 35, plate V, lanes 1-6).

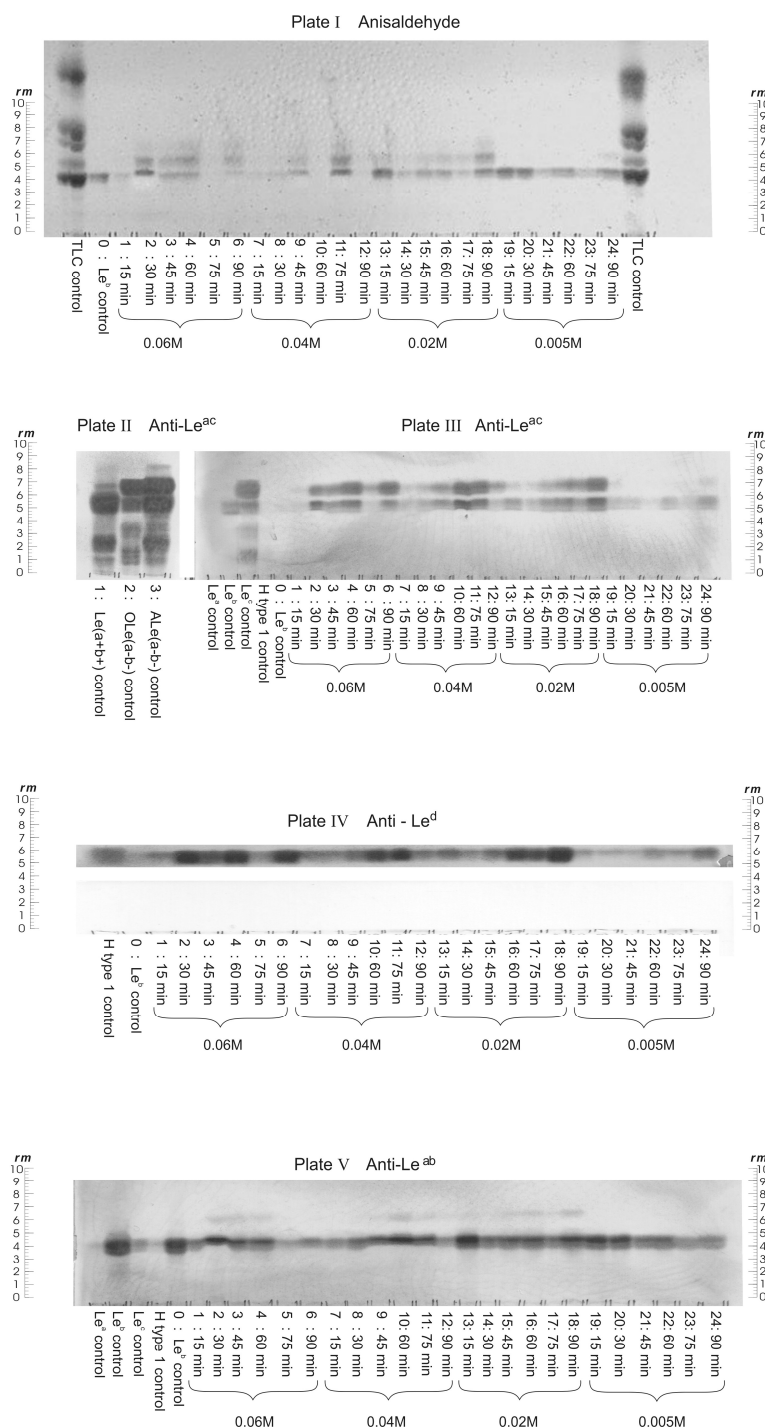


Figure 35: Effects of varying concentrations of HNO_3 on Le^b at 85°C over 90 minutes. Plate I is an anisaldehyde stain and plates II – V are immunostains with various antibodies. NOTE: Due to a limited amount of anti- Le^{d} available, only the strip in the region of Le^{d} migration was stained. The Le^{c} control had trace amounts of Le^{a} (plates II and III) and the Le^{c} and H-type 1 controls had trace amounts of Le^{b} structures (plate V).

0.04 M HNO₃

Degradation of Le^b with 0.04 M HNO₃ was even milder. The Le^b band at $rm\ 4.25 \pm 0.5$ persisted throughout the 90 minute incubation period (Figure 35, plate I, lanes 7-12). A secondary band at $rm\ 5.25 \pm 0.5$ was noticeable at 45 minutes of incubation which correlated to Le^a migration.

The Le^a and Le^c structures were formed after 45 minutes of incubation (Figure 35, plate III, lanes 9-12).

The Le^d or H-type 1 band at $rm\ 5.5 \pm 0.5$, were also observed upon immunostaining with the anti-Le^d reagent (Figure 35, plate IV, lanes 10-11).

0.02 M HNO₃

The mildest form of degradation was seen when Le^b sample was treated with 0.02 M HNO₃. The Le^b band at $rm\ 4.25 \pm 0.5$ was present throughout the incubation period indicating that Le^b did not undergo extensive degradation/hydrolysis. There was a very weak secondary band at $rm\ 5.25 \pm 0.5$ visible from 60 minutes of degradation and became most prominent only at 90 minutes of incubation (Figure 35, plate I, lane 18).

The positive Le^a and Le^c bands were most prominent from 45 minutes of incubation.

The Le^d structures were formed from about 45 minutes of incubation (Figure 35, plate IV, lanes 15-18) while the anti-Le^{ab} reagent stained positive for Le^b samples treated at all time intervals (Figure 35, plate V).

0.005 M HNO₃

Treatment of Le^b with 0.005 M HNO₃ resulted in nil degradation of the former. The Le^b band at $rm\ 4.25 \pm 0.5$ remained consistent throughout the incubation period without the formation of secondary bands (Figure 35, plate I, lanes 19-24).

The results are summarised in Table 28 below.

Summary

The data showed that increasing the concentration of HNO₃ degraded the Le^b glycolipid further into Le^a, Le^d and Le^c. There was no degradation/hydrolysis observed when Le^b was incubated with 0.005 M HNO₃. The Le^c glycolipid seemed to be a relatively robust structure that did not undergo further hydrolysis over the 90 minute incubation period. This was confirmed by the absence of multiple bands in the 3-, 2- and 1-sugar glycolipid regions on the TLC plate.

Table 28: Lewis antigens formed upon hydrolysis of Le^b with varying HNO₃ concentrations. Incubations were carried out at 85°C over 90 minutes. + and – indicate the presence or absence of specific structures respectively. These symbols are non-quantitative.

Time (min)	HNO ₃ Molarity (M)															
	0.06				0.04				0.02				0.005			
	Le ^b	Le ^a	Le ^d	Le ^c	Le ^b	Le ^a	Le ^d	Le ^c	Le ^b	Le ^a	Le ^d	Le ^c	Le ^b	Le ^a	Le ^d	Le ^c
0*	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-
15	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-
30	+	-	+	+	+	-	-	-	+	-	-	-	+	-	-	-
45	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-
60	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
75	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
90	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-

* Le^b had insignificant amounts of Le^a present and this was regarded as negative staining This could not be separated by the HPLC separation programme.

Le^b versus varying [methanolic hydrogen chloride]

Methanolysis of Le^b was also carried out with varying concentrations of anhydrous methanolic hydrogen chloride (HCl) i.e. 0.005 M, 0.02 M, 0.04 M and 0.06 M HCl as outlined in Protocol 9 v 1. Anhydrous methanolic hydrogen chloride was prepared as outlined in Protocol 8. The samples were incubated at 85°C over 40 minutes.

Upon methanolysis, glycosidic linkages are cleaved accompanied by the addition of a methanol molecule across the glycosidic linkage to form methyl glycosides. These methyl glycosides protect the sugar rings from further degradation by acids. In addition, the TLC migration of these methyl glycosides is somewhat similar to the underivatised glycosides as these glycosides are relatively large molecules and changing the functional group on one of the carbon atoms (of the sugar ring) would have minimal effect on the migration of such molecules.

The aim of this experiment was to compare the products generated by acid hydrolysis processes with the products of methanolysis.

0.005 M methanolic HCl

Degradation of Le^b sample with 0.005 M methanolic HCl was noticeable only after 30 minutes of incubation as shown by the presence of an additional band at $rm\ 5.25 \pm 0.5$ (Figure 36, plate I, lane 3). This secondary band at $rm\ 5.25 \pm 0.5$ was most likely due to the Le^a structure and the immunostain with anti-Le^{ac} in plate III confirms this with positive staining in the Le^a region (Figure 36, plate I, lane 3).

The anti-Le^{ac} reagent stained positive for both Le^a and Le^c structures at and after 30 minutes of incubation at $rm\ 5.25 \pm 0.5$ and 6.75 ± 0.5 respectively (Figure 36, plate III, lanes 2-4). Plate II shows the migration and staining of Le^a and Le^c controls with the anti-Le^{ac} reagent.

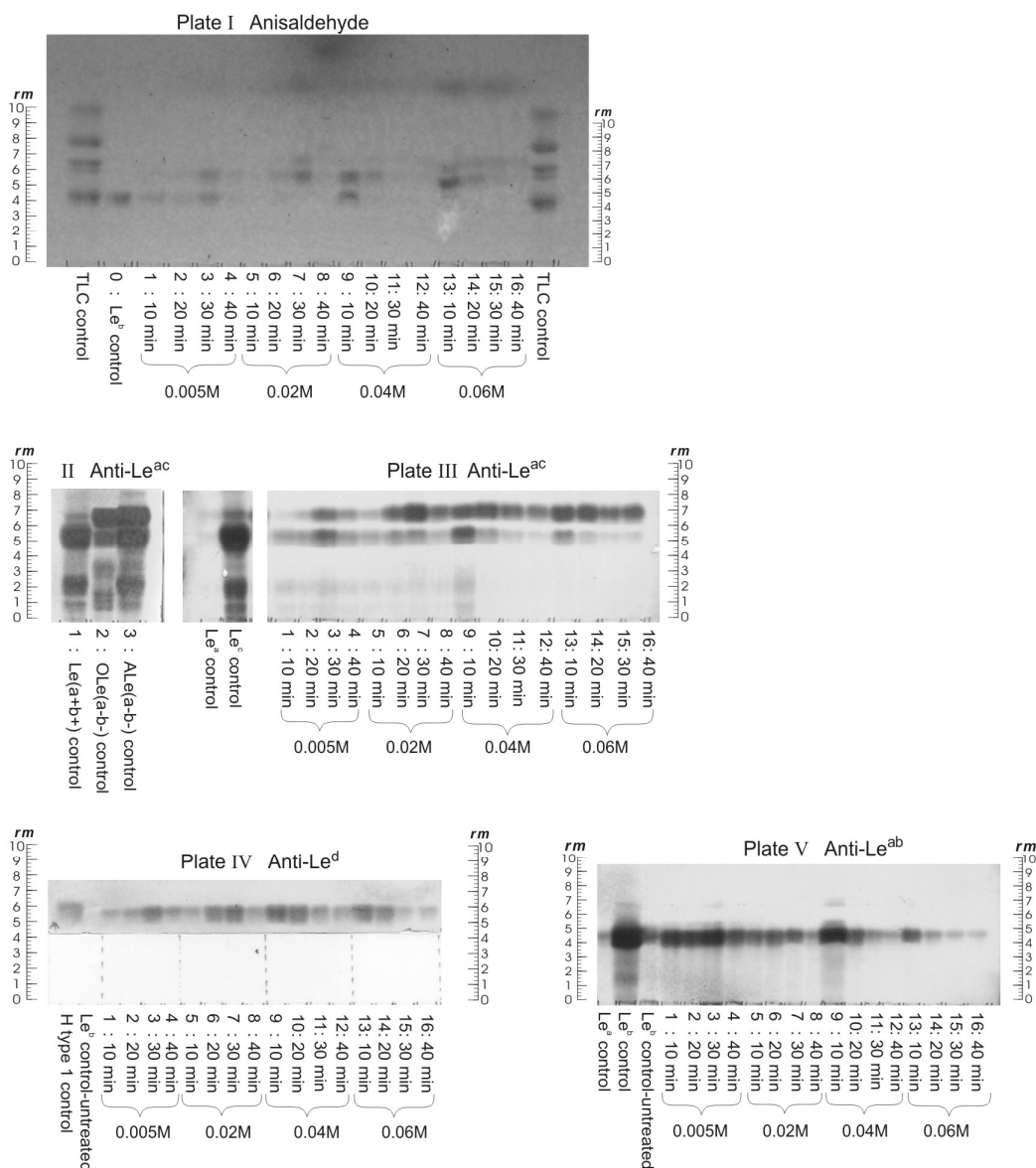


Figure 36: Effects of varying concentrations of methanolic HCl on Le^b. Incubations were carried out at 85°C over 40 minutes. Plate I is an anisaldehyde stain and plates II – V are immunostains with various antibodies. Note that the Le^c control had trace amounts of Le^a (plates II and III) and the Le^a control had trace amounts of Le^b structures (plate V).

The anti-Le^d reagent also stained positive for Le^d structures from 20 minutes (Figure 36, plate IV, lanes 2-4).

The Le^b band at $rm\ 4.25 \pm 0.5$, however, did not weaken suggesting that the rate of degradation of the Le^b molecule was quite slow. The Le^b bands stained significantly with the anti-Le^{ab} reagent at all time intervals suggesting that most of the Le^b molecules in the sample were not completely degraded (Figure 36, plates I & V, lanes 1-4).

In general very mild degradation was seen when Le^b was treated with 0.005 M HCl. The products Le^c and Le^d were produced in such minute quantities that they were not detected by the anisaldehyde staining but were picked up by the sensitive nature of the immunoassays.

0.02 M methanolic HCl

The Le^b molecule was able to degrade upon treatment with 0.02 M methanolic HCl. A secondary band appeared at 20 minutes of incubation at $rm\ 5.25 \pm 0.5$ which upon immunostaining with the anti-Le^{ac} reagent confirmed that it was the Le^a structure. At 30 minutes two bands appeared, one in the Le^a region ($rm\ 5.25 \pm 0.5$) and another at $rm\ 6.75 \pm 0.5$. The latter band was confirmed to be the Le^c structure upon immunostaining with the anti-Le^{ac} reagent (Figure 36, plate III, lanes 6-7). The Le^b band at $rm\ 4.25 \pm 0.5$ was absent at all time intervals indicating that most of the Le^b structure had undergone degradation. At 40 minutes there were no bands visible on the anisaldehyde stained plate (Figure 36, plate I, lane 8).

The anti-Le^{ac} gave a positive result for both Le^a and Le^c structures at 20 and 30 minutes but was positive only for Le^c structure at 40 minutes indicating that the Le^a generated upon hydrolysis was further reduced to the Le^c structure (Figure 36, plate III, lanes 6-8).

The Le^d structure at $rm\ 5.5 \pm 0.5$ stained positive at 20 and 30 minutes of incubation and this band had greatly weakened at 40 minutes suggesting that most of it could most probably be reduced to the Le^c glycolipid as well (Figure 36, plate IV, lanes 6-8).

The Le^b band at $rm\ 4.25 \pm 0.5$ stained consistently over 30 minutes of incubation (Figure 36, plate V, lanes 5-7). At 40 minutes, the Le^b band stained relatively weaker than the others (Figure 36, plate V, lanes 8). This could partly indicate the near complete degradation of all Le^b molecules treated with 0.02 M methanolic HCl and also explains the absence of the Le^b band in plate I, lane 8 (Figure 36, anisaldehyde stain).

0.04 M methanolic HCl

Treatment of the Le^b sample with 0.04 M methanolic HCl was able to cause degradation in the first 10 minutes of incubation evident by the presence of a secondary band at $rm\ 5.25 \pm 0.5$ in the anisaldehyde stain (Figure 36, plate I, lane 9). Immunostain with anti- Le^{ac} reagent confirmed that this band was the Le^a structure and another band, the Le^c band ($rm\ 6.75 \pm 0.5$), was also observed with this immunostain (Figure 36, plate II, lane 9). No further bands were observed at 30 and 40 minutes in the anisaldehyde stained plate indicating that most of the Le^b molecules had degraded. Further, extremely small quantities of Le^a were formed from 20 – 40 minutes upon hydrolysis of Le^b as shown by the immunostain with the anti- Le^{ac} reagent (Figure 36, plate, III, lanes 10-12).

Immunostain with the anti Le^d reagent gave strong bands at $rm\ 5.5 \pm 0.5$ from both 10 to 40 minutes of incubation (Figure 36, plate IV, lanes 9-12).

Staining with the anti- Le^{ab} reagent a strong Le^b band at $rm\ 4.25 \pm 0.5$ was observed in the first 10 minutes of degradation which then slowly became weak over time i.e. 40 minutes (Figure 36, plate V, lanes 9-12).

The anisaldehyde stain did not show bands in the Le^d or the Le^c regions on the TLC plate (at $rm\ 5.5 \pm 0.5$ and $rm\ 6.75 \pm 0.5$ respectively). This observation indicated that these products were made in very low quantities upon Le^b hydrolysis and therefore could not be detected by the quantitative anisaldehyde staining.

0.06 M methanolic HCl

As expected, 0.06 M methanolic HCl was able to degrade Le^b most severely. There were several bands observed at the first 10 minutes of incubation and the absence of the Le^b band at $rm\ 4.25 \pm 0.5$ indicated that most of the Le^b structures had already degraded. There were bands present at $rms\ 5.75 \pm 0.5$, 6.75 ± 0.5 and 5.25 ± 0.5 respectively (Figure 36, plate I, lane 13). The latter two bands were significantly weak.

Immunostain of this degraded Le^b sample (10 minute interval) with the anti- Le^{ac} reagent stained positive with both Le^a and Le^c at $rm\ 5.25 \pm 0.5$ and 6.75 ± 0.5 respectively (Figure 36, plate III, lane 13). Further, at 20 minutes the band at $rm\ 5.25 \pm 0.5$ had significantly weakened and had almost disappeared by 30 minutes (Figure 36, plate I, lanes 14 and 15). This observation is consistent with the immunostain against the anti- Le^{ac} reagent which stained negative for the Le^a band at 5.25 ± 0.5 but positive for the Le^c band at $rm\ 6.75 \pm 0.5$ (Figure 36, plate III, lanes 14 and 15).

The Le^d structure also stained positive at $rm\ 5.5 \pm 0.5$ when the treated Le^b sample was immunostained with the anti-Le^d reagent. The positive staining was especially visible at 10 and 20 minutes of incubation and the bands were rather weak at 30 and 40 minutes of incubation (Figure 36, plate IV, lanes 13-14 and 15-16).

Finally, the anti-Le^{ab} reagent was able to stain the Le^b band (at $rm\ 4.25 \pm 0.5$). However, this staining appeared to be much weaker than when Le^b was treated with less concentrated acids. This weak staining could be due to the glycan chain of the Le^b glycolipid being rapidly hydrolysed by 0.06 M methanolic HCl (Figure 36, plate V, lanes 13-16).

The results are summarised in Table 29.

Summary

The data obtained showed that more Le^c structures were produced from the Le^b degradation with more concentrated methanolic HCl. There were less Le^d structures formed with increased HCl concentrations. This indicated that the terminal fucose residue joined by an alpha (α) glycosidic bond was not very stable to a combination of both heat and acid although this Le^d structure was detectable when Le^b was treated with less concentrated methanolic HCl i.e. 0.005 M. The more concentrated acid would have most probably cleaved the terminal fucose (α 1-2) glycosidic bond from Le^b converting it into Le^a. This fucose is possibly more easily removed than the inner fucose residue bound by an α 1-4 glycosidic bond. Hence converting the Le^b glycolipid into Le^d is more difficult than converting it to the Le^a structure.

Table 29: Lewis antigens formed by methanolysis with varying methanolic HCl concentrations. Methanolysis of Le^b was carried out at 85°C over 40 minutes. + and – indicate the presence or absence of specific structures respectively. These symbols are non-quantitative.

Time (min)	Methanolic HCl Molarity (M)															
	0.005				0.02				0.04				0.06			
	Le ^b	Le ^a	Le ^d	Le ^c	Le ^b	Le ^a	Le ^d	Le ^c	Le ^b	Le ^a	Le ^d	Le ^c	Le ^b	Le ^a	Le ^d	Le ^c
0*	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-
10	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+
20	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+
30	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	+
40	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	+

* Le^b had insignificant amounts of Le^a present and this was regarded as negative staining This could not be separated by the HPLC separation programme.

At 0.005 M and 0.02 M methanolic HCl concentrations, the Le^b degraded more favourably into Le^a than Le^d. This bias in product formation was not observed towards Le^a product formation upon treatment with 0.04 M HCl. Treatment with 0.06 M HCl showed a rapid accumulation of the Le^c sample over time. This suggested that both fucose residues were removed at more or less the same rate resulting converting the Le^b into Le^c. It was also observed that Le^c was much more difficult to degrade once it was formed. One reason for this may be due to the strong beta (β) glycosidic bonds that hold the sugar residues in the Le^c structure.

The products of Le^b methanolysis were formed more rapidly than that observed with mild Le^b hydrolysis. This was due to the addition of a larger volume of acid to the Le^b sample in the methanolysis method. The methanolysis procedure was in a developmental stage and since this method generated identical products (observed with the anisaldehyde and immuno-stains) as those obtained from mild acid hydrolysis there appeared to be no advantage of using methanolysis over acid hydrolysis.

Acid Hydrolysis of A Lewis b (ALe^b) glycolipids

ALe^b versus varying [HNO₃], temperatures and incubation periods

This section investigated the effects of acid hydrolysis on the A Lewis b structure (ALe^b) at various concentrations of nitric acid (HNO₃), temperature and time. ALe^b is a 7-sugar glycolipid; GalNAc α 1-3Gal(Fuc α 1-2) β 1-3GlcNAc(Fuc α 1-4) β 1-3Gal β 1-4Glc β 1-1Cer, and its degradation into variable Lewis antigens by acid hydrolysis methods is expected to be relatively more complex than the Le^b glycolipid. The ALe^b molecule is a Le^b structure with an extra terminal GalNAc residue bound by an alpha (α) bond. Like the Le^b glycolipid, hydrolysis of the ALe^b glycan structure will involve cleavage of non reducing sugar residues bound by alpha (α) glycosidic bonds.

The aim of this set of experiments was to determine if the degradation process can be controlled to obtain a particular antigen of interest by manipulating one or more of the variables; acid concentration, temperature and time of incubation. The degradation experiments were carried out at various temperatures ranging from 50°C - 85°C and with 0.05 M and 0.10 M HNO₃.

The possible degradation pathways for the ALe^b molecule are shown in Figure 37. These pathways are based upon the behaviour of the de-oxy sugar, fucose and GalNAc (N-acetylated amino sugar) residues and as well as the stability of α and β bonds in an acidic environment. These chemical components make up the glycan chain of the ALe^b glycolipid. Literature and our studies of Le^b hydrolysis show that glycosidic bonds involving de-oxy sugars such as fucose are extremely labile in acid while those consisting of N-acetylated sugars are relatively more resistant to cleavage in acidic medium. It is also known that α 1-2 glycosidic bonds are less stable in acid than the α 1-4 bonds. Apart from forming the A-type 1 structure, the ALe^b glycolipid also has the potential to break down into the unnatural glycolipids namely 'GalNAc-Le^a' and 'linear A' by the cleavage of the Fuc α 1-4 or both Fuc α 1-4 and Fuc α 1-2 glycosidic bonds respectively. 'GalNAc-Le^a' being a 6-sugar glycolipid and having an identical molecular mass as the A-type 1 structure is also likely to have identical TLC mobilities as the latter. The, 'linear A' structure being a 5-sugar glycolipid would have a slightly higher migratory position on the TLC plate. However, specific antibodies to these unnatural structures were not available for detection by immunoassays and identification of these structures were mainly based on their relative migratory positions on the TLC plate.

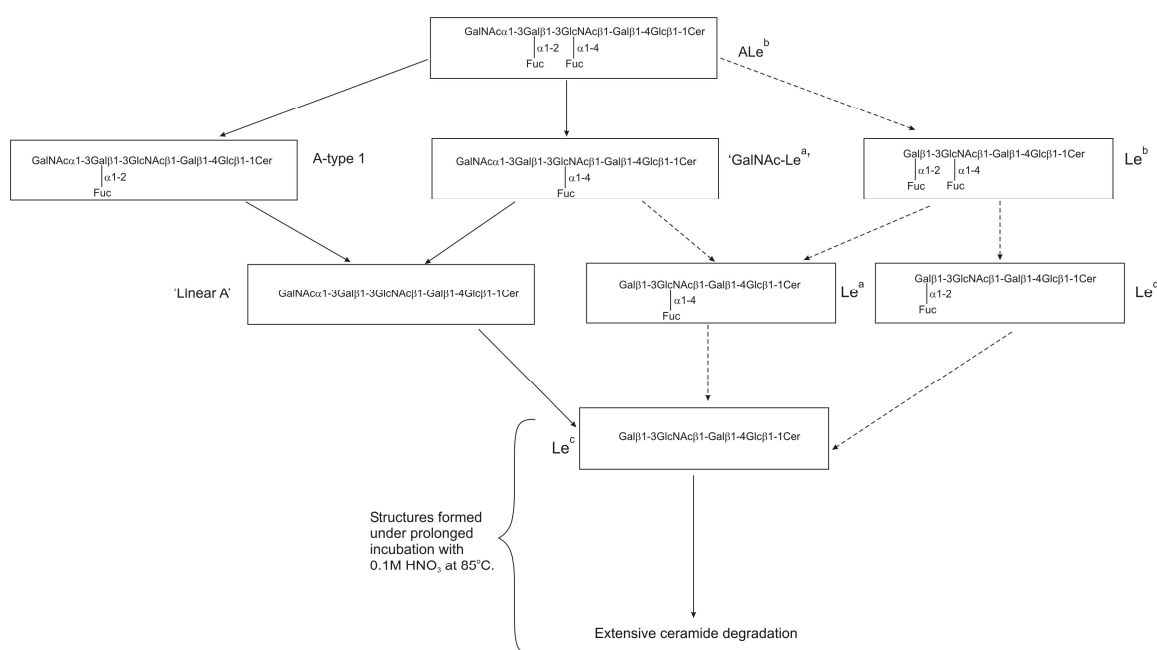


Figure 37: The possible degradation pathways of the ALe^b molecule. The solid arrows indicate the most probable pathway(s) while the arrows with broken lines represent less feasible pathways. Degradation route from Le^c to ceramide is not shown.

The terminal GalNAc residue on the ALe^b sugar is bound by stronger glycosidic bonds and is only expected to cleave under extremely low pH and high temperatures. In addition, this residue can only be removed after the cleavage of both fucosyl glycosidic bonds in the ALe^b structure.

The ALe^b sample also contained minute quantities of Le^b structures and even lesser quantities of A-type 1 structures that could not be separated by HPLC upon purification. This contaminating Le^b and A-type 1 structures were only detected by the sensitive immunostain assays. The interpretation of ALe^b hydrolysis experiments by acid has taken this into consideration as the 'contaminant Le^b' and the A-type 1 structure also have the potential to degrade into Lewis antigens.

It is also important to note that a single glycolipid component can have different ceramide compositions (e.g. hydroxylated and non hydroxylated) and therefore have slight differences in TLC mobilities thereby giving rise to two or more distinct bands on the TLC plate upon anisaldehyde/immuno-staining. This was observed in the immunostains of the 'contaminant Le^b' structures present in the ALe^b sample (see later). On the other hand, two different glycolipids with the same number of sugars also have the tendency to migrate together on the TLC plate. This concept will be discussed later.

The terms 'degradation' and 'hydrolysis' are used interchangeably in the thesis and refers to the hydrolysis of the glycosidic bonds in the glycan chain of the ALe^b molecule unless otherwise specified.

The immunostain assays were carried out as outlined in Protocol 14.

The relative migrations of various Lewis structures and glycolipids are listed in Table 30.

Table 30: Relative migrations (*rm*) of some glycolipids.
These were measured against a scale developed in-house.

Glycolipid	Number of sugar residues	Migration based the <i>rm</i> scale (± 0.5)
ALe ^b	7	3.5
Le ^b	6	4.25
A -6-1	6	5.0
Le ^a	5	5.25
Le ^d / H-type 1	5	5.5
Le ^c / type 1 precursor	4	6.75
Globoside	4	7.0
3-sugar glycolipid	3	9.0
2-sugar glycolipid	2	Off scale
1-sugar glycolipid	1	Off scale

ALe^b versus 0.05 M HNO₃ at 50°C over 120 minutes

The effects of 0.05 M HNO₃ on the ALe^b structure at 50°C over 120 minutes were investigated as outlined in Protocol 10, v 1. The ALe^b molecule migrated to an *rm* position of 3.5 ± 0.5 . The anisaldehyde stain revealed that the ALe^b molecules were not degraded by 0.05 M HNO₃. The controls for this experiment were ALe^b with HNO₃ set at room temperature for 120 minutes (Figure 38, plate I, lane 9) and ALe^b without HNO₃ set to incubate at 50°C for 120 minutes (Figure 38, plate I, lane 10). A consistent positive staining was observed with the anti-ALe^b reagent (Figure 38, plate VII). The anti-Le^{ab} reagent also stained a consistent Le^b band throughout the entire incubation period (Figure 38, plate VI). This ('contaminant') Le^b was present in the ALe^b sample in minute quantities as described earlier.

The presence of double Le^b bands in the immunostain against the anti-Le^{ab} reagent (Figure 38, plate V) was due to the different ceramide compositions of the Le^b glycolipid resulting in slightly different migrations on the TLC plate as described earlier.

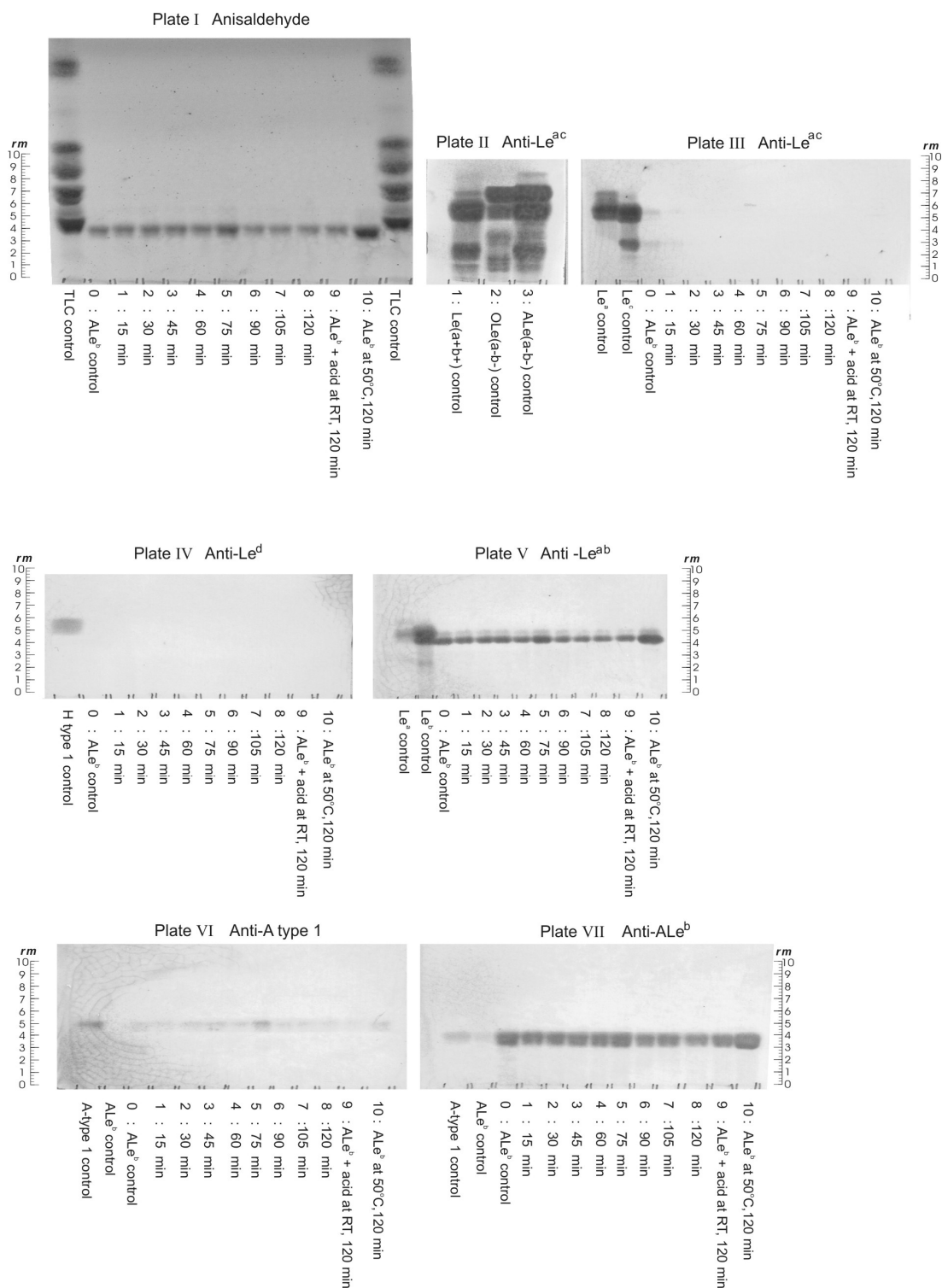


Figure 38: Acid hydrolysis of ALe^b with 0.05 M HNO₃ at 50°C over 120 minutes.

Plate I is an anisaldehyde stain and plates II –VII are immunostains with various antibodies. Note that the Le^c control had trace amounts of Le^a (plates II and III), the Le^a control had trace amounts of Le^b structures (plate V) and the A-type 1 control contained small amounts of ALe^b structures (plate VII).

The extremely weak band representing the A-type 1 structure in plate VI (Figure 38) is due to the presence of trace quantities of this structure present in the ALe^b sample.

The results are summarised in Table 31.

Summary

Table 31: Lewis antigens formed from hydrolysis of ALe^b glycolipid with 0.05 M HNO₃ at 50°C. Incubation was carried out over 120 minutes. + and – indicate the presence or absence of specific structures respectively and (?) indicates it is not known if these structures are reactive with the monoclonals used. (-) indicates that although immunostaining is present, it is not due to degradation of the ALe^b sample. These symbols are non-quantitative.

Incubation time (minutes)	Lewis Structures						
	ALe ^b	A-6-1*	GalNAc-Le ^a + Linear-A	Le ^b *	Le ^a	Le ^d	Le ^c
0	+	(-)	?	(-)	-	-	-
15	+	(-)	?	(-)	-	-	-
30	+	(-)	?	(-)	-	-	-
60	+	(-)	?	(-)	-	-	-
75	+	(-)	?	(-)	-	-	-
90	+	(-)	?	(-)	-	-	-
105	+	(-)	?	(-)	-	-	-
120	+	(-)	?	(-)	-	-	-
Control**	+	(-)	?	(-)	-	-	-
Control***	+	(-)	?	(-)	-	-	-

* ALe^b sample contained trace amounts of Le^b and A-type 1 structures that could not be separated by the HPLC separation programme.

Control** = ALe^b plus acid at room temperature for 120 minutes.

Control*** = ALe^b (nil acid treatment) at 60°C for 120 minutes.

ALe^b degradation into the A-type 1 and other Lewis structures was not observed with incubation at 50°C with 0.05 M HNO₃. It is also very likely that the 'GalNAc-Le^a' and 'linear A' structures were not generated by incubation at this temperature and acid concentration.

ALe^b versus 0.05 M HNO₃ at 60°C over 16 hours

The method is outlined in Protocol 10, v 2. The anisaldehyde stain in Figure 39, plate I, showed that the ALe^b sample underwent some degradation shown by the presence of an additional band at *rm* 5.75 ± 0.5. This band could most likely be due to A-type 1 structure (which has a migration of *rm* 5.0 ± 0.5 on the TLC plate) or could be due to the unnatural glycolipid 'GalNAc-Le^a'. This band could also represent the unnatural 'linear A' structure as its migration is also in the region of a 5-sugar structure.

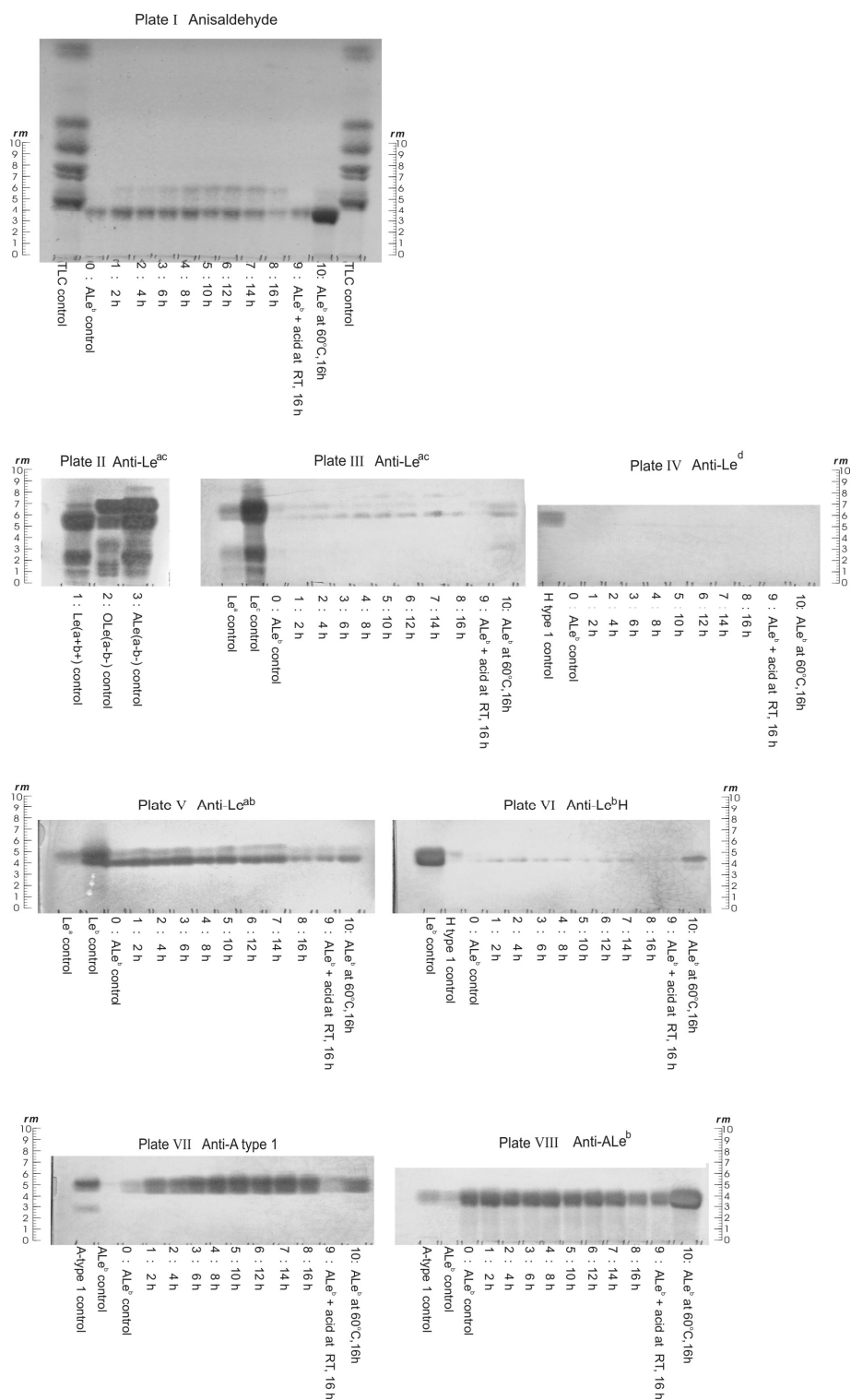


Figure 39: Acid hydrolysis of ALe^b sample with 0.05 M HNO₃ at 60°C over 16 hours. Plate I is an anisaldehyde stain and plates II –VIII are immunostains with various antibodies. Note that the Le^c control had trace amounts of Le^a (plates II and III), the Le^a control had trace amounts of Le^b structures (plate V) and the A-type 1 control contained small amounts of ALe^b structures (plate VIII).

It is unlikely that the Le^a molecule was generated from ALe^b hydrolysis under such mild acidic conditions (see later). This band was visible from the 2nd hour of degradation and became more prominent over time (Figure 39, plate I, lanes 1-8).

Immunostain of the treated ALe^b sample with the anti-Le^{ac} reagent gave an extremely weak positive band in the *rm* region of 5.75 ± 0.5 at all time intervals and the weak staining also indicated that this structure was generated in minute quantities (Figure 39, plate III, lanes 1-8). This is most likely the Le^a structure formed from the hydrolysis of the 'contaminant Le^b'. It is important to note that this Le^a structure migrated 0.5 *rm* units further than its normal position on the TLC plate (Figure 39, plate I, lanes 1-8). There was no staining visible in the region of Le^c migration (6.75 ± 0.5).

The anti-Le^d reagent also gave a negative staining result for the treated ALe^b samples.

In contrast, the anti-Le^{ab} and anti-Le^{bH} reagents stained positive at *rm* 4.25 ± 0.5 with all the treated and untreated ALe^b samples (Figure 39, plates V and VI, lanes 0-8). The appearance of two bands in the region of Le^b migration is due to, as mentioned earlier, a Le^b glycolipid with ceramides of different compositions (i.e. ceramide functionality). This positive staining in the Le^b region was due to the presence of minute quantities of Le^b in the ALe^b sample and is not likely to be a product of ALe^b hydrolysis. This is because, the cleavage of the terminal GalNAc residue on the ALe^b molecule to form the Le^b structure is not considered a possible pathway as glycosidic bonds involving the N-acetylated sugars are relatively more resistant to acid hydrolysis than the acid-labile fucosyl bonds. In other words, the terminal GalNAc residue can only be removed after the cleavage of fucose residues in very low pH conditions (Adams, 1965b) and elevated temperatures. Hence at 60°C and 0.05 M HNO₃, Le^b is unlikely to be generated.

Further, the anti-A-type 1 reagent showed a strong positive band at *rm* 5.0 ± 0.5 for all the treated ALe^b samples at all time intervals (Figure 39, plate VII, lanes 1-8 and 10). This band covered a more broad area on the TLC plate than usual. The positive result indicated that the Fuc α 1-4 bond in the ALe^b structure, GalNAc α 1-3Gal(Fuc α 1-2) β 1-3GlcNAc(Fuc α 1-4) β 1-R, was readily removed to form the A-type 1 glycolipid. The control ALe^b sample (at 60°C) showed a strong positive band in the A-type 1 region. This was entirely due to a high loading concentration of the ALe^b sample in this particular lane (Figure 39, plate VII, lane 10 compared to plate I, lane 10) and as the ALe^b sample contained trace quantities of the A-type 1 structure, the band due to the A-type 1 structure stained strongly with the anti-A reagent. Hence, heat alone was not able to generate A-type 1 from ALe^b structure.

Further, the formation of the unnatural structure 'GalNAc-Le^a' is also likely. In fact, literature states that the α 1-2 glycosidic bonds are more readily cleaved in acid than α 1-4 bonds; therefore theoretically, more 'GalNAc-Le^a' structures are expected to be generated than A-type 1 upon ALe^b hydrolysis. This structure has an oligosaccharide sequence of GalNAc α 1-3Gal β 1-3GlcNAc(Fuc α 1-4) β 1-R (a 6-sugar glycolipid) and would have an identical migration as A-type 1. Antibodies binding with this structure were not known at this stage (see later, Figure 42).

It is also possible for both fucose bonds (being extremely acid-labile) in the ALe^b molecule to be cleaved at about the same time to give rise to a glycolipid bearing a 'linear A' (defucosylated) oligosaccharide sequence, GalNAc α 1-3Gal β 1-3GlcNAc β 1-R. The anti-A-type-1 reagent could be detecting this structure.

The anti-A type 1 reagent has a specificity for the A-type 1 mono-fucosyl chain (GalNAc α 1-3Gal(Fuc α 1-2) β 1-3GlcNAc-) (Abe et al., 1984). However, it is possible that this reagent could be mildly reacting with the unnatural (mono-fucosylated) glycolipid 'GalNAc-Le^a' and as well as the (defucosylated 5-sugar) 'linear-A' structures as the glycan chains in all these glycolipids contain the A-determinant sugar, GalNAc and the linear type 1 chain.

The migration of the 5-sugar structure ('linear A') would be very similar to the 6-sugar (A-type 1) glycolipid, the former having a slightly higher TLC mobility. The band at $rm\ 5.75 \pm 0.5$ in the anisaldehyde stain in plate I (Figure 39, lanes 1-8) could most likely represent this linear (defucosylated) structure as the migration of the A-type 1 is $rm\ 5.0 \pm 0.5$.

The anti-ALe^b reagent stained positive for all the ALe^b samples treated and incubated with acid at 60°C. This was an expected result as minimal degradation of the ALe^b sample took place at lower temperatures.

The results are summarised in Table 32.

Summary

The results of ALe^b hydrolysis with 0.05 M HNO₃ at 60°C showed that A-like structures were the major products. The A-type 1 was seemingly the major product of hydrolysis of ALe^b with 0.05 M HNO₃ at 60°C. Based on the acid stabilities of glycosidic bonds, the unnatural glycolipid, 'GalNAc-Le^a' is also a likely product of degradation together with the defucosylated 'linear A' structure. However, specific antibodies to these structures are not known and their presence could not be ascertained with immunoassays.

On the other hand, chemical staining with anisaldehyde showed a dominant band at $rm\ 5.75 \pm 0.5$ which could most likely represent a 5-sugar molecule; this band could be most likely due to the 'linear A' structure.

Table 32: Lewis antigens formed upon hydrolysis of ALe^b glycolipid with 0.05 M HNO₃ at 60°C. Incubation was carried out over 16 hours. + and – indicate the presence or absence of specific structures respectively and (?) indicates it is not known if these structures are reactive with the monoclonals used. (-) indicates that although immunostaining is present, it is not due to degradation of the ALe^b sample. These symbols are non-quantitative.

Incubation time (hours)	Lewis Structures						
	ALe ^b	A-6-1*	GalNAc-Le ^a + Linear-A	Le ^b *	Le ^a	Le ^d	Le ^c
0	+	(-)	?	(-)	-	-	-
2	+	+	?	(-)	(-)	-	-
4	+	+	?	(-)	(-)	-	-
6	+	+	?	(-)	(-)	-	-
8	+	+	?	(-)	(-)	-	-
10	+	+	?	(-)	(-)	-	-
12	+	+	?	(-)	(-)	-	-
14	+	+	?	(-)	(-)	-	-
16	+	+	?	(-)	(-)	-	-
Control**	+	(-)	?	(-)	(-)	-	-
Control***	+	(-)	?	(-)	(-)	-	-

* ALe^b sample contained trace amounts of Le^b and A-type 1 structures that could not be separated by the HPLC separation programme.

Control** = ALe^b plus acid at room temperature for 16 hours.

Control*** = ALe^b (nil acid treatment) at 60°C for 16 hours.

There was also a small yet insignificant quantity of Le^a formed. It is likely that the Le^a formed from the trace quantities of Le^b structures present in the ALe^b sample by the cleavage of the α 1-2 fucose bond. Other Lewis structures were not generated.

ALe^b versus 0.05 M HNO₃ at 70°C over 16 hours

The degradative effects of 0.05 M HNO₃ on the ALe^b structure was investigated at 70°C over 16 hours as outlined in Protocol 10, v 3. The resulting immunostains of the acid-treated ALe^b sample with various antibodies revealed that the degradation of ALe^b was slightly more severe than those treated at 60°C previously.

From the anisaldehyde stain in Figure 40, plate I, degradation of the ALe^b structure was noticeable from almost the 2nd hour of incubation. The bands could most likely represent the A-like structures (A-type1, 'GalNAc-Le^a' and 'linear A') formed upon removal of one or both fucose residues from ALe^b. The ALe^b band at $rm\ 3.5 \pm 0.5$ persisted throughout the

entire degradation period indicating that most of the ALe^b sample was not degraded by the acid treatment over 16 hours (Figure 40, plate I, lanes 1-8).

The anti-A-type 1 reagent stained positive with the bands at $rm\ 5.0 \pm 0.5$ indicating the presence of the A-type 1 structures (Figure 40, plate VII, lanes 1-8). It is possible that the unnatural glycolipid, 'GalNAc-Le^a' is also migrating to the same position as the A-type 1 as both are 6-sugar structures. It is not known if the anti-A type 1 reagent is detecting this structure but it could be possible as described earlier. In addition, both fucose residues in the ALe^b glycan could be removed at about the same time thereby resulting in a 'linear A' structure. These predictions are based on the stability of α 1-2 and α 1-4 fucose bonds in acid.

Immunostaining with anti-Le^{ac} reagent gave weak positive staining with both Le^a and Le^c glycolipids at 5.25 ± 0.5 and $rm\ 6.75 \pm 0.5$ (Figure 40, plate III lanes 1-8). These are most likely to be products of 'contaminant Le^b' hydrolysis as they were not present in relatively larger quantities to be detected by the anisaldehyde stain.

In contrast, immunostaining with anti-H-type 1 reagent gave negative results (Figure 40, plate IV, lanes 1-8).

The Le^b band was also consistent throughout the degradation period as seen in plate V (Figure 40). Trace amounts of Le^b could not be removed by the purification of ALe^b structures by HPLC and as a result gave positive staining with the anti-Le^{ab} reagent. The control ALe^b sample with no acid or temperature treatment showed positive staining in the Le^b region of $rm\ 4.25 \pm 0.5$ (Figure 40, plate V, lane 0).

Plate VI shows immunostaining with the anti-Le^bH reagent which stained negative for all the treated ALe^b samples. This antibody was less sensitive to the Le^b structure than the anti-Le^{ab} reagent hence showed nil staining of the Le^b bands. The inability of this antibody to stain the Le^b structures also indicated that these structures were present in extremely minute quantities.

The anti-ALe^b reagent stained positive with all the ALe^b samples treated at various time intervals (Figure 40, plate VIII, lanes 0-8). This result was consistent with that seen in the anisaldehyde stain in plate I (Figure 40) implying that the ALe^b structure was not completely degraded into other Lewis antigens upon treatment with 0.05 M HNO₃ at 70°C over 16 hours.

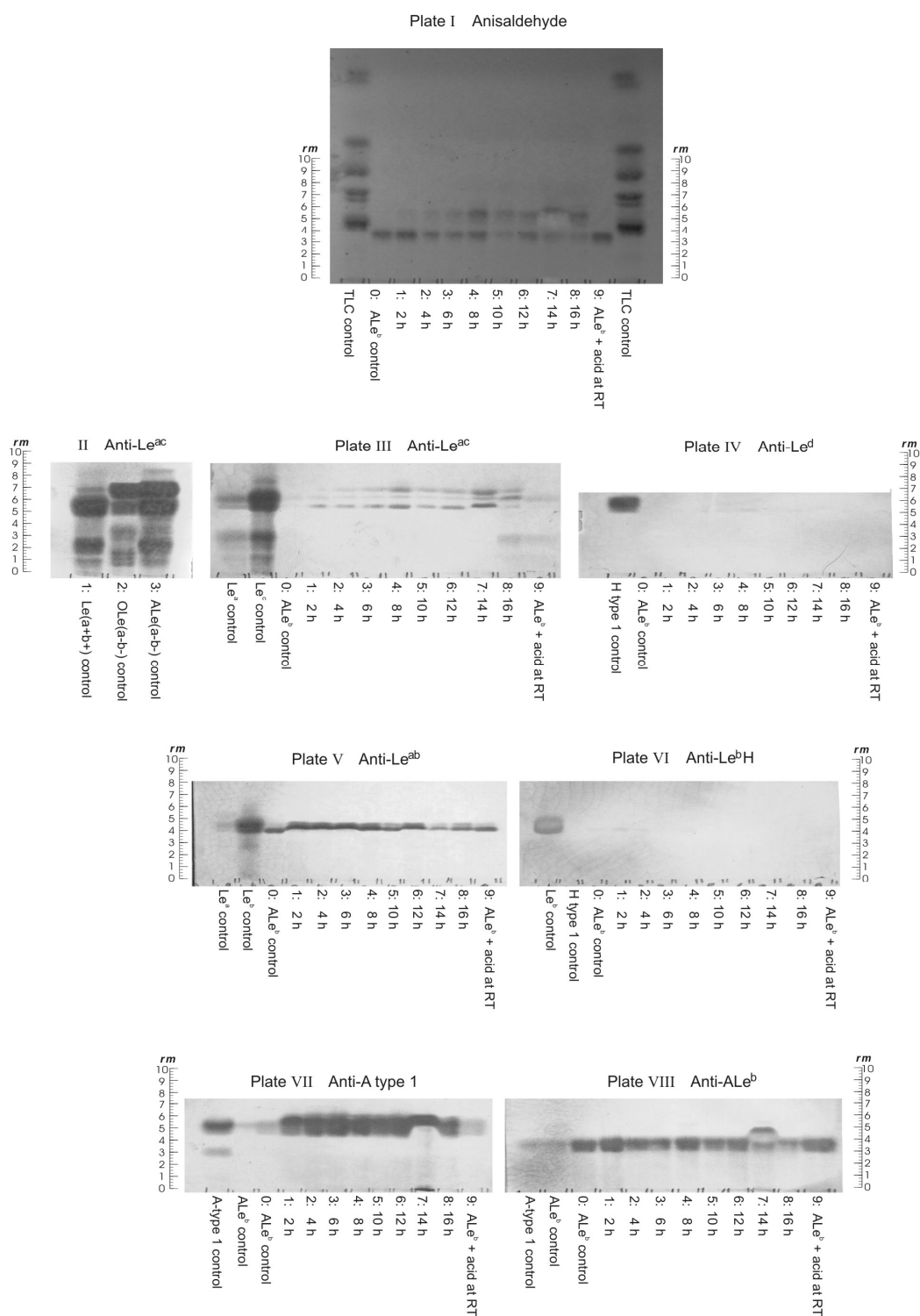


Figure 40: Acid hydrolysis of ALe^b with 0.05 M HNO₃ at 70°C over 16 hours. Plate I is an anisaldehyde stain and plates II –VIII are immunostains with various antibodies. Note that the Le^c control had trace amounts of Le^a (plates II and III), the Le^a control had trace amounts of Le^b structures (plate V) and the A-type 1 control contained small amounts of ALe^b structures (plate VIII).

The results are summarised in Table 33.

Summary

Table 33: Lewis antigens formed hydrolysis of ALe^b molecule with 0.05 M HNO₃ at 70°C. Incubation was carried out over 16 hours. + and – indicate the presence or absence of specific structures respectively and (?) indicates it is not known if these structures are reactive with the monoclonals used. (-) indicates that although immunostaining is present, it is not due to degradation of the ALe^b sample. These symbols are non-quantitative.

Incubation time (hours)	Lewis Structures						
	ALe ^b	A-6-1*	GalNAc-Le ^a + Linear-A	Le ^b *	Le ^a	Le ^d	Le ^c
0	+	(-)	?	(-)	-	-	-
2	+	+	?	(-)	(-)	-	(-)
4	+	+	?	(-)	(-)	-	(-)
6	+	+	?	(-)	(-)	-	(-)
8	+	+	?	(-)	(-)	-	(-)
10	+	+	?	(-)	(-)	-	(-)
12	+	+	?	(-)	(-)	-	(-)
14	+	+	?	(-)	(-)	-	(-)
16	+	+	?	(-)	-	-	(-)
Control**	+	(-)	?	(-)	-	-	-

* ALe^b sample contained trace amounts of Le^b and A-type 1 structures that not be separated by the HPLC separation programme.
Control** = ALe^b + acid at room temperature for 16 hours.

The data obtained showed that the A-type 1 structure was more readily formed upon treatment of ALe^b with 0.05 M HNO₃ at 70°C. Based on the stability of Fucα1-2 and Fucα1-4 glycosidic bonds in acid, it is very likely that unnatural glycolipids 'GalNAc-Le^a' and 'linear-A' structures may have also resulted from the hydrolysis of the ALe^b glycan chain.

The Lewis antigens (Le^a, Le^b, Le^c and Le^d) were not generated from the hydrolysis of the ALe^b glycolipid and the Le^a and Le^c structures detected were most likely to be products of 'contaminant Le^b' hydrolysis.

ALe^b versus 0.10 M HNO₃ at 60°C and 70°C over 24 hours

The degradative effects of 0.10 M HNO₃ on the ALe^b structure was investigated at 60°C and 70°C simultaneously over an incubation period of 24 hours as outlined in Protocol 10, v 4. The results obtained were compared to that of ALe^b treated with 0.05 M HNO₃.

From the anisaldehyde stain in Figure 41, plate I, the ALe^b structure had completely degraded (lanes 1 and 3). The control for this experiment was ALe^b sample incubated at 60°C and 70°C separately without any acid treatment. These controls did not undergo any form of degradation.

Both, anti-Le^{ac} and anti-Le^d reagents gave negative staining with the treated and control ALe^b samples (Figure 41, plates III and IV respectively) suggesting that the corresponding Lewis structures were not formed upon ALe^b hydrolysis.

The anti-Le^{ab} reagent gave positive staining in the *rm* region of 4.25 ± 0.5 (Figure 41, plate V, lanes 0-4). This Le^b band was due to the trace amounts of 'contaminant Le^b' present in the ALe^b sample (Figure 41, plate V, lanes 0-4). In contrast, the anti-Le^bH reagent was not able to detect this trace level of Le^b glycolipids as this reagent was not as sensitive in detecting trace Le^b quantities as the anti-Le^{ab} serum (Figure 41, plate VI, lanes 0-4).

The anti-A ALBA clone reagent (a reagent reactive with all A determinants of all chain types) was able to detect both ALe^b and the A-like structures (at *rm* 3.5 ± 0.5 and *rm* 5.0 ± 0.5 and 6.0 ± 0.5 respectively) generated upon hydrolysis of ALe^b with 0.10 M HNO₃ (Figure 41, plate VII, lanes 1 -4). There are three distinct bands present as shown in Figure 42 (which provides a closer look at plate VII in Figure 41). The band with the highest TLC mobility (present at *rm* 6.0 ± 0.5) most likely represents the 5-sugar glycolipid, 'linear A' which is the defucosylated A-type 1 chain. In addition, the staining of the 6-sugar band at *rm* 5.0 ± 0.5 could be most likely due to both the A-type 1 and/or the unnatural glycolipid, 'GalNAc-Le^a'. The A-type 1 control stained in a much wider region including that of the 5-sugar molecule. This was entirely due to a high loading concentration of this sample.

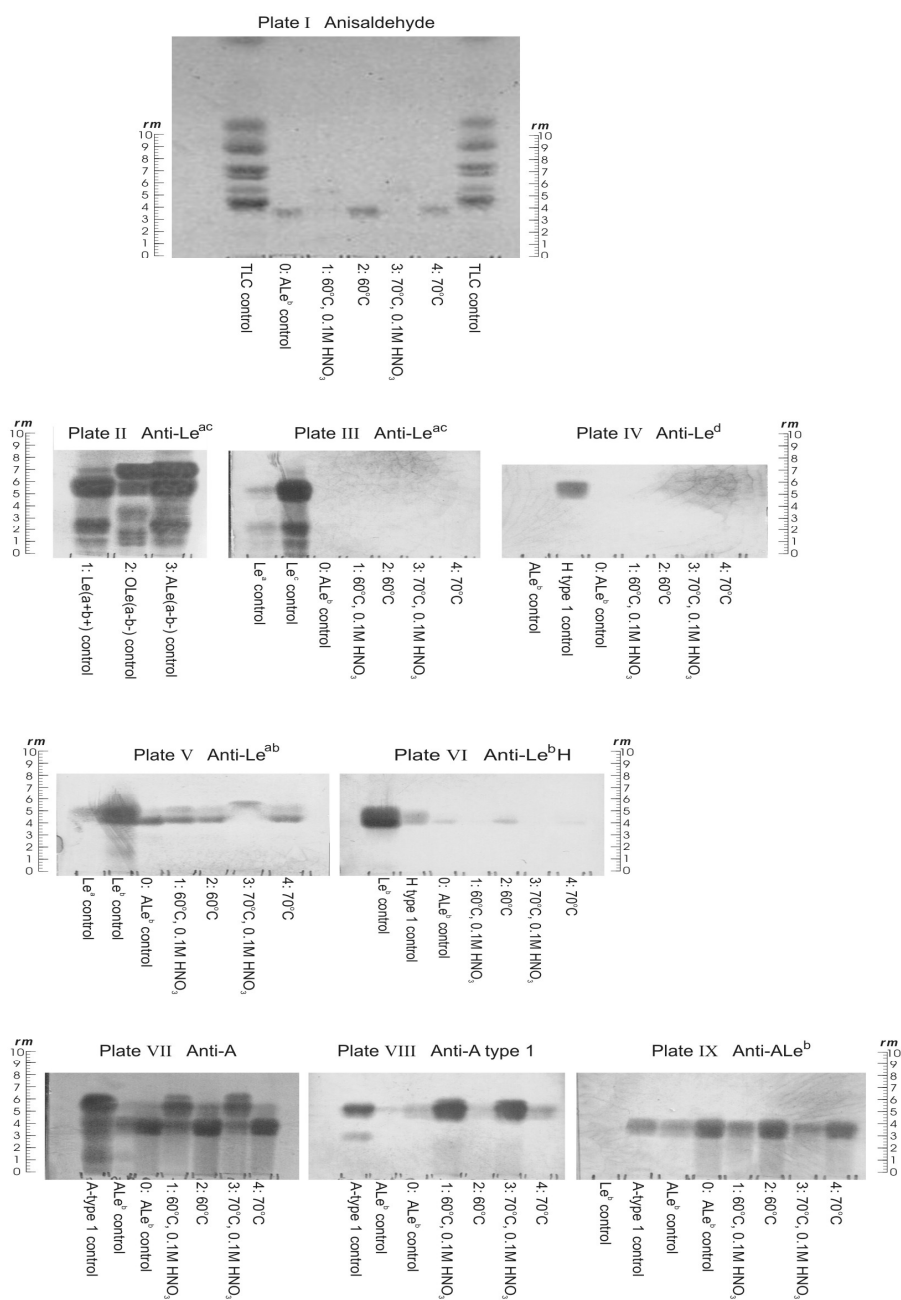


Figure 41: Acid hydrolysis of ALe^b with 0.10 M HNO₃ at 60°C and 70°C. Incubation time was 24 hours. Plate I is an anisaldehyde stain and plates II –IX are immunostains with various antibodies. See also Figure 42 for an expanded version of plate VII. Note that the Le^c control had trace amounts of Le^a (plates II and III), the Le^a and H-type 1 controls had trace amounts of Le^b structures (plate V and VI) and the A-type 1 control contained small amounts of ALe^b structures (plate VII and IX).

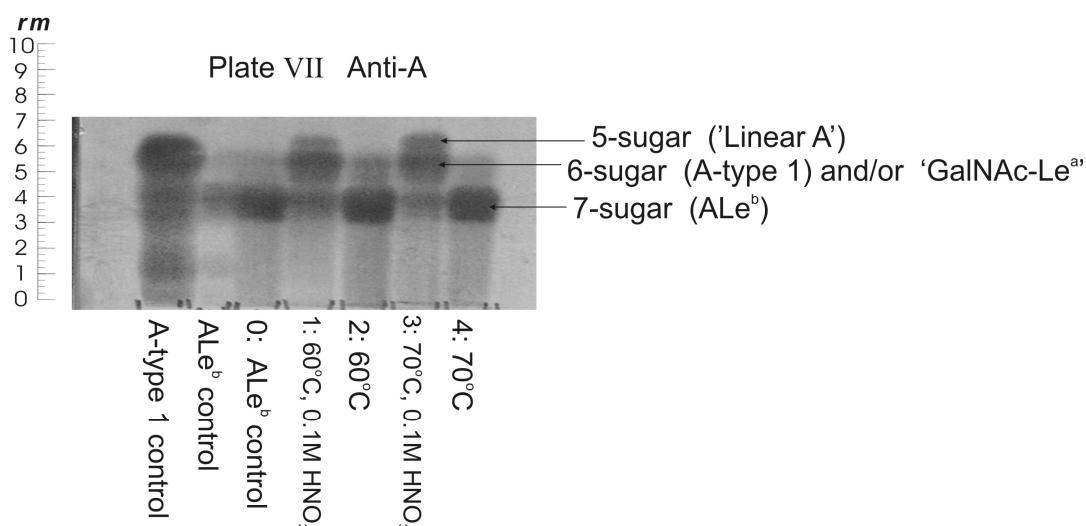


Figure 42: A close-up view of ALe^b staining with anti-A ALBA clone reagent.

As expected, the anti-A type 1 reagent also stained positive for the A-type 1 structure at $rm\ 5.0 \pm 0.5$ (Figure 41, plate VIII, lanes 1 and 3). This showed that A-type 1 was readily formed with 0.10 M HNO₃ treatment at both 60°C and 70°C. This reagent could be detecting the unnatural structures as described earlier.

The anti-ALe^b reagent stained positive for ALe^b samples treated at both 60°C and 70°C suggesting that the entire ALe^b sample was not completely degraded by treatment with 0.10 M HNO₃ over 24 hours (Figure 41, plate IX, lanes 1 and 3). However the absence of the ALe^b in the chemically stained plate (plate I) indicates that only trace quantities of the ALe^b structures were undegraded (as anisaldehyde staining is a quantitative assay).

The results are summarised in Table 34.

Summary

Table 34: Lewis antigens formed upon hydrolysis of ALe^b with 0.05 M HNO₃ at 60°C & 70°C. Incubation was carried out over 24 hours. + and – indicate the presence or absence of specific structures respectively and (-) indicates that although immunostaining is present, it is not due to degradation of the ALe^b sample. These symbols are non-quantitative.

Incubation time (hours)		Lewis Structures						
		ALe ^b	A-6-1*	GalNAc-Le ^a + Linear-A	Le ^{b*}	Le ^a	Le ^d	Le ^c
60°C	0	+	(-)	-	(-)	-	-	-
	24	+	+	+	(-)	-	-	-
	C1*	+	(-)	-	(-)	-	-	-
70°C	0	+	(-)	-	(-)	-	-	-
	24	+	+	+	(-)	-	-	-
	C2*	+	(-)	-	(-)	-	-	-

* ALe^b sample contained trace amounts of Le^b and A-type 1 structures that could not be separated by the HPLC separation programme.
C1* and C2* = ALe^b controls set at 60°C and 70°C respectively without any acid treatment for 24 hours.

The results indicated that treatment of ALe^b with 0.10 M HNO₃ over a 24-hour period resulted in the formation of A-like structures. The anti-A-type 1 reagent stained positive for the A-type 1 structures. However, it is also possible that the 'GalNAc-Le^a', a 6-sugar glycolipid was a product of ALe^b hydrolysis. The anti-A ALBA clone reagent was able to positively identify these structures as shown in Figure 42. Further, because fucosyl glycosidic bonds are extremely acid-labile, the 'GalNAc-Le^a' was most likely further degraded into the 'linear A' structure by the cleavage of the Fucα1-4 glycosidic bond. This resulting defucosylated 'linear A' structure is a 5-sugar glycolipid with a slightly higher migration on the TLC plate (Figure 42). The anti-A ALBA clone reagent appears also to be detecting this product as shown in Figure 42.

Other Lewis antigens were not formed in the acid hydrolysis of ALe^b glycolipids.

ALe^b versus 0.10 M HNO₃ at 70°C over 120 minutes

This experiment dealt with incubating the ALe^b sample with 0.10 M HNO₃ at 70°C over 120 minutes in order to obtain degradation patterns over a shorter incubation period. The results obtained from degrading ALe^b with varying concentration of HNO₃ and over longer time intervals (as in previous experiments) did not allow thorough examination of the hydrolytic process. The method is outlined in Protocol 10, v 5.

The anisaldehyde stain in Figure 43, plate I, showed a more gradual breakdown of the ALe^b molecule. There was no degradation visible in the first 15 minutes of incubation with 0.10 M HNO₃ and a single ALe^b band existed at $rm\ 3.5 \pm 0.5$. Degradation of ALe^b was visible from 30 minutes as shown by a weak band (rm position of 5.0 ± 0.5) present in the relevant lane which gradually darkened over time i.e. 120 minutes (Figure 43, plate I, lanes 2-8). This band was most prominent at 90 and 105 minutes. The ALe^b band at $rm\ 3.5 \pm 0.5$ was much weaker at 120 minutes and was due to a lower loading volume of sample in this lane (Figure 43, plate I, lane 8). The secondary band at $rm\ 5.0 \pm 0.5$ is most likely an A-like structure. This most likely represents the A-type 1 glycolipid and/or the unnatural glycolipid, 'GalNAc-Le^a'. The fucosyl bonds in both glycolipid structures can be further cleaved to form the (defucosylated) 5-sugar 'linear A' glycolipid.

Plate II is an immunostain of Le^a and Le^c controls against the anti-Le^{ac} reagent for comparison of migrations of the degraded samples against these controls. The immunostain of the treated ALe^b sample against the anti-Le^{ac} reagent also showed that both Le^c and Le^a (at $rm\ 5.25 \pm 0.5$ and $rm\ 6.75 \pm 0.5$ respectively) were formed. These were most likely to be products of hydrolysis of the 'contaminant Le^b' molecule (Figure 43, plate III, lanes 0-8).

The anti-Le^d reagent gave a negative staining result for the acid-treated ALe^b sample. (Figure 43, plate IV, lanes 5-7).

The Le^b band at $rm\ 4.25 \pm 0.5$ as described earlier, was due to the trace levels of this structure present with the ALe^b sample (Figure 43, plate V, lanes 0-8). Immunostain with the anti-Le^{bH} reagent also stained the Le^b structure but the staining was extremely weak compared to the Le^b control shown in plate VI (Figure 43). This antibody is less sensitive than the anti-Le^{ab} reagent hence was not able to detect extremely small quantities of Le^b.

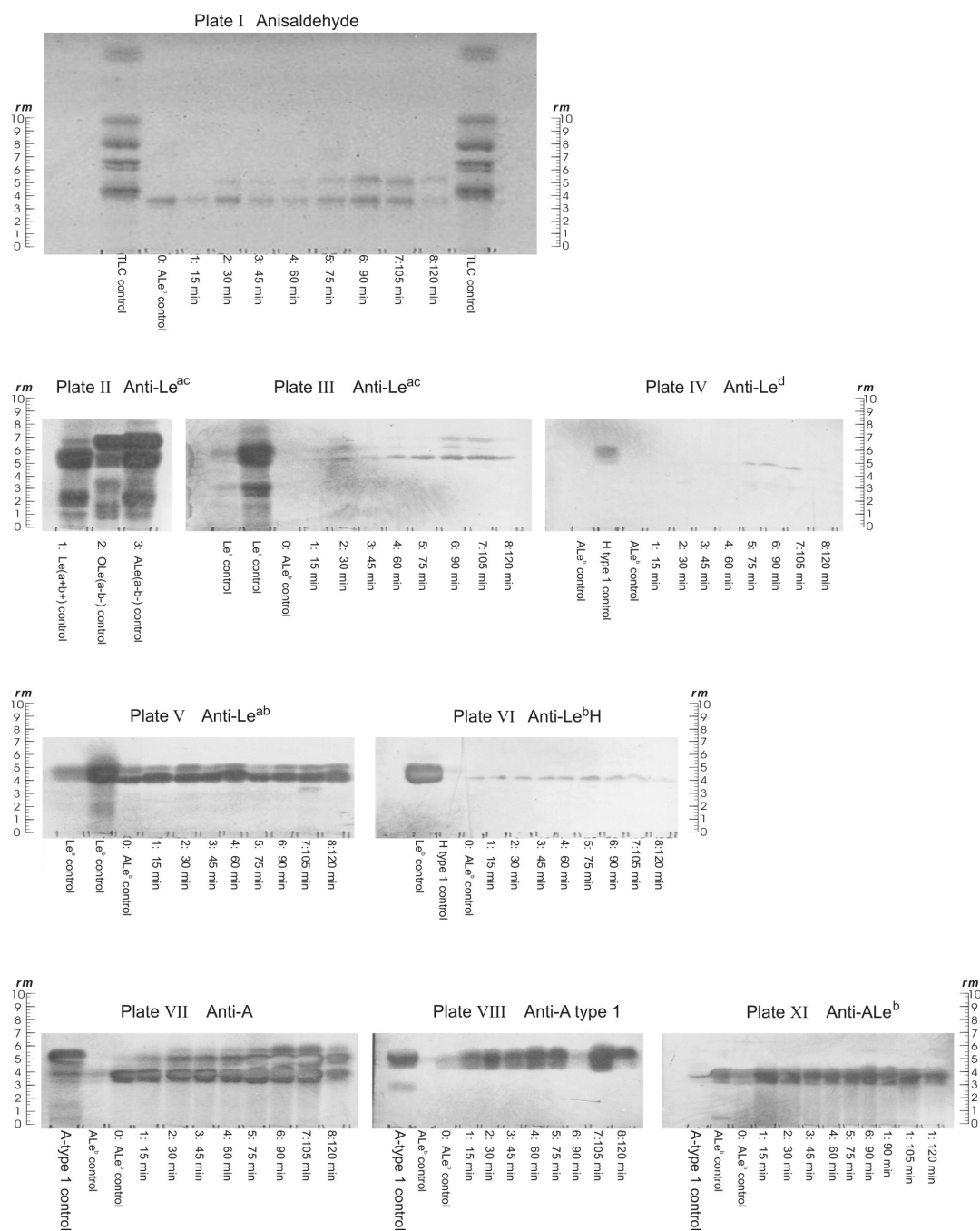


Figure 43: Acid hydrolysis of ALe^b sample with 0.10 M HNO₃ at 70°C over 120 minutes. Plate I is an anisaldehyde stain and plates II –XI are immunostains with various antibodies. See Figure 44 for an enlargement of plates VII and VIII. Note that the Le^c control had trace amounts of Le^a (plates II and III), the Le^a and H-type 1 controls had trace amounts of Le^b structures (plate V and VI) and the A-type 1 control contained small amounts of ALe^b structures (plate VII and IX).

Immunostain with the anti-A ALBA clone reagent revealed an interesting and important pattern of degradation. This reagent seemed to have the ability to detect the linear defucosylated type-A chain together with the ALe^b and mono-fucosylated type-A structures as shown by positive staining in the appropriate regions. The plates in Figure 44 show a close-up view of staining with the ALBA clone reagent shown in (Figure 43). It shows the gradual conversion of the 7-sugar ALe^b glycolipid into a 6-sugar A-type 1 (and/or-'GalNAc-Le^a') which is then further reduced to a 5-sugar 'linear A' structure as shown by the migration of a band in the 5 sugar region as marked in Figure 44, plate VII, lanes 5-8. The weak bands in plate VII, lane 8 are due to a lower loading concentration of sample in this lane.

Further, the anti-A type 1 reagent was also able to stain the A-type 1 structures formed from the hydrolysis of the ALe^b sample (Figure 43, Figure 44, plate VIII, lanes 1-8). This band migrated over a broad region on the TLC plate and it is probable that it may be detecting the 'linear A' structure. As described earlier, this reagent may also be weakly detecting the unnatural 'GalNAc-Le^a' structure.

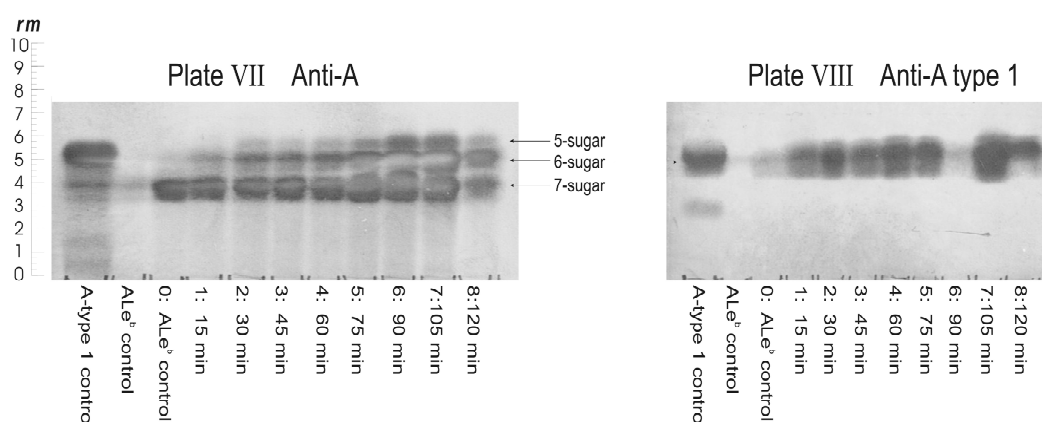


Figure 44: A close-up view of ALe^b staining with anti-A ALBA clone and anti A-type 1 reagent. The staining on plate VII shows the migrations of 5-, 6- and 7-sugar glycolipids relative to one another. The anti-A type 1 reagent is staining the A-type 1 and probably the 'GalNAc-Le^a' and 'linear A' structures as well shown by the broad bands.

Plate XI (Figure 43, lanes 1-8) is an immunostain against the anti-ALe^b reagent and shows that the ALe^b sample was not entirely degraded by incubating ALe^b with 0.10 M HNO₃ at 70°C over a period of 24 hours.

Hence from these results, it is more than likely that the cleavage of the fucose glycosidic bonds in the ALe^b sample is random. Literature states that α 1-2 bonds are less stable in acid than α 1-4 and are therefore cleaved before the latter. However, this rule may not follow when glycosidic bonds involve de-oxy sugars such as fucose, as they further tend to amplify the weakness of the glycosidic bonds in acid.

The results are summarised in Table 35.

Summary

Table 35: Lewis antigens formed upon hydrolysis of ALe^b with 0.10 M HNO₃ at 70°C. Incubation was carried out over 120 minutes. + and – indicate the presence or absence of specific structures respectively and (-) indicates that although immunostaining is present, it is not due to degradation of the ALe^b sample. These symbols are non-quantitative.

Incubation time (minutes)	Lewis Structures						
	ALe ^b	A-6-1*	GalNAc-Le ^a + Linear-A	Le ^b *	Le ^a	Le ^d	Le ^c
0	+	(-)	-	(-)	-	-	-
15	+	+	+	(-)	-	-	-
30	+	+	+	(-)	(-)	-	-
45	+	+	+	(-)	(-)	-	-
60	+	+	+	(-)	(-)	-	-
75	+	+	+	(-)	(-)	-	-
90	+	+	+	(-)	(-)	-	(-)
105	+	+	+	(-)	(-)	-	(-)
120	+	+	+	(-)	(-)	-	-

* ALe^b sample contained trace amounts of Le^b and A-type 1 structures that could not be separated by the HPLC separation programme.

As expected, the ALe^b molecule was only able to degrade into the A-like structures; A-type 1 together with 'GalNAc-Le^a' and 'linear A'. Other Lewis antigens were not formed from the hydrolysis of ALe^b as shown by negative staining with the relevant antibodies.

A similar experiment was carried out where ALe^b was treated with 0.05 M HNO₃ at 70°C and over a period of 120 minutes. ALe^b degradation/hydrolysis was not observed (results not shown).

ALe^b versus 0.10 M HNO₃ at 85°C over 120 minutes

The hydrolysis effects of a higher temperature on the ALe^b glycolipid molecule were investigated in the current section. It is thought, that an elevated temperature together with 0.10 M HNO₃ will be able to cleave more glycosidic bonds in the ALe^b molecule to generate Lewis structures apart from A-type1 and/or 'GalNAc-Le^a'.

Incubation of ALe^b sample with 0.10 M HNO₃ at 85°C was carried out according to the method outlined in Protocol 10, v 6.

The anisaldehyde stain in Figure 45, plate I, showed a more intense degradation of ALe^b upon incubation at 85°C as compared to 70°C. Degradation was visible from the first 15 minutes of incubation with a secondary band appearing at *rm* 4.25 ± 0.5 (Figure 45, plate I, lanes 1-8). This band could not be identified by the immunostains and may represent an unspecified product. The ALe^b band had significantly weakened by 90 minutes of incubation and had completely disappeared by 105 minutes (Figure 45, plate I, lanes 6 and 7). The ALe^b control in lane 0 appeared as a very weak band due to a low loading concentration in this particular lane.

Plate II is an immunostain of Le^a and Le^c controls (Figure 45).

Immunostaining with the anti-Le^{ac} reagent gave positive Le^a bands from 15 to 60 minutes of incubation. On the other hand, the Le^c band at *rm* 6.75 ± 0.5 was present throughout the incubation period (Figure 45, plate III, lanes 1-8). The intensity of the Le^c band indicated that it could not be solely generated by the hydrolysis of trace quantities of the 'contaminant Le^b'. It is possible that the terminal GalNAc residue on the 'linear A' (as described earlier) was cleaved under the extremely low pH and high temperature (85°C) employed in the current experiment to give rise to the 4-sugar, Le^c glycolipid. The degradation pathway is illustrated in Figure 37.

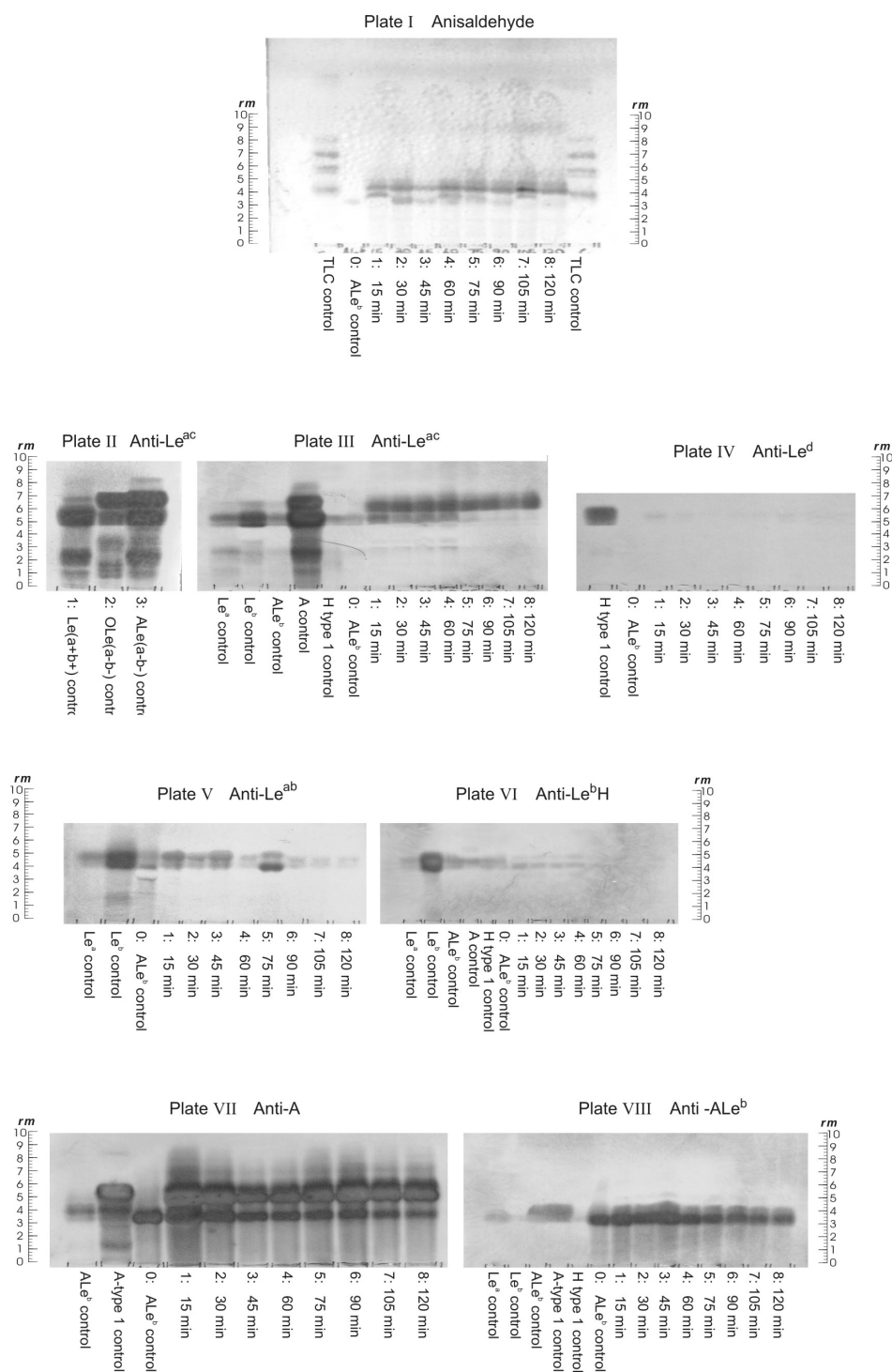


Figure 45: Acid hydrolysis of ALe^b sample with 0.10 M HNO₃ at 85°C over 120 minutes. Plate I is an anisaldehyde stain and plates II–VIII are immunostains with various antibodies. Note that the Le^c control had trace amounts of Le^a (plates II and III), the Le^a and H-type 1 controls had trace amounts of Le^b structures (plate V and VI) and the A-type 1 control contained small amounts of ALe^b structures (plate VII and VIII).

The anti-Le^d reagent was not able to detect any Le^d/H-type 1 structures (Figure 45, plate IV). The H-type 1 structures were expected to be a product of 'contaminant Le^b' hydrolysis (but not a product of ALe^b hydrolysis) upon treatment with 0.10 M HNO₃ at 85°C. This observation implied that the Le^d molecule was likely to be a fleeting product. The Le^d structure has a terminal Fuc α 1-2 glycosidic bond which is readily cleaved upon incubation with 0.10 M HNO₃ at a high temperature of 85°C. On the other hand, Le^a, which has a fucose residue bound to the sub-terminal GlcNAc residue by an α 1-4 bond has a greater 'shielding effect' (within the glycolipid) from the external environment than the α 1-2 fucose in the Le^d structure. Consequently, Le^d has a higher tendency to lose its α 1,2 fucose residue than for the Le^a molecule to lose its α 1,4 fucose in the fairly acidic conditions created by 0.1 M HNO₃. Literature also supports this theory by suggesting that the α 1-2 glycosidic bonds are more acid labile than α 1-4 bonds. The immunoassay, hence, stained positive for Le^a and negative for Le^d. Hence, the formation of Le^a and as well in-part Le^c structures was due to the hydrolysis of the 'contaminant Le^b' molecule. The Le^c, as described earlier, is also very likely to be a product of ALe^b hydrolysis. The immunostain with the anti-Le^{ab} reagent showed positive staining of Le^b structures (at *rm* 4.25 \pm 0.5) as well but this staining appeared to be much weaker than when ALe^b was treated at temperatures below 85°C (Figure 45, plate V). As mentioned earlier, the positive staining with the anti-Le^{ab} in all the immunostains of ALe^b experiments was due to the presence of 'contaminant Le^b'. The weak staining of Le^b bands indicated that the 'contaminant Le^b' also underwent hydrolysis and possibly formed the Le^a and Le^c structures. Weakening of this band, as shown in the immunostain was most likely due to the hydrolysis of Le^b into Le^a and Le^c structures as shown in Figure 45 plate III. The anti-Le^bH was able to weakly detect Le^b structures because this reagent was not as sensitive as the anti-Le^{ab} reagent (Figure 45, plate VI).

Staining with the anti-A ALBA clone reagent showed a gradual weakening of the ALe^b band at 3.5 \pm 0.5 together with a simultaneous appearance of a 6-sugar glycolipid band at *rm* 5.0 \pm 0.5 (Figure 45, plate, VII, lanes 1-8) as this reagent was able to stain both ALe^b (at *rm* 3.5 \pm 0.5) and the A-like structures (at *rm* 5.0 \pm 0.5). The ALe^b band at 120 minutes was significantly reduced. As with previous ALe^b hydrolysis experiments, it is likely that both A-type 1 and 'GalNAc-Le^a' were also formed. These structures may have then further degraded into the 'linear A' glycolipid (5-sugar glycolipid) which may be recognised by the anti-A ALBA clone reagent. A band in the 5-sugar region (*rm* 6.0 \pm 0.5) as seen in plate VII, Figure 44, was not observed and this could be due to a heavy loading of sample as seen in plate VII, Figure 45). The intense bands due to the A-type 1 (or 'GalNAc-Le^a'

structures may have masked the 5-sugar band (due to the 'linear A' glycolipid) as seen in Figure 42 and Figure 44.

Immunostain with the anti-ALe^b reagent revealed positive staining for all the samples treated at all time intervals. Upon closer observation, the ALe^b band at *rm* 3.5 ± 0.5 at 90-120 minutes of incubation stained relatively less strongly than those treated between 15 to 75 minutes (Figure 45, plate VIII, lanes 6-8 and 1-5 respectively). Trace quantities of ALe^b sample had not degraded at 120 minutes. Immunoassays being a more qualitative technique were able to detect and stain these ALe^b structures strongly without being able to give an indication of the quantity of the antigens present. In contrast, the anisaldehyde stain was not able to detect the ALe^b structures after 90 minutes indicating that most of the ALe^b sample was hydrolysed.

The results are summarised in Table 36.

Summary

Table 36: Lewis antigens formed upon hydrolysis of ALe^b with 0.10 M HNO₃ at 85°C. Incubation was carried out over 120 minutes. + and – indicate the presence or absence of specific structures respectively and (-) indicates that although immunostaining is present, it is not due to degradation of the ALe^b sample. These symbols are non-quantitative.

Incubation time (minutes)	Lewis Structures						
	ALe ^b	A-6-1*	GalNAc-Le ^a				Le ^{c**}
			+	Le ^b *	Le ^{a**}	Le ^d	
			Linear-A				
0	+	(-)	-	(-)	-	-	-
15	+	+	+	(-)	+	-	+
30	+	+	+	(-)	+	-	+
45	+	+	+	(-)	+	-	+
60	+	+	+	(-)	+	-	+
75	+	+	+	(-)	-	-	+
90	+	+	+	-	-	-	+
105	+	+	+	-	-	-	+
120	+	+	+	-	-	-	+

* ALe^b sample contained trace amounts of Le^b and A-type 1 structures that could not be separated by the HPLC separation programme.

**Le^c and Le^a could be a product of both ALe^b and 'contaminant Le^b' degradation in this situation.

The data obtained showed that the ALe^b sample underwent extensive degradation when incubated with 0.10 M HNO₃ at 85°C over a period of 120 minutes. The hydrolysis of the ALe^b molecule was able to generate the A-type 1 glycolipid together with Le^c structure. Further, it is possible that the 'GalNAc-Le^a' and 'linear A' structures were also products of

ALe^b hydrolysis. The Le^c may have most likely formed from the cleavage of the terminal 'GalNAc' residue of the 'linear A' glycan (see Figure 37).

In addition, Le^a was also generated and could be the product of hydrolysis of 'contaminant Le^b'. Le^c, in-part, was also a product of 'contaminant Le^b' hydrolysis.

In addition, the H-type 1 structure was not generated (from the 'contaminant Le^b' structure). This observation confirmed that the Fuc α 1-2 bond in this glycolipid was extremely labile in low pH.

The original ALe^b glycolipid had not entirely degraded over the 120-minute incubation period. Ceramide degradation was also not observed.

ALe^b versus 0.10 M HNO₃ at 85°C over 96 hours

The effects of 0.10 M HNO₃ on ALe^b sample was investigated over a longer period of time (96 hours) in order to compare the degradation patterns of the ALe^b sample treated with acid at 120 minutes. This experiment was also set out to determine if the ceramide portion of the glycolipid was also affected by acid hydrolysis over prolonged incubation periods. The method is outlined in Protocol 10, v 7.

The anisaldehyde stain in plate I showed that degradation of the ALe^b molecule was quite extensive within the first 8 hours of incubation at 85°C (Figure 46, plate I, lane 1). The ALe^b band at *rm* 3.5 ± 0.5 was not visible but additional bands at *rm* 5.25 ± 0.5 together with another band off the *rm* scale was enough evidence to suggest that the glycan chain of the ALe^b glycolipid had undergone extensive acid hydrolysis. This pattern was consistent until 48 hours of incubation (Figure 46, plate I, lanes 1-3) with the ALe^b band disappearing at 72 hours (lane 4).

However there was a more diffuse band spread across a mean *rm* value of 6.0 ± 0.5 between 8 and 96 hours (Figure 46, plate I, lanes 1-5). In addition, bands (off the *rm* scale) in the region of 2- and 1-sugar glycolipids were also observed suggesting that ALe^b glycolipid sample was significantly degraded. However, ceramide degradation was not observed.

Plate II is an immunostain of Le^a and Le^c controls against the anti-Le^{ac} reagent.

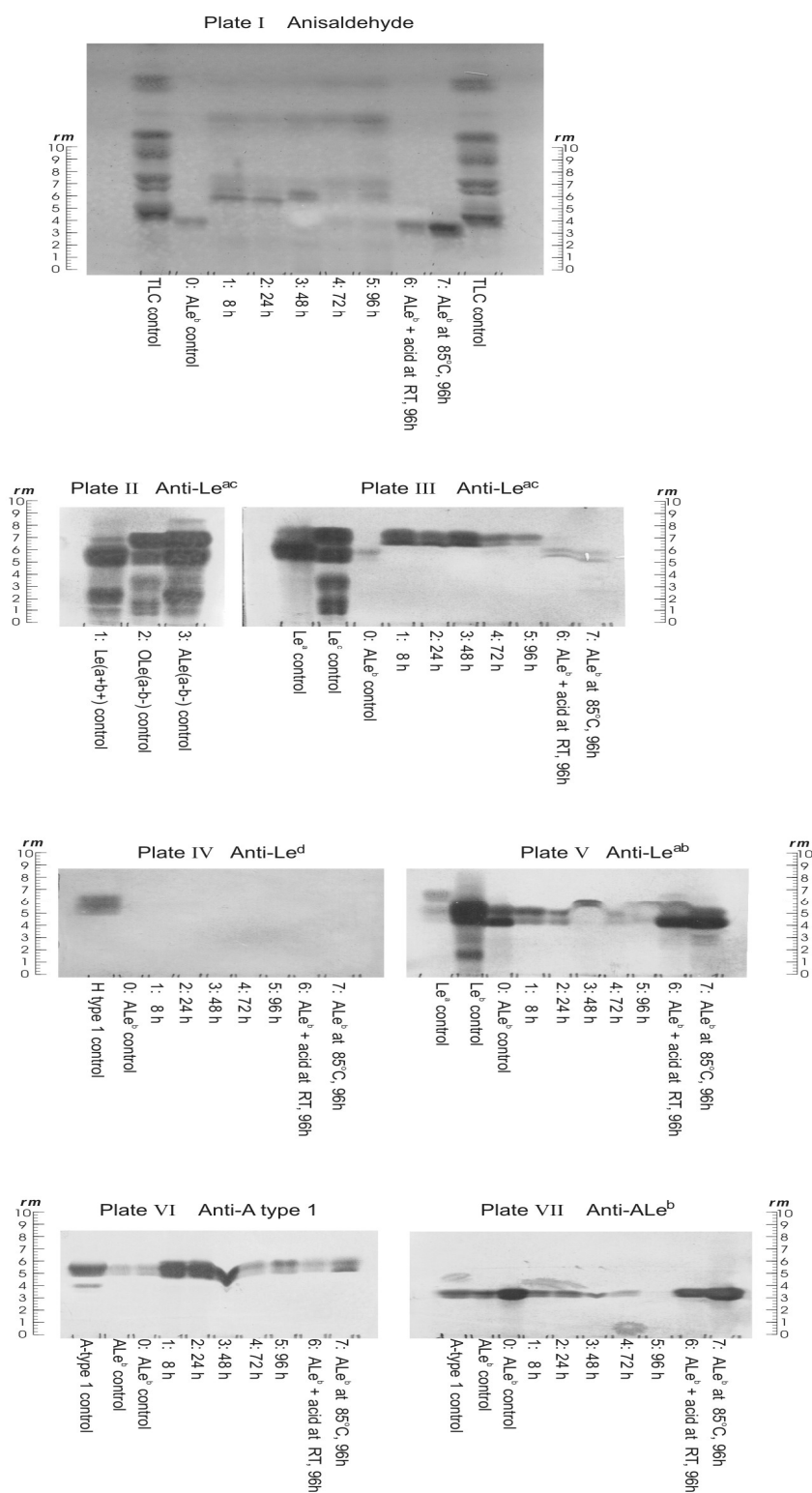


Figure 46: Acid hydrolysis of ALe^b sample with 0.10 M HNO₃ at 85°C over 96 hours. Plate I is an anisaldehyde stain and plates II –VII are immunostains with various antibodies. Note that the Le^c control had trace amounts of Le^a (plates II and III), the Le^a control had trace amounts of Le^b structures (plate V) and the A-type 1 control contained small amounts of ALe^b structures (plate VII).

Plate III (Figure 46) is an immunostain of the treated ALe^b against the anti- Le^{ac} reagent and shows that the Le^c precursor molecules were readily formed upon the first 8 hours of incubation. This is evident by the presence of a single band in the region of $rm\ 6.75 \pm 0.5$, the migratory position of a 4-sugar glycolipid such as Le^c . There was no staining observed in the region of Le^a migration ($rm\ 5.25 \pm 0.5$). The low pH environment may have most likely caused the cleavage of the acid-labile fucosyl linkages which could explain the absence of Le^a structures. The Le^c most likely formed from the cleavage of the terminal GalNAc residue of the 'linear-A' structure (formed upon ALe^b hydrolysis) and as well as from the hydrolysis of the 'contaminant Le^b ' sample. With due consideration to loading, it is interesting to note that the Le^c band had slightly lightened at 72 and 96 hours of incubation (Figure 46, plate III, lanes 4-5). This Le^c structure could be further degrading into 3-, 2- and 1-sugar glycolipid molecules during these times (Figure 46, plate I).

Negative staining was obtained with immunostains of the acid-treated ALe^b samples against the anti- Le^d or anti-H type 1 reagent (Figure 46, plate IV).

Plate V (Figure 46) is an immunostain against the anti- Le^{ab} reagent and showed positive staining in the Le^b region ($rm\ 4.25 \pm 0.5$) from 8 - 48 hours (lanes 1-3). This Le^b was present in trace quantities with the ALe^b structure as described earlier and was not a product of ALe^b hydrolysis. The slight irregularity in the migration pattern in lane 3 is probably due to the presence of salt impurities. After 48 hours, the staining was observed to be less strong indicating that the Le^b sample also underwent extensive degradation possibly being reduced to the Le^a (fleeting) and Le^c structures.

The acid-treated ALe^b samples stained strongly with the anti-A type 1 reagent at the rm region of 5.0 ± 0.5 indicating the formation of A-type 1 structure upon hydrolysis of the ALe^b glycan. The 'GalNAc- Le^a ' and 'linear A' structures are also possible products of ALe^b hydrolysis and could be weakly detected by this reagent as their glycan chains also consist of the type 1 chain with the A-determinant sugar (GalNAc) as described earlier. The irregularity in migration shown in lane 3 could be due to salt impurities (sodium nitrate [$NaNO_3$] formed upon the neutralisation process) present in the hydrolysed sample (Figure 46, plate VI). The A-type 1 band was weakly stained after 48 hours of incubation suggesting that it was further degraded possibly into "linear A' glycolipid and then into the Le^c structure by the cleavage of the terminal GalNAc residue as described earlier (Figure 46, plate VI, lanes 4-5). The pathway for ALe^b degradation is shown in Figure 37.

A positive band in the region of the A-type 1 sugar was observed upon staining the controls (Figure 46, plate VI, lanes 6 and 7) with the anti-A type 1 reagent. This was

entirely due to the presence of trace levels of the A-type 1 molecules in the ALe^b and a higher concentration of ALe^b loaded into the respective lanes showed a positive staining for the contaminant A-type 1 structure as well. Hence, heat alone did not contribute towards ALe^b degradation/hydrolysis.

The anti-ALe^b reagent showed a strong positive result with the original untreated ALe^b sample in lane 0 (Figure 46, plate VII). However the treated samples from 8-48 hours stained less significantly with this reagent (Figure 46, plate VII, lanes 1-4). At 96 hours, there was negative staining observed (Figure 46, plate VII, lane 5) suggesting that the ALe^b sample had completely degraded.

The results are summarised in Table 37.

Summary

The ALe^b sample underwent extensive degradation when incubated with 0.10 M HNO₃ over 96 hours. The A-type 1 structures were readily formed. The unnatural glycolipids namely 'GalNAc-Le^a' and linear-A are also possible products of ALe^b degradation.

The Le^c structures could have formed from the hydrolysis of both the 'linear A' structure and as well as the 'contaminant Le^b'. In extremely low pH and elevated temperatures, this Le^c has a tendency to further degrade into the more simple (3-, 2- and 1-sugar) glycolipids. However, ceramide degradation was not observed upon incubating ALe^b with 0.10 M HNO₃ over 96 hours. This observation suggested that the Glc-ceramide bond was extremely stable to acid. In addition, the presence of the Le^c structure throughout the incubation period indicated that it was relatively robust in nature due to its β glycosidic bonds although it must be noted that the low pH and high temperature used to generate Le^c from ALe^b would cause some form of degradation to the other sugar residues in the Le^c structure.

Table 37: Lewis antigens formed upon hydrolysis of ALe^b with 0.10 M HNO₃ at 85°C. Incubation was carried out over 96 hours. + and – indicate the presence or absence of specific structures respectively, ± indicates trace quantities and (-) indicates that although immunostaining is present, it is not due to degradation of the ALe^b sample. These symbols are non-quantitative.

Incubation time (hours)	Lewis Structures						
	ALe ^b	A-6-1*	GalNAc-Le ^a + Linear-A	Le ^b *	Le ^a	Le ^d	Le ^c *
0*	+	(-)	-	(-)	-	-	-
8	+	+	+	(-)	-	-	+
24	+	+	+	(-)	-	-	+
48	±	+	+	(-)	-	-	+
72	±	±	+	-	-	-	+
96	-	±	+	-	-	-	+
Control 1**	+	(-)	-	(-)	(-)	-	-
Control 2***	+	(-)	-	(-)	(-)	-	-

* ALe^b sample contained trace amounts of Le^b and A-type 1 structures that could not be separated by the HPLC separation programme.

* Le^c could be a product of both ALe^b and 'contaminant Le^b' degradation in this situation.

** Control 1 = ALe^b + acid at room temperature for 96 hours. This sample had trace levels of A-type 1 structures present.

*** Control 2 = ALe^b (nil acid treatment) at 85°C for 96 hours. This sample had trace levels of A-type 1 structures present.

On the other hand, Lewis structures consisting of the acid-labile fucose bonds, Le^a and Le^d were not formed from the hydrolysis of 'contaminant Le^b' sample as these bonds may have been readily cleaved due to the high incubation temperature and low pH of the solution mixture. In other words, the Le^d and Le^a would have been fleeting products of ('contaminant') Le^b hydrolysis.

The original ALe^b glycolipids had entirely degraded over the 96-hour incubation period.

The possible pathways for ALe^b hydrolysis is illustrated in Figure 37.

4 Discussion

Very little is known on methods based on the controlled degradation of glycolipids by acid hydrolysis. The major factors contributing towards the degradation of carbohydrates include acid concentration, temperature, and time. It is possible that these variables can be simultaneously manipulated in such a way that a chosen acid concentration is able to cleave certain glycosidic bonds at a given temperature and time without cleaving any other glycosidic bonds or degrading any monosaccharide residues. Exactly how this can be achieved has not been described.

Glycosidic bonds differ in their lability towards acids depending on the type of anomeric linkage, its position and the type (or nature) of monosaccharides involved in the linkage. Any given neutral oligosaccharide chain in a blood group glycolipid has a range of glycosidic bonds (α versus β bonds together with 1-2, 1-3, 1-4 and 1-6 branching linkages) and monosaccharides (neutral, de-oxy sugars and acetylated forms). Because the glycosidic bonds and monosaccharide residues have varying lability in acidic conditions, it is possible to selectively cleave bonds to modify glycolipid structures.

The aim of the project was to carry out systematic degradation of blood group glycolipids by acid hydrolysis in order to selectively cleave glycosidic bonds to obtain precursor and underlying structures that are not readily available.

Part of this project also involved determining the influence of metal ions acting as catalysts in the degradation of glycolipids because of the potential catalytic effect in cellulose degradation.

Preliminary degradation work was carried out on the more readily available globoside (G-4-4) glycolipids before valuable blood group glycolipids were used. The intention of the experiments was to cleave glycosidic bonds from the non reducing end of the oligosaccharide chain by acid hydrolysis. In doing so, the underlying precursor structure, as revealed, would be identified by their migration positions on the TLC plate by anisaldehyde staining and their ability to react or not to react with appropriate monoclonal antibodies. In other words, this study was trying to investigate if the removal of sugar residues on the blood group active glycolipid molecule by controlling the acid hydrolysis processes would change the entire blood group specificity of a glycolipid of interest. Should this be the case, it would be possible to use common blood group glycolipids to obtain rare blood group precursor molecules in desired yields for use in many biological assays.

The general methodology was based around the acid hydrolysis/partial acid hydrolysis processes. A known amount of glycolipid was suspended in a fixed quantity of either metal ion or acid solution, incubated at 85°C for a period of time, neutralised and finally the degradation products were isolated by partitioning in chloroform. These were then loaded onto Thin Layer Chromatography (TLC) plates, developed with the mobile phase and then chemically stained with anisaldehyde. The migration of the degraded products was then compared with that of reference samples on the same TLC plate. Based on these observations, the number of sugar residues of the degraded products was identified by their migrations relative to those of reference glycolipids. The degraded blood group products (from Le^b and ALe^b degradation) were further subjected to immunostain assays against various monoclonal antibodies to confirm the presence of certain precursor or underlying structures. The migration and staining of the degraded samples from both the anisaldehyde staining and immunostain assays were used to confirm the structures present. Analysing these degraded structures by mass spectrometry (MS) methods would have been ideal but were not readily available. However, the identity of the degraded products can be confidently based on the two staining techniques used. The comparison of migrations and staining of the degraded products with the reference molecules and monoclonal antibodies provided exceptionally good structural information on the degraded products.

The anisaldehyde stain can identify a few structural features of glycolipid samples represented in bands. They are as follows;

- § Glycolipids stain as green bands
- § Ceramide stain as blue bands and degraded ceramides stain as intense blue bands
- § The presence of fucose residues in the glycolipid produces a distinct green band.

The specific monoclonal antibodies utilized to identify degradation products are also very sensitive to the antigenic oligosaccharide sequences hence would only detect structures that are intact. Antibodies operate on a 'lock and key' mechanism; the ability of the appropriate monoclonal antibodies to react with the products of hydrolysis suggests that the glycosidic bonds are cleaved in an orderly manner that does not disrupt the antigenic structures.

Establishing degradation concepts of glycolipids - globoside structure

Using globoside, a 4-sugar glycolipid, $\text{GalNAc}\beta 1-3\text{Gal}\alpha 1-4\text{Gal}\beta 1-4\text{Glc}\beta 1-1\text{Cer}$, a series of experiments was performed to establish some concepts of glycolipid degradation.

From the results of initial experiments with the globoside structure, it could be established that;

Metal ions are not involved in catalysing the cleavage of the glycosidic bonds in the globoside molecule. Globoside degradation was only noticed with those salt solutions forming mild and strong acidic solutions with a pH of less than 3. It was observed that there was no globoside degradation when incubated with lower concentrations of these (acidic) salts in a solution of pH greater than 3. Conversely, salts that formed neutral solutions were able to degrade globoside only when acidified with HNO_3 (Results, Section A, Part IV and Part V).

From the range of acids used, HNO_3 gave visually 'clean' degradation patterns of the globoside molecule and its pattern of the cleavage of the glycosidic bonds within the oligosaccharide chain in globoside could be easily interpreted (Results, Section A, Part VI). Based on its ready availability and relatively stable nature at low concentrations, HNO_3 was chosen as the degrading acid for all future hydrolysis experiments.

In addition, simultaneously varying the concentrations of both metal ions and pH on a fixed concentration of globoside clearly pointed out that low pH and not metal ions were responsible for globoside degradation. Both FeCl_3 and SnCl_2 form acidic solutions and are therefore expected to degrade globoside. However, at low concentrations of these salts, degradation was not observed unless the pH of the same degrading environment was lowered by the addition of acid. Therefore acidity is solely responsible for catalysing globoside degradation (Results, Section A, Part V).

The ceramides (both hydroxy and non hydroxy ceramides) were found not to be susceptible to degradation when incubated with 0.1 M HNO_3 over a period of 24 hours. In contrast, globoside was degraded into 3-, 2- and 1-sugar glycolipids upon identical treatment.

The ceramides, however, were severely degraded by 1.75 M HNO_3 as visualised by distinct blue bands migrating towards the top of the TLC plate. These bands were absent when globoside was treated with acid in previous experiments and is an important indication that the sphingolipid (or ceramide) portion of glycolipids is not affected by HNO_3 concentrations less than 0.10 M. The degraded glycolipids (consisting of 3-, 2- or 1-sugar)

migrated to the same position as their corresponding reference molecules on the TLC plate (Results, Section A, Part VI.).

The optimal temperature for globoside degradation was found to be 85°C (Results, Section A, Part VII).

The re-investigation of the catalytic effect in degrading the globoside structure by Fe^{3+} and Cu^{2+} showed that there was some degradation of globoside when a sample was incubated with 0.005 M solutions of these ions (Results, Section A, Part VIII.). The pH of the degrading environment was between 6 and 6.5. Mild globoside degradation was only noticeable after 16 days of incubation. This indicated that metal ions may have catalysed the long-term breakdown of the glycolipid. Alternatively, the presence of chloride ions (from FeCl_3 and CuCl_2) in solution may have also contributed towards the acidity of the degrading environment thereby causing subsequent degradation of globoside.

Analysis of globoside hydrolysis

The removal of the non reducing terminal N-acetylated sugar, GalNAc, from globoside ($\text{GalNAc}\beta 1\text{-3Gal}\alpha 1\text{-4Gal}\beta 1\text{-4Glc}\beta 1\text{-1Cer}$), and cleaving off the terminal $\beta 1\text{-3}$ glycosidic bond does not occur readily as compared with neutral sugars bound by α glycosidic bonds (Shallenberger, 1982; Wolfrom et al., 1963). Nonetheless, the reduction of the globoside to a 3-sugar molecule was observed in solutions of pH less than or equal to 3 and at 85°C. The removal of sugars was observed to be sequential with cleavage of glycosidic bonds occurring from the non reducing end of the oligosaccharide chain. It is also possible that the acid may also be cleaving the terminal disaccharide structure, $\text{GalNAc}\beta 1\text{-3Gal}$, giving rise to a 2-sugar glycolipid as, according to literature, it is easier to cleave the sub terminal α glycosidic bond binding two neutral sugars together compared to a β bond involving the terminal acetylated sugar. This is in agreement with the work carried out by Schiffman and co-workers who were able to isolate a disaccharide, $\text{GalNAc}\alpha 1\text{-3Gal}$, from the non reducing end of A blood group substance by partial acid hydrolysis (Schiffman et al., 1962). However, the co-existence of the 3-sugar ceramide contaminant with the globoside made it difficult to identify this product as this 3-sugar glycolipid would have also been reduced to the 2-sugar moiety at the same time.

On the other hand, it is also possible that the terminal GalNAc residue may have cleaved when the pH was low enough to induce degradation and the incubation period long enough to eventually cleave this glycosidic bond.

The initial product of globoside degradation observed during the first hour of incubation was a 3-sugar glycolipid whose migration was identical to the 3-sugar glycolipid standard. This was followed by a 2-sugar and eventually a 1-sugar glycolipid structure with prolonged incubation (seen especially when globoside was incubated with salts that formed highly acidic solutions). This degradation pathway is illustrated in Figure 47.

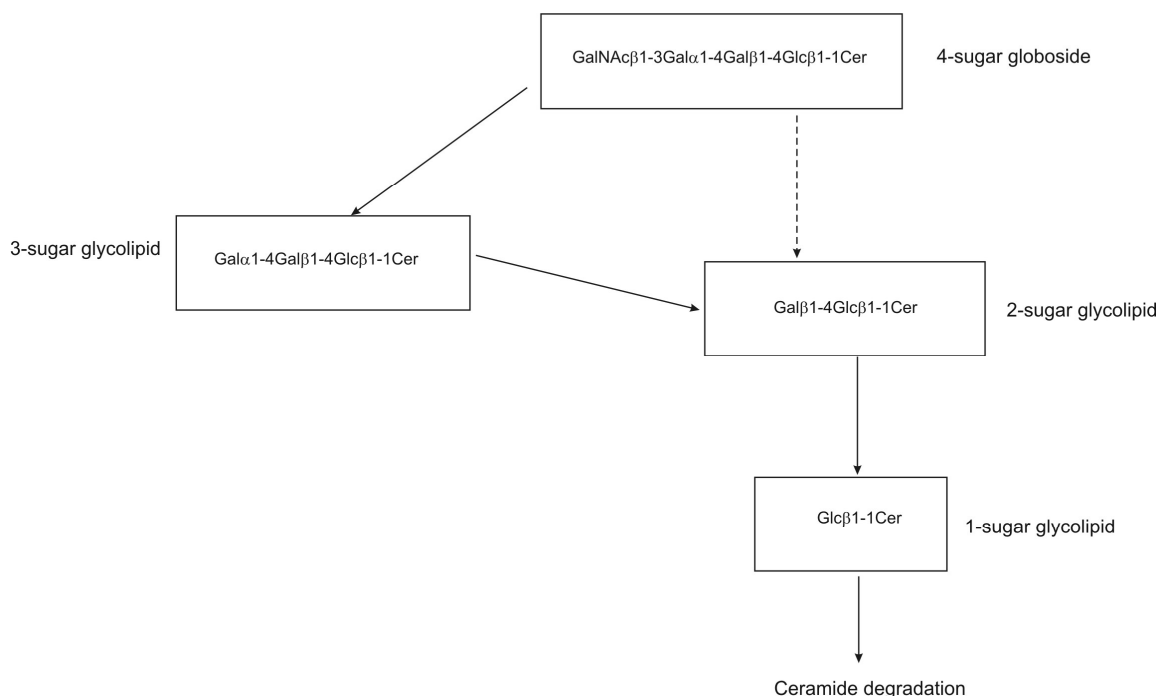


Figure 47: Probable degradation pathways of the globoside molecule. The arrow represent by broken line indicates a possible but less likely pathway.

It was found that the ceramide tail was not degraded until the 1-sugar glycolipid was reduced to the sphingolipid structure seen when globoside was incubated with concentrated acids or salt solutions that formed highly acidic solutions. The degraded products of globoside i.e. 3-, 2- and 1-sugar glycolipids (formed upon treatment with dilute acid and salts that formed acidic solutions) migrated to positions on the TLC plate corresponding to reference glycolipids suggesting that the ceramide tails were intact and undamaged by the action of acids. This also indicated that the sugars 'protect' the ceramide tail from the influence of acid by concentrating the initial cleavage and degradation reactions at the oligosaccharide portion of the glycolipid.

In addition, the degraded ceramide-based products being soluble in chloroform when partitioned with water and chloroform indicated that the reduced oligosaccharide chain (bearing 3-, 2- or 1-sugar(s)) was still attached to the ceramide tail in order to be soluble in

the non polar chloroform phase upon partitioning. In contrast, the sugar residues cleaved from the glycolipid tail would be found in the water phase due to their high polarity and therefore are not seen by TLC analysis which used only the chloroform partition.

Another indication that only the glycosidic bonds were cleaved by acidic solutions and that the sphingolipid tail of the globoside did not undergo degradation by 0.1 M HNO_3 over 24 hours was the absence of intense blue bands migrating towards the top-half of the TLC plate. These bands were indicative of ceramide (sphingolipid) degradation as seen in Figure 25, plate I, lanes 7 & 8.

Further evidence to suggest that the ceramide tail of the glycolipids were intact when incubated with an acid (HNO_3) concentration of 0.1 M or less was seen when no degradation was observed upon incubating ceramide (hydroxy and non hydroxy) samples with 0.1 M HNO_3 as shown in Figure 25, plate II, lanes 8 & 9.

Metal ions did not seem to catalyse the short term degradation of the globoside sample. Long term incubation of globoside with very low concentrations of Fe^{3+} and Cu^{2+} (from FeCl_3 and CuCl_2 respectively) gave positive results for degradation. It is possible that metal ions may be involved in catalyzing the degradation reaction. It is also possible that the G-4-4 degradation may be due to a composite effect induced by both metal ions and a slightly acidic medium.

Overview of Le^b acid hydrolysis

Using the information gained from the degradation of globoside, a series of experiments were planned for the more precious blood group active glycolipids, Le^b and ALe^b , previously isolated from the small intestines of human cadavers. Le^b is a relatively simple structure ($\text{Gal}(\text{Fuc}\alpha 1-2)\beta 1-3\text{GlcNAc}(\text{Fuc}\alpha 1-4)\beta 1-3\text{Gal}\beta 1-4\text{Glc}\beta 1-1\text{Cer}$) with antibodies available for most of the degradation products forecasted. These blood groups were investigated with a limited number of variables as suggested by the globoside experiments.

This time, we were able to demonstrate that;

The Le^b molecule, upon incubation with 0.1 M HNO_3 at 85°C over a period of 48 hours, underwent extensive degradation. The Le^c precursor and Le^a structures were readily formed upon the 1st hour of incubation. The Le^d glycolipid was also a likely product of Le^b hydrolysis but could not be detected at this stage due to the anti- Le^d reagent not being available at this time. Many investigators have acknowledged that even the mildest acidic conditions can readily cleave the fucosyl bonds (Baer et al., 1950; Rege et al., 1964).

However, the degradation of Le^b at 85°C over a 90-minute period with 0.1 M HNO₃ gave a clearer pattern of degradation with no further degradation of the Le^c or ceramide entity (Figure 32 vs. Figure 31). Compared with globoside (Figure 33), the Le^b is much more labile to hydrolysis by 0.1 M HNO₃ than globoside at 85°C (see Figure 32 and Figure 33). This lability is entirely due to the presence of α fucose bonds in Le^b.

The HPLC programme used to purify the Le^b glycolipids was also able to separate the hydrolysates of Le^b (Le^a + Le^c). This was further indication that the ceramide tails of the degraded glycolipids suffer minimal or no damage within 90 minutes of incubation with 0.1 M HNO₃. The products of degradation namely Le^a and Le^c stained with their corresponding antibodies and had *rm* values identical to the reference molecules.

Degrading the Le^b molecule over a range of HNO₃ concentrations led to the observation that higher concentrations of acid resulted in more rapid degradation of the glycolipid into the underlying structures, Le^a, Le^c and H-type 1, as seen in Figure 36.

Methanolysis of the Le^b sample with a range of methanolic HCl concentrations identical to the HNO₃ concentration range used earlier yielded the same products. The difference between acid hydrolysis and methanolysis is that the latter process cleaves glycosidic bonds to form methyl glycosides instead of sugars (or oligosaccharides) with non reducing ends. The methyl groups protect the sugars from undergoing any further hydrolysis and this is a major advantage of using the methanolysis method. The methanolysis method was not carried out in any other future experiments as it did not seem necessary to use this relatively more expensive method over acid hydrolysis with HNO₃. The methanolysis method is especially useful when analysing and identifying the monosaccharide residues and their anomeric linkages in the oligosaccharide chain using MS techniques.

Detailed Analysis of Le^b hydrolysis

The two fucose residues that occur as end groups (one terminal and the other internal) on the Le^b structure are readily removed when incubated with acid at 85°C. The resulting products, as a result, are H-type1 (Le^d), Le^a and Le^c with the Le^c structure being formed readily.

The probable degradation pathways for Le^b degradation are shown in Figure 48.

The results were visualised with anisaldehyde and immunostaining assays. The linear oligosaccharide chain (the Le^c type 1 precursor), after de-fucosylation, remained unchanged throughout most of the degradation process as seen by the positive Le^c bands in the immunostain plates. This suggested that the sugars in the Le^c structure bound by

β glycosidic bonds are relatively stronger than those binding the fucose residues. Le^c has the structure $\text{Gal}\beta 1-3\text{GlcNAc}\beta 1-\text{R}$ and according to the work of Wolfrom, β glycosidic bonds are more resistant to acid hydrolysis than the α counterparts (except for $\alpha 1-6$ linkage which is quite resistant to acid hydrolysis) (Wolfrom et al., 1963). The presence of an internal GlcNAc residue also very likely contributed towards the stability of the Le^c molecule in acid (Adams, 1965a).

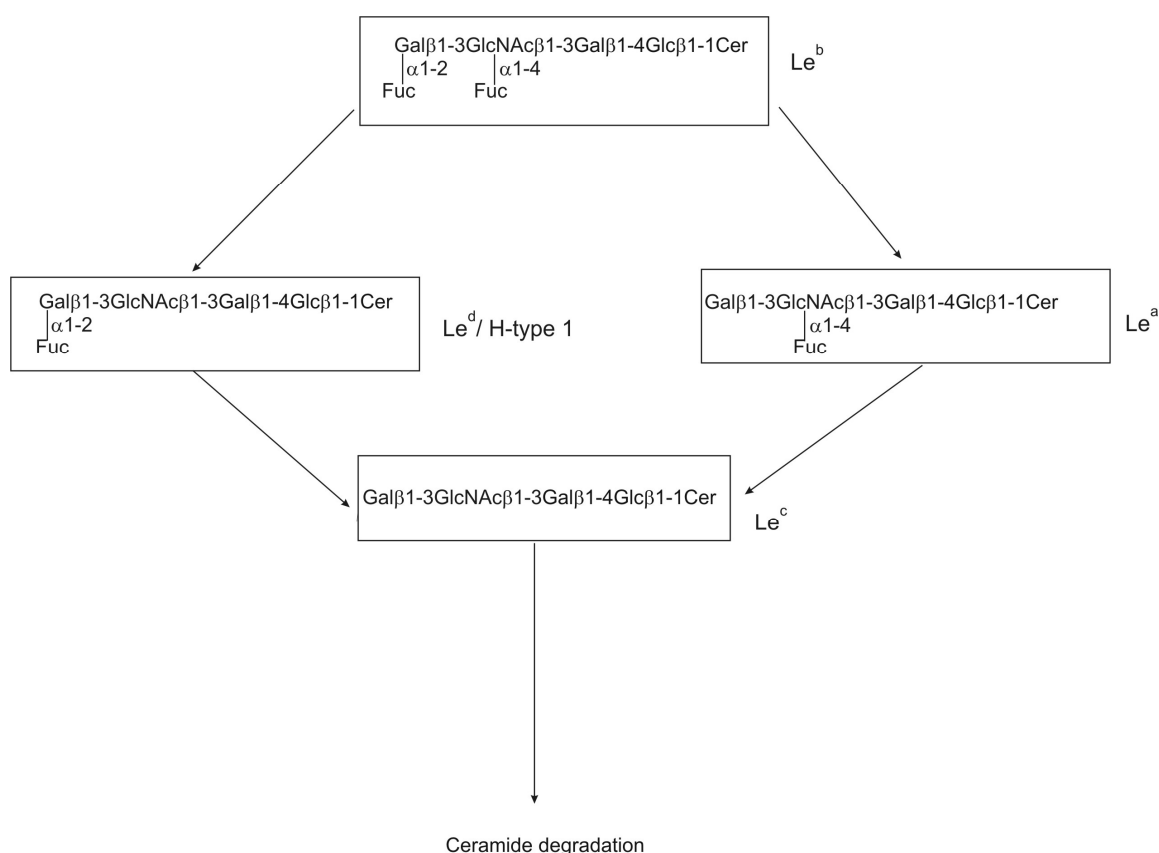


Figure 48: Probable degradation pathways of the Le^b upon hydrolysis with HNO_3 at 85°C .

The only noticeable extensive degradation of the Le^c molecule was observed when Le^b was treated with 0.1 M HNO_3 over a period of 48 hours. It is likely that the Le^c structure further degraded into the 3-, 2- and 1-sugar glycolipid moieties as shown by the presence of multiple bands with high TLC mobilities (Figure 31, plate I).

Hence, the results of Le^b degradation showed that after the removal of fucose residues, the cleavage of monosaccharide units occurred sequentially from the non reducing end of the oligosaccharide unit.

However, this project was not able to fully demonstrate the rate of production of either Le^a or Le^d; i.e. the preference of the acid to cleave the Fuc α 1-2 bond and the Fuc α 1-4 bond in the Le^b structure could not be established. It is nonetheless speculated that the α (1-2) bond may be more easily cleaved as it is situated on a more 'exposed' site on the oligosaccharide chain compared to the α (1-4) which has surrounding protective groups providing the 'steric hindrance' effect. This, in turn, makes it more difficult for radicals to reach and react with the α 1-4 fucosyl bond thereby cleaving it. This proposal is in agreement with Shallenberger whose study demonstrated that the (1-4) glycosidic bonds are more stable than the (1-2) bonds in acid solutions (Shallenberger, 1982). His rationale for the relative stabilities of glycosidic bonds in acidic solutions is that linkages are formed depending on the reactivity of the hydroxyl groups present on the different carbon atoms or are formed in order of their relative stabilities in an acidic solution. He demonstrated this concept with the glycosidic bonds formed between aldohexoses. This property can be extrapolated to glycosidic bonds formed between aldohexoses and de-oxy aldohexoses (in glycolipids) to predict degradation pathways/hydrolysis of glycosidic bonds in the glycan chains of glycolipids.

In addition, the Fuc α 1-4 residue may be possibly cleaved after the cleavage of the Fuc α 1-2 bond in the Le^b chain as the former is bound to an N-acetylated residue (GlcNAc) which forms relatively stable bonds in acid. In saying so, the Fuc α 1-4 is readily cleaved but as it is bound to an N-acetylated sugar, its cleavage may occur after the hydrolysis of the Fuc α 1-2 bond.

The degradation of the 'contaminant Le^b' present in ALe^b sample was better able to demonstrate the preference of cleavage of the two fucosyl bonds discussed above (see later). The presence of Le^a (5-sugar glycolipid) but the absence of the H-type 1 (5-sugar glycolipid) was a fair indication that the Fuc α 1-2 glycosidic bonds were cleaved before the Fuc α 1-4 bond and these results are consistent with the findings of a few researchers who studied the acid stabilities of aldohexoses in acids (Shallenberger, 1982; Wolfrom et al., 1963). This indicates that the Fuc α 1-4 bonds are relatively more stable than the Fuc α 1-2 glycosidic bonds in acid conditions. This preference in cleaving the two different glycosidic bonds binding the fucose residues was not seen with Le^b degradation experiments discussed earlier where the Le^a and the H-type 1/(Le^d) structures were observed to be generated at about the same rate with these experiments.

Hence, the attempt to control the acid hydrolysis of the Le^b molecule was not entirely successful; incubating the Le^b structure with varying concentrations of HNO₃ resulted in a

mixture of degraded products. However, the degraded components of the mixture can be separated by HPLC as shown in Figure 34. Another advantage of HPLC separation is that the components of interest can be readily separated from the unspecified products formed during the hydrolysis method.

However, it is not known how the points of attachment of the fucose residues to the Gal and GlcNAc residues are affected by the acid. It is reasonable to state that upon hydrolysis, an element of water is added across the glycosidic bond and the site of attachment (carbon 2 of Gal or carbon 4 of GlcNAc) of the fucose residue most probably resumes its hydroxyl group. From the immunostain assay, the ability of the specific antibodies to readily detect the products of Le^b degradation is an indication that the precursor molecules obtained from acid degradation, at worst, are only minimally disrupted in antigenic structure.

Overview of the acid hydrolysis of the ALe^b structure

The ALe^b entity, GalNAc α 1-3Gal(Fuc α 1-2) β 1-3GlcNAc(Fuc α 1-4) β 1-3Gal β 1-4Glc β 1-1Cer, being a 7-sugar glycolipid structure added more complexity to the degradation pathway.

The terminal sugar residues (GalNAc and two fucose residues occurring as terminal end groups) are bound to the oligosaccharide sequence by α glycosidic bonds. The effects of varying concentrations of acids over a range of temperatures were investigated to establish if these glycosidic bonds were cleaved according to some pattern or if the bonds were randomly broken. The following have been observed from the many acid hydrolysis processes the ALe^b structure was subjected to;

A temperature of 50°C and 0.05 M HNO₃ was not able to degrade the ALe^b sample over a period of 120 minutes. Slight degradation of the ALe^b into an A-like structure was observed at 60°C with the same acidic treatment after 16 hours as detected by the anti-A serum (Figure 39). There was no evidence of sphingolipid degradation as intense blue bands migrating towards the top half of the TLC plate were absent.

Similarly, at 70°C, and treatment with 0.05 M HNO₃ over 16 hours, the A-type 1 structure was detected by the anti-A reagent. The Le^a, Le^c and Le^d structures were not generated (Figure 40). Again there was no evidence of sphingolipid degradation.

However, treatment of the ALe^b sample with 0.10 M HNO₃ at 70°C over a period of 120 minutes led to the formation of both A-type 1 and A-like structures from as early as 30 minutes. Again, other Lewis structures were not formed and most of the ALe^b structure remained intact (Figure 43). There was no evidence of sphingolipid degradation.

At 85°C and incubation with 0.1 M HNO₃, ALe^b readily degraded into the A-type 1 and A-like structure (5- or 6-sugar) over 120 minutes. The Le^c structure was also generated. There were also bands, non reactive with anti-A, migrating to a position similar to that of 2-sugar glycolipids at around 90 minutes on the TLC plate (Figure 45). There was no evidence of sphingolipid degradation.

Identical treatment of ALe^b over a period of 96 hours caused further degradation of the glycolipid. The entire ALe^b sample had degraded within 96 hours. The presence of Le^c (type 1 precursor) at 96 hours of incubation indicated that the terminal disaccharide sequence, Galβ1-3GlcNAc in this structure was relatively stable unlike the other Lewis structures. This precursor molecule is likely to be the degradation product of both the ALe^b and the 'contaminant Le^b' molecule. There was also evidence of 1-sugar and 2-sugar glycolipids forming from these parent structures as shown by bands migrating to the appropriate positions on the TLC plate (Figure 46). The intense blue bands representing ceramide degradation were not present.

The acid hydrolysis of the ALe^b structure did not generate the H-type 1(Le^d) structure.

Detailed analysis of ALe^b degradation

Reducing the ALe^b structure, a seven-sugar glycolipid, into the simpler structures provided a more complex scenario for the glycolipid degradation process. This structure has two terminal fucose residues present as side groups that are bound by α glycosidic bonds and as well as a terminal non reducing GalNAc residue bound to the oligosaccharide chain by an α (1-3) glycosidic bond. All results obtained from ALe^b degradation experiments predominantly yielded A-like structures but it is possible that Le^c structure was also generated under extensive degradation conditions as shown in Figure 45 and Figure 46, plate III.

An overview of the ALe^b degradation pathway observed is shown in Figure 49 and is discussed in detail as follows.

One or both fucose residue(s) in the ALe^b glycolipid were observed to be readily removed in acidic conditions (see Results, Section B, Acid Hydrolysis of A Lewis b (ALe^b) glycolipids). However the order of removal of the fucose residues in the ALe^b structure could not be established.

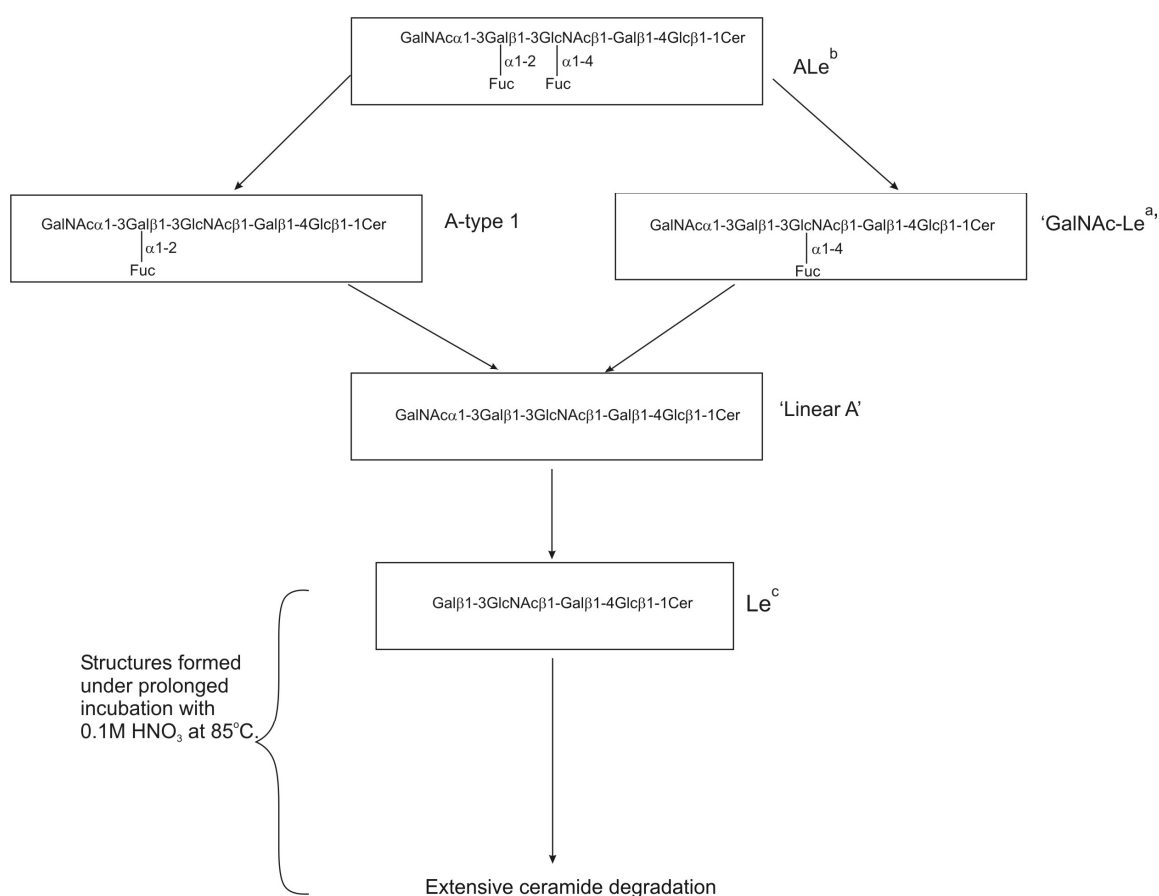


Figure 49: Probable degradation pathways of ALe^b upon hydrolysis with HNO₃ at 85°C.

The A-type 1 structure was recognised as one of the first products of ALe^b degradation. It is also possible that the hydrolysis of the ALe^b glycan can generate two other unnatural glycolipids 'GalNAc-Le^a' and 'linear A'. These products were detected by the anti-A ALBA clone reagent which has specificity for all A-determinants of all chain types (Figure 42 and Figure 44).

The anti-A-type 1 reagent has a specificity for the GalNAcα1-3Gal(Fucα1-2)β1-3GlcNAc-sequence and was therefore readily able to detect the A-type 1 structure formed upon hydrolysis of the ALe^b glycan. Because the other two possible products (unnatural glycolipids, 'GalNAc-Le^a' and 'linear A') also consist of the linear A-type 1 chain, it is possible that the anti-A-type 1 reagent may be reacting with these structures albeit the reaction may be mild due to the absence of the fucose (in 'linear A' structure) and the presence of fucose in a slightly different position (in 'GalNAc-Le^a'). A study carried out by Yakota's group was able to isolate a novel 5-sugar A-like glycolipid from a blood group O individual with hepatocarcinoma; this A-like glycolipid was found to have a linear sugar

sequence of HexNAc-Hex-HexN-R which was proposed to be the pentasaccharide GalNAc α 1-3Gal β 1-3GlcNAc-R sequence (R is the common inner disaccharide). An anti-A reagent was able to react with this defucosylated linear A sequence suggesting that the absence of fucose does not completely inhibit an anti-A reagent's ability to detect structures with the linear A-determinant chain (Yokota et al., 1981). Based on this observation, it is very likely that the anti-A-type 1 reagent used in the current project was able to detect both 'GalNAc-Le^a' and 'linear A' structures to some extent.

The fucose residue, although not a primary determinant of the A and B specificity, contributes towards the complete determinant structure. Painter's group demonstrated that the presence of fucose as a side group increased an anti-B reagent's ability to agglutinate than when the fucose was not present (Painter et al., 1965). They were also able to demonstrate that an anti-A reagent had the same ability to agglutinate both fucosylated and defucosylated type-A oligosaccharide sequences (di- tri and tetrasaccharides). Hence antibody binding could be observed both in the presence and absence of the fucose side groups. This is also supported by Schiffman's study which reported that the linear (defucosylated) trisaccharide sequence, GalNAc α 1-3Gal β 1-3GlcNAc-, is the antigenic determinant of blood group A specificity. (Schiffman et al., 1962).

Hence both the anti-A ALBA-clone and the anti-A-type 1 reagents could be detecting the linear defucosylated structure, (GalNAc α 1-3Gal β 1-3GlcNAc β 1-R) and/or the mono-fucosylated structures, GalNAc α 1-3Gal(Fuc α 1-2) β 1-3GlcNAc β 1-R and/or GalNAc α 1-3Gal β 1-3GlcNAc(Fuc α 1-4) β 1-R.

The results of the ALe^b can be analysed as follows;

Upon acid hydrolysis, the labile fucosyl glycosidic bonds in the ALe^b molecule can be cleaved to form 3 possible structures that could be binding with the anti-A reagent and are listed as follows;

Molecule 1 = GalNAc α 1-3Gal(Fuc α 1-2) β 1-3GlcNAc β 1-R = (A-type 1),

Molecule 2 = GalNAc α 1-3Gal β 1-3GlcNAc(Fuc α 1-4) β 1-R = (GalNAc-Le^a) or

Molecule 3 = GalNAc α 1-3Gal β 1-3GlcNAc β 1-R = ('linear A')

From the results, it was observed that the removal of fucose residues in the ALe^b sample was random and consequently generated any of the three structures (molecules 1, 2 & 3) mentioned above at any given time. However, because the different glycosidic bonds involving sugar residues of varying properties have varying bond labilities in acid, there must be some form of order of cleavage of these bonds. The most logical pattern of

cleavage has been described in Figure 49 and is discussed as follows.

The cleavage of the Fuc α 1-4 glycosidic bond in the ALe^b structure would form the A-type 1 structure (molecule 1). This was shown by positive staining with the anti-A-type 1 reagent with most immunostains.

On the other hand, cleavage of the Fuc α 1-2 bond would result in the formation of the unnatural 'GalNAc-Le^a' structure (molecule 2). This, like the A-type 1, is also a 6-sugar glycolipid and therefore would have an identical TLC mobility (*rm* value) as the A-type 1 structure. However, it is not known whether this structure is recognized by the anti-A reagents but it is speculated that it could be due to the presence of the linear A-determinant oligosaccharide sequence, GalNAc α 1-3Gal β 1-3GlcNAc β 1- .

Further, the glycosidic bonds involving both fucose residues can be cleaved at about the same time owing to the fact that the fucosyl bonds are extremely labile in acidic environment. This would generate a 5-sugar (unnatural) glycolipid, 'linear A' (molecule 3) which was possibly recognized by the anti-A ALBA clone reagent as shown in Figure 44, plate VII. The lability of fucose bonds in the ALe^b glycolipid upon acid treatment is in agreement with many researchers who have shown that these bonds are readily cleaved upon incubation in mild acidic conditions (Adams, 1965a; Biermann, 1989; Kennedy et al., 1988).

According to the work of Shallenberger and Wolfrom, α 1-2 glycosidic bonds are most labile to acid hydrolysis (Shallenberger, 1982; Wolfrom et al., 1963). The fact that the α 1-2 bond involves a de-oxy residue (de-oxy aldohexose namely fucose) may further contribute towards the lability of this particular bond in acid solutions. Hence, molecule 2 is most likely to be readily formed upon acid hydrolysis of ALe^b. Its migration on the TLC plate will be identical to that of molecule 1 as both are 6-sugar glycolipids hence would be difficult to tell apart from the A-type 1 structure.

Another rationale for the fucose bound by the α 1-2 glycosidic bond to be removed first is that this fucose residue is present at a more 'exposed' site which is easily accessible by acid radicals compared to the other fucose residue bound to the inner GlcNAc by α 1-4 bond. This inner fucose residue is sterically hindered hence protected from the acid radicals. The Fuc α 1-4 bond, as a result is possibly cleaved after the removal of the fucose bound to the sub terminal Gal residue.

In addition, the inner fucose residue being bound to an N-acetylated sugar, GlcNAc, most likely makes the Fuc α 1-4GlcNAc bond slightly more stable to acid conditions (than the

other fucose bound to a neutral sugar, Gal) as glycosidic bonds involving N-acetylated sugars are more stable in acidic conditions (Biermann, 1989).

However, the Fuc α 1-4 bond is also eventually cleaved on the basis that it also involves a de-oxy sugar which adds towards the susceptibility of the α glycosidic bond. This cleavage most likely occurs once the terminal (α 1-2) fucose residue is removed and results in molecule 3. This structure being a 5-sugar glycolipid is expected to migrate slightly further up the TLC plate compared to the 6-sugar A structures molecule 1 and molecule 2. This is clearly shown in Figure 42 and Figure 44.

Despite the rationale for the cleavage of the varying fucosyl bonds in the ALe^b glycolipid, the results obtained showed a random cleavage of these linkages. The presence of the A-type 1 structure consisting of the 'more labile' Fuc α 1-2 glycosidic bond is sufficient evidence to suggest that the molecule 2 ('GalNAc-Le^a' consisting of the relatively 'more stable' Fuc α 1-4 bond) was also formed. These structures, as discussed earlier, would have been readily reduced to the common 5-sugar glycolipid ('linear A') as detected by the anti-A ALBA clone reagent (Figure 44, plate VII).

The bands stained with anisaldehyde in the region of 6-sugar glycolipid (A-type 1 structure) did not necessarily show the distinct green colour which indicates the presence of fucose residues in the glycolipid. It must be noted, however, that the anisaldehyde-stained TLC plates were over baked in order to make each band representing a degraded product more visible. In doing so, this green colour may have been masked by a deeper brown-black band present at *rm* 5.0 \pm 0.5 (region of 6-sugar glycolipid band staining).

Further, the Le^b structure Gal(Fuc α 1-2) β 1-3GlcNAc(Fuc α 1-4) β 1-R is unlikely to be a possible degradation product of ALe^b since the terminal non reducing GalNAc residue is bound by more acid resistant glycosidic bonds (N-acetylated sugars form relatively more acid resistant bonds). This bond can only be cleaved under relatively strong acidic conditions and after the deacetylation of the GalNAc residue. On the other hand, the glycosidic bonds including the fucose residues are extremely labile to even the mildest acidic environment and are readily cleaved under such conditions. In other words, the GalNAc bond cannot be cleaved before the acid labile fucosyl bonds. Hence Le^b, Gal(Fuc α 1-2) β 1-3GlcNAc(Fuc α 1-4) β 1-R, is not a viable product of ALe^b degradation. Based on this same explanation, the H-type 1 and Le^a structures were not generated from ALe^b hydrolysis.

These results are in agreement with the findings of many researchers who state that the presence of an N-acetyl group makes the glycosidic bond relatively resistant to acid hydrolysis and often become de-acetylated in extremely acidic environment while glycosidic bonds linking fucose residues are acid-labile and are cleaved in the mildest acidic conditions (Adams, 1965a, , 1965b; BeMiller, 1967; Biermann, 1989; Kennedy et al., 2004; Kennedy et al., 1988; Lindberg et al., 1975; Rege et al., 1963).

It is important to note that the terminal GalNAc (bound by an α glycosidic bond) residue in the ALe^b structure was relatively difficult to cleave due to the milder acid hydrolysis conditions employed compared to those used for the hydrolysis of the globoside structure. However, upon treatment with 0.1 M HNO₃ at 85°C over 120 minutes and 96 hours may have cleaved this residue (after the removal of fucose residues) from the 'linear A' structure (molecule 3) to form the Le^c structure. It is also possible that upon extreme acidic conditions, the Le^c may have further reduced to 3-, 2- and 1-sugar glycolipids. From the anisaldehyde stain, it can be seen that the oligosaccharide chain of the ALe^b underwent extensive degradation indicated by the multiple bands present on the TLC plate (Figure 46, plate I).

The cleavage of the Glc-ceramide bond was not observed and this would have resulted in extensive degradation of the sphingolipid tail. This was not observed as the intense blue bands indicating ceramide degradation were absent. Many studies have suggested that this Glc-ceramide bond is not readily cleaved in the relatively milder acidic conditions employed in this project (Hakomori et al., 1961; Kannan et al., 1974). Such a cleavage would require treatment of glycolipids with 1M HCl at 100°C over 12 hours (Kannan et al., 1974). Other researchers have utilized more robust methods of releasing the oligosaccharide sequence from the ceramide and this included osmium-catalysed periodate oxidation followed by mild alkali treatment (Hakomori, 1966; Yang et al., 1971) and ozone oxidation (Morrison, 1994). The β 1-1 linkage between the glycan and ceramide can also be hydrolysed by using a strong alkali catalyst (Biermann, 1989).

Further, the immunostain of the degraded ALe^b samples against the anti-B serum was negative (results not shown) and this indicated that the terminal GalNAc residue of the ALe^b glycolipid did not undergo de-acetylation and deamination processes to reduce the terminal GalNAc into Gal thereby forming the B-like structures.

The degradation patterns obtained from the acid hydrolysis of a BLe^b structure would have been ideal to compare with the ALe^b degradation. The difference between the two structures is that the B molecule has a terminal non reducing Gal residue instead of a

GalNAc residue. The terminal Gal being a neutral sugar is thought to be relatively easily cleaved from the oligosaccharide sequence compared to the GalNAc residue (A blood group determinant) after the cleavage of the two fucosyl bonds. The product of the cleavage of the Gal and fucose residues from the BLe^b structure would be the precursor, Le^c structure. An investigation into the isolation and characterization of A and B glycolipids by Hakomori and co-workers showed that the terminal Gal residue on type B glycolipids is removed much more easily than the terminal GalNAc residue on the type A glycolipids (Hakomori et al., 1961). A comparison study in degradation mechanisms between ALe^b and BLe^b was not carried out due to the unavailability of the BLe^b structures. However, this study is highly recommended as valuable information on glycolipid degradation mechanisms by acid hydrolysis can be obtained.

Degradation of 'contaminant Le^b' structure in ALe^b sample

The ALe^b sample, as mentioned earlier, contained some Le^b that could not be separated upon purification. The Le^c structure may have been the product of acid hydrolysis of both the 'contaminant Le^b' and ALe^b as discussed earlier (plate III, Figure 45 and Figure 46). The Le^b degradation is evident from the gradual darkening of positive Le^a and Le^c bands with subsequent and simultaneous weakening of the Le^b and ALe^b bands in their respective immunostains as seen in Figure 45 and Figure 46.

It is also likely, that the Le^c formed may have been further degraded into 3-, 2- and 1-sugar glycolipids.

General analysis of results

Stability of ceramides

The ceramide entity of globoside and Lewis glycolipids did not seem to undergo any extensive degradation as the noticeable blue bands representing the degraded ceramide products were absent in the anisaldehyde stain.

There are three distinct sites on a ceramide chain that can 'attract' acid radicals to cause the ceramide tail to degrade (Solomons, 1996). These are;

- § The amide linkage that joins the long chain base to the fatty acid molecule which is fairly stable but can undergo hydrolysis under extreme basic or acidic conditions. These conditions were not used in the current project.
- § The hydroxyl groups present on the long chain base that can undergo oxidation to form carbonyl groups and

§ The alkene bonds (double bonds) of unsaturated sphingolipids can also undergo oxidation reactions thereby cleaving the bonds in the aliphatic tail.

Any cleavage in the aliphatic ceramide tail would cause the entire glycolipid to migrate differently on the TLC plate. This was not observed with the products of the acid hydrolysates of the globoside and Lewis structures. The migration of glycolipids on a TLC plate is largely dependent upon the sugar sequence. However, the sphingolipid portion also has some influence on the migratory distance; i.e. a shorter ceramide tail would make the glycolipid structure more polar hence would migrate to a position closer to the point of origin while a glycolipid with an identical oligosaccharide sequence but a longer ceramide tail would result in an overall relatively less polar structure and migrate further up the TLC plate.

The polar groups (hydroxyl groups) in a glycolipid are concentrated on the glycan unit. Hence the polar acid radicals must be initially 'attracted to' and consequently interacting and cleaving glycosidic bonds at the glycan end, in particular. The sugar chain, consequently, seems to protect the sphingolipid chain and only undergoes degradation by acid hydrolysis after the complete breakdown of the oligosaccharide chain. This was proved by the action of 0.1 M HNO₃ on ceramides and globoside at 85°C over a period of 24 hours. Ceramides did not degrade under these conditions but the globoside was readily broken down into 3- and 2-sugar glycolipid molecules (Figure 25, plate II). The removal of the sugar residue bound to the hydrophobic ceramide tail must make the entire ceramide entity susceptible to degradation by acid hydrolysis. However, the sugar-ceramide bond is very strong and is not readily cleaved. Upon cleavage of this bond, the hydroxyl group, amide linkage between the fatty acid and long chain base together with the double bonds in the aliphatic chain become exposed and are consequently degraded by the acid.

Alkaline degradation and stability of fucose and N-acetylated sugars in alkali

Early studies carried out by Kabat on glycoprotein structures showed that the treatment of blood group active oligosaccharides with alkali selectively degrades the internal GalNAc residues together with the serine and threonine residues (Kabat et al., 1965). They were not able to establish if these internal GalNAc residues were bound to the amino acids but proposed that it was a distinct possibility. In other words, he suggested that these amino acids may be linked to the internal GalNAc residue by glycosidic bonds and the destruction of these amino acids by alkali resulted in the release of this sugar by a β -carbonyl elimination reaction. The oligosaccharide chains liberated by this reaction are further

degraded by a 'peeling' reaction from the reducing end. Another possibility was that the amino acids may be attached to some other sugar and the peeling degradation of the of the oligosaccharide may occur and proceed to include the internal GalNAc residue (Kabat et al., 1965). Lloyd further reported that the sugars in blood group substances (glycoproteins) are bound to serine and threonine residues by alkali-labile glycosidic bonds and that the GalNAc residue was mostly found to be involved in such linkages (Lloyd et al., 1966)

Another study by Painter's group on glycoproteins suggested that the 1-3 linkage at the reducing end of the type 1 blood group active A and B tetrasaccharide sequence is extremely alkali-labile and undergoes subsequent degradation (Painter et al., 1965). This was also suggested by Morgan and Bemiller (BeMiller et al., 1962; Morgan, 1960). Bemiller in his investigations (with polysaccharides containing amino sugars) showed that the 1-3 glycosidic bond formed between an acetylated amino sugar and a neutral sugar (Glc β 1-3GalNAc) was readily cleaved to form Gal and an unknown product while the 1-4 glycosidic bond in Glc β 1-4GalNAc was stable in dilute alkali (BeMiller et al., 1962). This study suggested that it is the linkage rather than the acetylated monosaccharide that contributes towards lability in alkaline solutions.

However, Karlsson's investigations have shown that the glycosidic bonds in the glycan unit of glycolipids are relatively resistant to alkali (Karlsson, 1970). His methods of extracting glycolipids involves a step where the crude glycolipids are subjected to 2 Molar potassium hydroxide (KOH) solution over 24 hours which removes the esters of phospholipids and triglycerides. Although this saponification step removes major lipid contaminants from glycosphingolipids, it can readily degrade alkali-labile structures such as the O-acyl groups (Schnaar, 1994). Hence mild alkaline degradation should be avoided when dealing with such glycolipids (Schnaar, 1994). Morgan also supported this argument by suggesting that strongly acidic and alkaline reagents should be avoided when isolating and purifying active blood group substances. This, in turn, will prevent the molecules of interest from undergoing undesirable irreversible reactions (Morgan, 1960).

In contrast to acidic conditions, fucose residues have been found to be stable to alkaline hydrolysis and blood group active oligosaccharide chains consisting of fucose residues have been isolated using alkaline hydrolysis (Karlsson, 1970; K. Lloyd et al., 1966; Rege et al., 1964). The blood group substances employed by these researchers were glycoproteins.

Alkaline degradation techniques hence can be utilised to preserve fucose residues and at the same time cleave the alkali-labile 1-3 glycosidic bond involving N-acetylated sugars such as GalNAc and GlcNAc in glycoproteins as discussed earlier. Much is not known about the nature and behaviour of monosaccharides and their glycosidic bonds in glycolipids and further investigation into this would be highly recommended. The methods can be adapted from those used in glycoprotein and polysaccharide analyses.

For example, the Le^b structure can be obtained from the ALe^b glycolipid by cleaving the GalNAc residue by alkaline hydrolysis (based on 1-3 glycosidic bonds being alkali labile) thereby forming the Le^b structure and then further subjecting this Le^b to mild acid hydrolysis to remove the fucose residues to form the valuable Le^c /type 1 precursor.

Cleavage of sugars by acid hydrolysis

Glycosidic bonds have varying susceptibility to hydrolysis which is dependent on a combination of factors such as the anomeric configuration of the glycosidic linkages, position of the glycosidic bonds and the type of sugar residues involved in the linkage (e.g. de-oxy sugars, neutral sugars or N-acetylated sugars).

To date there has been no systematic study carried out based on the acid hydrolysis of Lewis blood group antigens. The results obtained from this project showed that Le^a, Le^d and Le^c can be obtained from simple and economical mild acid hydrolysis of the Le^b structure. On the other hand, hydrolysis of the ALe^b glycan generated the A-type1 glycolipid together with two other unnatural glycolipids discussed earlier. The Le^c glycolipid was only formed from ALe^b hydrolysis after prolonged treatment with 0.1 M HNO₃. This intensive treatment may have caused some degradation of the of the Le^c glycolipid.

Over the years, researchers have, employed several hydrolysis methods carefully engineered to isolate and degrade oligosaccharide structures from glycoconjugates containing a range of monosaccharides bound by glycosidic bonds of varying acid and alkaline lability. Their main objective was to use the techniques to assist in structural analysis.

In acid hydrolysis/partial acid hydrolysis, it has also been reported that the sugar residues in the oligosaccharide chain of a glycoconjugate are cleaved in a stepwise or sequential manner (Kabat et al., 1948). The cleavage of the glycosidic bonds in glycolipids was also observed to occur from the terminal non reducing end in the current research. There are no reported studies stating that the cleavage of monosaccharide residues in the oligosaccharide chain occur from the reducing end upon acid hydrolysis but many studies have isolated monosaccharides and disaccharide units from the non reducing end using

acid hydrolysis procedures. One example is the study carried out by Kabat and workers who showed that there is a loss of blood group activity upon heating A and B substances in an acidic medium and the degraded substance (the glycoconjugate) had the ability to agglutinate Type XIV anti-pneumococcal serum (Kabat et al., 1948). This serum binds with the (inner) Gal β 1-3GlcNAc disaccharide sequence (Barker et al., 1961) which is revealed after the cleavage of the terminal (non reducing) GalNAc (or Gal) and fucose residues when the A (or B) substance is treated with acid (Kabat et al., 1948).

The ability to remove the terminal non reducing A determinant (GalNAc residue) from the oligosaccharide sequence was achieved by treating the A substance with 1 M HCl at 100°C for 2.5 hours (Kabat et al., 1948). In contrast, milder acid hydrolysis conditions were utilised in the current project (0.1 M HNO₃, 85°C) and the Le^c was still observed to be a product of ALe^b hydrolysis. This is also further evidence suggesting that the outermost, non reducing blood-group determining sugar residues are removed to 'expose' the inner defucosylated structure common to both A and B blood groups i.e. the Le^c oligosaccharide sequence. Kabat also suggested that the fucose residues are readily removed from the oligosaccharide sequence by acid hydrolysis of the glycoconjugates and that the removal of fucose residues does not affect the hydrolysis of the main carbohydrate chain; fucose occurs as end groups and does not form an integral part of the main polysaccharide chain (Kabat et al., 1948). He showed that (partial) acid hydrolysis of the oligosaccharide chain of blood group active A and O substances was found to cleave sugars in an orderly fashion with the fucose residues being split off readily.

Limitations of this study

There were a few limitations experienced during the course of the project. This led to the inability to establish a few concepts. They are as follows;

- § The structural studies of the degraded products were not carried out due to the unavailability of equipment in the lab. However, the relative migrations of the degraded products were compared with and found to be almost identical to the reference molecules. The ability of antibodies to react with and identify the products of the Lewis glycolipid degradation further proved that the antigenic oligosaccharide sequence was undergoing chemical modification by 'clean' scission of the glycosidic bonds. In other words, the cleavage of glycosidic bonds removes sugars from the non reducing end with minimal damage to the adjacent sugar residues. The immunostain assays showed that these degraded products migrated to identical positions on the TLC plate and stained appropriately with the

corresponding antibody. Structural studies, at this point, would have been ideal to confirm the results of the immunostain assays but were not necessary.

- § The glycolipids were purified in-house from biological samples and there were trace amounts of 'contaminant' glycolipids in the mixture i.e. the globoside sample contained 3-sugar precursor glycolipids while the ALe^b sample had trace amounts of Le^b and A-type 1 'contaminants'. In an ideal experiment, absolute pure molecules would have been used. The presence of contaminants would interfere with the results as they have the tendency to breakdown in the same manner as the glycolipids of interest thereby interfering with the degradation products. However, these contaminant molecules were present inevitably in the sample and the interpretation of the results took these small interferences into consideration.
- § Specific antibodies against the products of globoside degradation (3, 2- and 1- sugar glycolipids) and those of ALe^b hydrolysis namely 'GalNAc-Le^a' and 'linear A' were not available to positively identify the presence of these structures. The anti-A ALBA clone reagent seemed to react with these novel structures.
- § The glycolipid molecules were classed by their known TLC migrations. The exact migrations of unnatural glycolipids (such as 'linear A' and 'GalNAc-Le^a') are not known. It must be recognised that some glycolipids may have unexpected TLC migrations and hence their identification cannot be based solely on TLC migrations e.g. the para-Forssman glycolipid migrates to a 6-sugar region even though it is a 5-sugar glycolipid (M.Breimer, personal communication).
- § The acid hydrolysis experiments required large quantities of glycolipids and hence were restricted to those available.
- § It should be borne in mind that due to the developmental nature of methods in this project the amount of acid used for degradation experiments varied from earlier to later experiments. Future experiments will need to ensure that the concentration of the acid is kept consistent in order to closely compare, analyse and control the glycolipid degradation pathways and products.
- § The relative acid liabilities of the two fucosyl bonds could not be thoroughly established although it is known that the α 1-2 glycosidic bond in aldohexoses is more labile than the α 1-4 glycosidic bond. This property could be extrapolated to glycosidic bonds involving de-oxy aldohexoses.

- § The concepts developed in the project do not apply to acidic, sulphated and other types of derivatised sugar residues present in glycolipids. The bond cleavage mechanisms in these structures still need to be investigated.

Summary

From the results of the degradation experiments of the type 1 Lewis blood group structures Le^b and ALe^b and as well as the erythrocyte-membrane glycolipid, globoside, the following principles of glycolipid breakdown have been established;

The degradation of glycolipid is not catalysed by the metal ions or at least their contribution towards the degradation process is minimal; rather acids are responsible for the cleavage of glycosidic bonds linking the sugars in the oligosaccharide unit in the glycolipid

The cleavage of sugars occurs in an orderly fashion. The fucose bonds being most labile to acid are cleaved readily followed by cleavage of the sugars from the non reducing end. The removal of fucose residues from the oligosaccharide chain does not affect the appreciable hydrolysis of the latter (Kabat et al., 1948). Conversely, N-acetylated residues present as the terminal non reducing sugar (in ALe^b) were seemingly resistant to the acid hydrolysis conditions utilised in this project and were only cleaved upon prolonged incubation with acid at 85°C. There is sufficient evidence shown by the work of Kabat and Barker that suggest that the bonds binding these terminal N-acetylated sugars can be cleaved by employing extremely acidic conditions and higher temperatures (Barker et al., 1961; Kabat et al., 1948).

The sphingolipid tails of glycolipids are not affected by HNO_3 concentrations at and below 0.1 M but are readily degraded at higher concentrations.

The defucosylation of Le^b by acid seemed to occur randomly although Le^a and Le^c were more predominantly produced. There is a rapid appearance of Le^c , H-type 1 and Le^a ; products that can be readily separated and purified by HPLC. The Le^c structure seemed to be a relatively robust structure as the sugar residues are bound by stronger β glycosidic bonds.

In contrast, some of the results obtained from Le^b degradation indicated that the $\text{Fuc}\alpha 1-2$ bonds are cleaved before the $\text{Fuc}\alpha 1-4$ bonds. This may be due a number of reasons such as

- § steric hindrance experienced by the internal fucose,

- § the internal fucose bound to an N-acetylated residue (GlcNAc) which is relatively stable in acid and
- § the α 1-2 glycosidic bonds are more readily cleaved in acidic solutions than the α 1-4 bonds (Shallenberger, 1982; Wolfrom et al., 1963).

The acid hydrolysis of ALe^b degradation seemed to occur in a different manner and was able to generate A-like structures; namely, A-type 1, 'GalNAc-Le^a' and 'linear A' (molecules 1, 2 and 3 respectively). The 6-sugar A-type 1 and 'GalNAc-Le^a' glycolipids can be further separated by HPLC and utilised in a number of biological assays. The 'linear A' structure GalNAc α 1-3Gal β 1-3GlcNAc β 1-R (molecule 3), is of unknown biological importance but, if desired, a fucosyl transferase enzyme can be employed to conjugate a fucose residue to the sub-terminal Gal by an α 1-2 glycosidic bond to form the A-type 1 structure.

The behaviour of glycolipids in varying acidic conditions investigated in this experiment have shown that slight changes in critical factors such a temperature and acid concentrations can result in structural modifications of the glycolipids of interest. The variables need to be carefully monitored to avoid unwanted degradation of the glycolipids.

This project was able to bring together a few degradation concepts of blood group glycolipid by the way of acid hydrolysis. Very little is known about the degradation mechanisms of blood group active glycolipids. Investigations into their breakdown mechanisms have not been carried out since the 1980s and the ability to degrade blood group antigens into their precursor forms by acid hydrolysis processes has not gained much attention.

The methods of acid hydrolysis used here highlighted that certain glycosidic bonds, sugar residues and the ceramide entities of glycolipids are sensitive to a range of chemical conditions.

Although this thesis focussed on preparing precursor by acid hydrolysis it is tempting to theorise that some of the unnatural molecules described in this work may actually occur naturally. For example, although 'linear A' and 'GalNAc-Le^a' are not known to occur naturally, in the stomach where the pH is less than 2, these structures could easily result from ALe^b. Additionally, the formation of Le^c from Le^b, although a natural molecule is also possible in phenotypes which do not normally express significant levels of this antigen. This raises some interesting questions. Is it possible that over exposure to an acid

environment (e.g. during stomach ulceration) may create new receptors which may have an increased binding for a microbial pathogen? Could such a mechanism be part of the basis for progression of a disease into more serious forms? It is interesting to note that the best established disease association with cancer is blood group A and stomach cancer (Henry et al., 2000; Mourant et al., 1978). Although the above is purely speculative that possibly that stomach acid may hydrolyse carbohydrate blood groups into novel microbial receptors remains a possibility worthy of further study.

Overall, the methods described here established some principles of glycolipid degradation and show that controlled acid hydrolysis can be used to yield Le^a and Le^c and to some extent, Le^d from Le^b . On the other hand, mild acid hydrolysis of the ALe^b molecule does not yield any common precursors apart from the A-type 1 glycolipid together with some unusual A-like structures. These structures may be of use in many biological assays.

5 Protocols

Protocol 1

Metallic salt degradation of globoside

OBJECTIVE

To observe the effects of various metal ions on globoside.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

- § Globoside isolated from human erythrocytes

Reagents

- § De-ionised (DI) water
- § Chloroform 02405E21, SDS, France
- § Methanol 5METHL, ASCC
- § Chloroform: methanol mix CM 2:1
- § Methanol: water mix MW 1:1
- § 0.02 M concentrations of solutions were made with the following salts in MW 1:1
- § Ammonium ferric oxalate A189/18/66, May & Baker, UK
- § Ferric ammonium chloride 27162, BDH, England
- § Aluminium sulphate 270905K, BDH, England
- § Ammonium nickel sulphate 10029, BDH, England
- § Ammonium cupric chloride BDH, England
- § Aluminium chloride 11016, Riedel-De-Haen ag seelze Hannover, Germany
- § Ammonium ferric sulphate 1409, Merck, Germany
- § Ammonium aluminium sulphate BDH, England
- § Ammonium chloride A69/18/67, M&B, UK
- § Ammonium cobaltous sulphate 27156, BDH, England
- § Calcium sulphate C94/18/67, May & Baker, UK
- § Ferric sulphate 28400 5E, BDH, England
- § Cuprous chloride D3247, Ajax, Australia
- § Ferric chloride 101104R, BDH, England
- § Ferrous chloride, 103967, Merck, Germany
- § Barium chloride B102/18/66, May & Baker, UK
- § Caesium chloride 1006750, BDH, England
- § Cadmium sulphate 10066, BDH, England
- § Cadmium chloride 27548, BDH, England
- § Potassium chloride 10198, BDH, England
- § Lithium sulphate D3247, Ajax Australia
- § Lead chloride 29025, BDH, England
- § Nickel chloride, 1.06717.0250, BDH, England
- § Cobalt chloride 10082, BDH, England
- § Chromium chloride 4161778, Merck, Germany
- § Magnesium nitrate 10309, BDH, England
- § Sodium sulphate 30223 4T, BDH, England
- § Zinc sulphate 31665, Riedel-De-Haen ag seelze Hannover, Germany
- § Zinc chloride 30605 4K, BDH, England
- § Tin (II) chloride 62729, Ajax, Australia
- § Silver nitrate 30095, BDH, England
- § Ammonium ferrous sulphate A7145/255/A, May & Baker, UK
- § Potassium nitrate Ajax, Australia
- § Magnesium nitrate D3247, Ajax, Australia
- § Sodium nitrate Ajax, Australia
- § Cadmium nitrate 27554, BDH, England
- § Lead nitrate 10145, BDH, England
- § Barium nitrate 27299, BDH, England
- § Cupric nitrate D3247, Ajax, Australia
- § Ferrous nitrate 416079, JT Baker, Holland

- § Aluminium nitrate A164/18/66, May & Baker, UK
- § Chromium nitrate 1606-1, JT Baker, Holland
- § Zinc nitrate 10335, BDH, England
- § Nickel nitrate N36/18/89, May & Baker, UK
- § Silver nitrate Johnson Matthey Aust Ltd
- § Cobalt nitrate 10083, BDH, England
- § Chromium (III) oxide K268802944942, BDH, England
- § Ferric oxide 83938W46658, Hopins and Williams
- § Chromium(II) sulphate 27759, BDH, England
- § Ferrous sulphate 28400, BDH, England
- § Chromium potassium sulphate 510810, BDH, England
- § Aluminium oxide 15139 4U, BDH
- § Aluminium potassium sulphate SSCR12626500, Scientific Supplies, NZ
- § Chromium (VII) oxide 5190030, Merck, Germany
- § Copper (II) nitrate 72747, Ajax, Australia

Equipment

- § Timer
- § Serology tubes
- § Immufuge® II Centrifuge, Serial # B5055-3X
- § Glass Pasteur pipettes
- § Water Bath, Contherm, Serial # 300 310 320 330
- § Whatman's pH paper, pH range 4-6, 038120, England
- § Whatman's pH paper, pH range 1-4, 318502, England
- § Thermometer

GENERAL METHOD

1. In a screw –top Kimax tube, suspend 240 µl of 0.02 M globoside solution into 120 µl of each 0.02 M metal ion solution.
2. Cap the tubes tightly, place the tubes in an 85°C water bath and incubate for 1, 2, 4, 8, 24 and 48 hour intervals.
3. At each time interval, remove the tubes from the water bath and let the tubes cool before opening the cap. This ensures that the entire sample inside the reaction tube has condensed. Stop the timer at the same time.
4. Draw 40 µl of solution out of the reaction tube into a clearly labelled serology tube. Cap the reaction tube and replace in the water bath
5. Partition this solution with chloroform and DI water and centrifugate for 1 minute.
6. After centrifugation, remove the upper water layer using a glass Pasteur pipette. The upper water layer contains the metal cations together with the anions that formed the salt. The lower chloroform layer contains the glycolipid sample; some of which may be degraded due to the action of heat and acid.
7. Add more DI water to the serology tube and spin.
8. Repeat steps 6 and 7 two more times. A total of three sets of washing ensure that most of the ions are removed from the solution.
9. The glycolipid sample left behind in the chloroform layer is then dried under vacuum.
10. The controls for this experiment are native globoside without any treatment with metal ions at room temperature and at 85°C.
11. A thin layer chromatography is carried out at the end of each experiment using anisaldehyde as the chemical stain.

METHOD VARIATIONS

Variation 1. Varying salt concentrations versus globoside.

1. Suspend 280 µl of 0.02 M globoside solution into 560 µl, 280 µl, 140 µl, 70 µl, 35 µl, 18 µl, 9 µl, 4 µl and 2 µl of 0.02 M salt solutions. Record the pH.
2. The salt solutions to use are Aluminium chloride (AlCl₃), Chromium (VII) oxide (Cr₂O₇), Copper (II) nitrate (Cu(NO₃)₂), Ferric chloride (FeCl₃) and Tin (II) chloride (SnCl₂).
3. Cap the tubes tightly, place the tubes in an 85°C water bath and incubate for 1, 2, 4, 8, 24 and 48 hour intervals.
4. Repeat steps 3-11 as detailed in the 'Method' section above.

Variation 2. Concentrated HNO₃ versus fixed salt and globoside concentration.

1. Add 3 µl of 1.75 M of HNO₃ to each tube containing 280 µl of 0.02 M globoside and 140 µl of metal ion solution as prepared in step 1 above. This lowers the pH of each degrading medium to at least 2 or lower.
2. The salts to be tested are listed below.

Chromium sulphate	Ferric chloride	Aluminium potassium sulphate	Nickel sulphate
Tin (IV) chloride pentahydrate	Ferrous nitrate	Ammonium ferrous sulphate	Manganese chloride
Tin (II) chloride	Ferric ammonium chloride	Chromium (III) nitrate	Calcium chloride
Chromium (VII) oxide	Ferric oxide	Aluminium ammonium sulphate	Sodium chloride
Aluminium chloride	Ferrous sulphate	Chromium (III) chloride	Potassium chloride
Chromium (III)oxide	Ferric ammonium sulphate	Aluminium sulphate	Copper (II) chloride
Copper(I) nitrate	Ferric sulphate	Potassium nitrate	Tin (II) sulphate
Aluminium oxide	Ferrous chloride	Zinc nitrate	Aluminium nitrate
Tin (IV) oxide	Chromium potassium sulphate	Lead chloride	Sodium nitrate

3. Some salts such as SnCl₂ will form acidic solutions when dissolved in water. 3 µl of 1.75 M of HNO₃ is still added to such solutions to keep the experimental procedures uniform.
 4. Record the individual pH of solutions in each tube prior to degradation.
 5. Cap the tubes tightly, place the tubes in an 85°C water bath and incubate for 1, 2, 4, 8, 24 and 48 hour intervals.
 6. Repeat steps 3-11 as detailed in the 'Method' section above.
 7. The control for this experiment is native globoside without any acid and heat.
-

Variation 3. Varying [HNO₃] versus fixed salt and globoside concentration.

1. Suspend 280 µl of 0.02 M globoside solution and 280 µl solutions of 0.02 M metal ion solution with 560 µl, 280 µl, 140 µl, 70 µl, 35 µl, 18 µl, 9 µl, 4 µl and 2 µl of 0.1 M HNO₃.
 2. Record the pH of each solution prior to incubation.
 3. The salt solutions to use are aluminium chloride (AlCl₃), chromium (III) oxide (Cr₂O₃), copper (II) nitrate (Cu(NO₃)₂), ferric chloride (FeCl₃) and tin (II) chloride (SnCl₂).
 4. Cap the tubes tightly, place the tubes in an 85°C water bath and incubate for 1, 2, 4, 8, 24 and 48 hour intervals.
 5. Repeat steps 3-11 as detailed in the 'Method' section above.
 6. The control for this experiment is native globoside without any metal ions and heat.
-

Variation 4. Decreasing pH and salt concentrations versus globoside.

1. Suspend 50 µl of 0.02 M globoside with 50 µl of varying HNO₃ concentrations(0.02 M, 0.04 M, 0.06 M, 0.08 M and 0.1 M) and 50 µl of varying metal ion concentration(0.002 M, 0.004 M, 0.006 M, 0.008 M, 0.01 M).
 2. Record the pH of each solution prior to incubation.
 3. The salt solutions to be used are 0.02 M of FeCl₃ and SnCl₂.
 4. Cap the tubes tightly, place the tubes in an 85°C water bath and incubate for 24 hours.
 5. Repeat steps 3-11 as detailed in the 'Method' section above.
 6. The control for this experiment is native globoside subjected to heat but without any treatment, globoside with each concentration of metal ions subjected to heat and globoside with each concentration of acid and subjected to heat.
-

Protocol 2

Acid degradation of Globoside

OBJECTIVE

To observe the effects of acids on globoside.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

§ 0.02 M globoside

Reagents

§ De-ionised (DI) water	
§ Chloroform 02405E21, SDS, France	
§ Methanol 5METHL, ASCC	
§ Chloroform: methanol mix CM 2:1	
§ Methanol: water mix MW 1:1	
§ Nitric acid 101686E, BDH, England	
§ 0.01 M, 0.02 M, 0.04 M, 0.06 M, 0.08 M, 0.1 M, 0.2 M, 0.4 M, 0.8 M, 0.1 M and 1.6 M Nitric acid	Concentration
§ Hydrochloric acid, Ac0741, Sharlau Spain	0.1 M
§ Phosphoric acid, UN1805, Sharlau, Spain	0.1 M
§ Acetic acid, 1.00063.2500, Merck, Germany	0.1 M
§ Sulphuric acid, 30743, Riedel de-Haen, Germany	0.1 M
§ Nitric acid, 101686E, BDH, England	0.1 M
§ Hydrochloric acid, Ac0741, Sharlau, Spain	0.1 M
§ Phosphoric acid, UN1805, Sharlau, Spain	0.1 M
§ Sodium hydroxide R.06203, Scientific supplies, New Zealand	0.1 M

Equipment and Consumables

- § Timer
- § Serology tubes
- § Immufuge® II Centrifuge, Serial # B5055-3X
- § Glass Pasteur pipettes
- § Water Bath, Contherm, 300 310 320 330
- § pH paper
- § Thermometer
- § Whatman's pH paper, pH range 4-6, 038120, England
- § Whatman's pH paper, pH range 1-4, 318502, England
- § Heating block Wealtec Corp HB-2, Serial # E02W04M0139

METHOD

Variation 1. Varying [HNO₃] versus globoside.

1. In a screw-top Kimax tube, suspend 250 µl of 0.02 M globoside solution into 125 µl of each concentration of HNO₃ ranging from 0.01 M, 0.02 M, 0.04 M, 0.06 M, 0.08 M, 0.1 M, 0.2 M, 0.4 M, 0.8 M and 1.6 M.
2. Record the pH of each solution prior to incubation.
3. Cap the tubes tightly, place the tubes in an 85°C water bath and incubate for 1, 2, 4, 8, 24 and 48 hour intervals.
4. At each time interval, remove the tubes from the water bath and let the tubes cool before opening the cap. This ensures that the entire sample inside the reaction tube has condensed. Stop the timer at the same time.
5. Draw 40 µl of solution out of the reaction tube into a clearly labelled serology tube. Cap the reaction tube.
6. Partition this solution with chloroform and DI water and centrifuge for 1 minute.
7. After centrifugation, remove the upper water layer using a glass Pasteur pipette. The upper water layer contains the metal cations together with the anions that formed the salt. The lower chloroform layer contains the glycolipid sample; some of which may be degraded due to the action of heat and acid.

8. Add more DI water to the serology tube and spin.
9. Repeat steps 6 and 7 two more times. A total of three sets of washings ensure that most of the hydrogen ions are removed from the solution.
10. The glycolipid sample left behind in the chloroform layer is then dried under vacuum.
11. A thin layer chromatography is carried out at the end of each experiment using anisaldehyde as the chemical stain.
12. Control for this experiment is native globoside subject to heat (85°C) with no acidic treatment.

Variation 2. Effects of various 0.1 M acids versus globoside.

1. Suspend 50 µl of 0.02 M globoside into a Kimax tube with 100 µl of 0.1 M acid so that the pH of the solution at least 2 or lower. Test with pH paper.
2. Acids to be used are 0.1 M of Hydrochloric acid, 0.1 M Phosphoric acid, 0.1 M Acetic acid, 0.1 M Sulphuric acid and 0.1 M Nitric acid
3. Incubate these solutions overnight in an 85°C water bath.
4. Test as in steps 3-11 outlined in the Method (Variation 1) section above.
5. The control for this experiment is native globoside with acid set at room temperature and native globoside subjected to heat (85°C) over 24 hours.

Variation 3. Effects of acid on globoside over 90 minutes.

1. Make 10 mg/ml of pure globoside solution and aliquot 0.2 ml (2mg) into 7 screw-top Kimax tubes labeled 15, 30, 45, 60 75 and 90 minutes.
2. Add 0.2 ml of 0.1 M HNO₃ to each tube. Check the pH with Litmus paper. pH should be at least 2.
3. Cap the tubes and place them in a heating block set at 85°C.
4. Start the timer.
5. Remove the appropriate tubes at their respective time intervals.
6. Let cool for a few minutes and then uncap the tube. Add 0.2 ml of 0.1 M NaOH to neutralize the acid. Check with pH paper that the solution is neutral.
7. Dry down the neutralized mixture using nitrogen gas.
8. Once the contents of the tube are dry, reconstitute in CM 2:1
9. Then, using glass Pasteur pipettes, draw out this sample into a clean glass serology tube. Continue with two more washings of the dry sample with CM2:1 into the same glass serology tube. This ensures that the entire degraded sample is washed into the serology tube.
10. Add water to the tube that contained the original degraded mixture. This step will dissolve the salt formed upon neutralisation of the acid with the base. Add an equal amount of chloroform to form a two phase system. The upper layer will be water containing dissolved salts and hydrophilic substances while the lower chloroform layer will contain dissolved degraded globoside and other hydrophobic substances.
11. Discard the upper water layer using glass Pasteur pipettes. Add more water, swirl gently and remove the water using glass Pasteur pipettes. This washing with water should be done at a total of three times. Washing with water ensures that all the salts are removed and the bottom chloroform layer had the last traces of glycolipids
12. Wash the remaining chloroform layer thrice into their respective serology tubes and dry down using nitrogen gas.
13. Reconstitute in CM2:1, load onto Thin Layer Chromatography (TLC) plates and chemically stain with anisaldehyde.
14. Control for this experiment was globoside without any treatment with acid at room temperature and globoside at 85°C for 90 minutes.

Variation 4. Validation of optimal temperature for globoside degradation with 0.1 M HNO₃.

1. Suspend 240 µl of 0.02 M globoside in a Kimax tube and enough 0.1 M HNO₃ so that the pH of the mixture is between 1.5 and 2.
2. Cap the tubes tightly and place in a heating block set at 85°C, 60°C, 40°C and 20°C and incubate for 2, 4, 8, 24 and 48 hours.
3. Repeat steps 3-10 as detailed in Method, Variation 1 above.
4. Control for this experiment is native globoside set at room temperature with no acidic treatment.

Protocol 3

Acid Degradation of Hydroxy and Non-Hydroxy Ceramide

OBJECTIVE

To observe the effects of 0.1 M and 1.75 M nitric acid on hydroxy and non-hydroxy ceramide.

SAMPLES, REAGENTS AND EQUIPMENT

Samples	Concentration
§ Hydroxy ceramide Matreya Inc 1323	5 mg/ml
§ Non hydroxy ceramide Matreya Inc 1322	5 mg/ml
§ Globoside	5 mg/ml
Reagents	
§ De-ionised (DI) water	
§ Chloroform 02405E21, SDS, France	
§ Methanol 5METHL, ASCC	
§ Chloroform: methanol mix CM 2:1	
§ Nitric acid 101686E, BDH, England	0.1 M
§ Nitric acid 101686E, BDH, England	1.75 M
§ Nitric acid 101686E, BDH, England	0.5 M
§ Sodium hydroxide R.06203, Scientific supplies, New Zealand	0.1 M
Equipment	
§ Timer	
§ Serology tubes	
§ Glass Pasteur pipettes	
§ Heating block Wealtec Corp HB-2 , Serial # E02W04M0139	
§ Whatman's pH paper, pH range 4-6, 038120, England	
§ Whatman's pH paper, pH range 1-4, 318502, England	
§ Kimax tubes	

METHOD

1. In a screw-top Kimax tube, suspend 50 µl of 5 mg/ml hydroxy /non hydroxy ceramide solutions into 50 µl of each concentration of HNO₃ , 0.1 M and 1.75 M
2. Cap the tubes tightly, place the tubes in an 85°C heating block and incubate for 24 hours.
3. Stop the reaction by taking the capped tubes out of the heating block. Let cool for a few minutes and then add an appropriate amount of base, preferably sodium hydroxide to neutralize the acid. Check with pH paper that the reaction mixture is neutral.
4. Dry down this neutralized mixture using nitrogen gas.
5. Once the contents of the tube containing the neutralized mixture are dry, reconstitute the dried sample with CM 2:1 solvent mix. The hydrophobic structures i.e. the ceramide and degraded ceramide structures would be dissolved in this solvent mix.
6. Using glass Pasteur pipettes, draw out this sample into a glass serology tube. Continue with two more washings of the dry sample with CM 2:1 and transfer this washing into the same glass tube each time. This ensures that the entire degraded sample is washed into the serology tube.
7. Then add DI water to the Kimax tube that contained the degraded sample (now containing the salt [NaNO₃] residue at the bottom). This will dissolve the salt. Add an equal amount of chloroform to form a two phase system (upper layer would be water and lower layer would be chloroform). Discard the upper layer (containing dissolved salts) using glass Pasteur pipettes. Repeat this washing with water two more times. This washing with water ensures that all the salts are removed and the bottom chloroform layer has the last traces of ceramides.
8. Wash the remaining (bottom) chloroform layer thrice into their respective serology tubes and dry under nitrogen gas.
9. Dry the sample under nitrogen, reconstitute in CM2:1 and load onto TLC plates using the general lab TLC protocol. Stain chemically with anisaldehyde.
10. The control for this experiment is hydroxy and non hydroxy ceramide subject to 85°C heat for 24 hours.

METHOD VARIATIONS

Variation 1. Effects of 0.1 M HNO₃ on ceramides and globoside over 24 hours.

1. In a screw-top Kimax tube, suspend 50 µl of 5 mg/ml hydroxy /non hydroxy ceramide and globoside solutions into 50 µl of 0.1 M HNO₃ solution.
 2. Cap the tubes tightly, place the tubes in an 85°C heating block and incubate for 24 hours.
 3. Repeat steps 3-9 as outlined in the "Method" section above.
 4. The controls for this experiment are
 - hydroxy and non hydroxy ceramide and globoside - untreated
 - hydroxy and non hydroxy ceramide subject to 85°C heat for 24 hours.
-

Variation 2. Effects of 0.2 M and 0.5 M HNO₃ on ceramides and globoside

1. In a screw-top Kimax tube, suspend 50 µl of 5 mg/ml hydroxy /non hydroxy ceramide solutions into 50 µl of 0.1 M and 0.5 M HNO₃ solutions in clearly labelled Kimax tubes.
 2. Cap the tubes tightly, place the tubes in an 85°C heating block and incubate for 24 hours.
 3. Repeat steps 3-9 as outlined in the "Method" section above.
 4. The controls for this experiment are
 - a. hydroxy and non hydroxy ceramide -untreated
 - b. hydroxy and non hydroxy ceramide subject to 85°C heat for 24 hours.
-

Protocol 4

HPLC Purification of Glycolipids

OBJECTIVE

To purify and separate glycosphingolipids based on the size of the sugar component using High Performance Liquid Chromatography (HPLC).

SAMPLES, REAGENTS AND EQUIPMENT

Samples

- § Glycolipid sample

Reagents

- § HPLC-grade isopropanol MKT3043-10, Mallinckrodt, USA
- § HPLC-grade chloroform MKT4443-68, Mallinckrodt, USA
- § HPLC-grade methanol 8402, JT Baker, Holland
- § Solvent A ; Chloroform: methanol: water 65:25:4
- § Solvent B; Chloroform: methanol: water 40:40:12
- § 2,2 dimethoxypropane D136808-500ml, Sigma, Germany
- § glacial acetic acid , 1.0000632500, Merck , Germany
- § n-hexane, 1043672500, Merck, Germany
- § 2.5% 2,2 dimethoxypropane and 2.5% glacial acetic acid in n-hexane

Equipment

- § Safety glasses and gloves
- § HPLC Liquid Chromatograph (Shimadzu LC-10AT)
- § System controller (Shimadzu LC-10A)
- § Degassex DG-4400
- § Fraction collector (Shimadzu FRC-10A)
- § Fraction collector racks – type 2A
- § HPLC-F column
- § 2 x short blue coloured peek tubing
- § Long natural coloured peek tubing
- § Collection vessels (various)
- § Mettler Toledo electronic balance, Serial # 13278 (0.01g accuracy)
- § HPLC tubes (20 mL)
- § Injection valve
- § 5 mL sample loop
- § Glass syringe (10 mL)
- § Hamilton Gastight® 5mL syringe with luer lock fitting
- § Stainless steel needles
- § Injection valve needles with plastic luer lock fitting
- § Timers
- § Bain marie
- § Test-tube racks
- § Thermometer

METHOD

1. Separate the glycolipid mixture using the following gradient program at a flowrate of 2 ml/min and start fraction collection at 67 minutes until 180 minutes.

2.

Time	Mobile phase	Mobile phase concentration (%)
0	CMH 65:25:4	100
180	CMH 40:40:12	100

Protocol 5

Packing of Buchi Columns

OBJECTIVE

To pack Buchi cartridges with Silica 60.

SAMPLES, REAGENTS AND EQUIPMENT

Equipment

§ Buchi cartridge C-670, Serial # CH9230

Consumables and Glassware

§ 40mm(ID) x 150mm Cartridge packed with Silica 60 (40-63um)

§ Silica 60, 2050027, SDS, France

METHOD

1. **Connecting the Cartridge to a vacuum source** - The cartridge has two connections on the back side. The upper connection is for the vacuum. Connect the vacuum- pump tube to this securing the connection with a plastic cable tie.
2. **Connecting the Cartridge to compressed air or gas supply** - The lower connection on the back side of the cartridge should be connected to compressed air or gas supply. (We use nitrogen gas). The outlet pressure should be a maximum of 140 kiloPascals. Secure the connection on both ends with plastic cable ties. The use of in-house compressed air is not recommended as they can easily contaminate the stationary phase with water and oils. Inert gases that can be used as an alternative to compressed air are nitrogen, argon, helium or technical air).
3. **Checking suction output** - Before packing a column the suction output of the cartridge must be checked. This can be done by screwing in the supplied cartridge with glass-ball and opening the vacuum valve found below the container containing the stationary phase/silica. The ball should quickly rise to the top and remain there once the vacuum is switched on. If the glass ball does not rise or rises very slowly and falls to the bottom again, then there is not enough vacuum created to pack a column. Check the vacuum seal of the system and inspect and replace safety frits. Cartridges can only be packed once the suction output is adequate.
4. **Selecting and Filling the Stationary Phase** - Select the stationary phase, in this case Silica 60, and fill it into the glass container attached to the Buchi cartridge C-670 upto or just below the maximum mark.
5. **Fluidization of the stationary phase** – Fluidization allows the flow of gas (or air) through the stationary phase. The gas passes through a frit fitted to the bottom of the glass vessel. The optimal gas flow is dependent upon the grain size of silica and can be set on the right side using the needle valve. Fill the glass vessel with the stationary phase and open the gas very slowly until the first air bubbles appear on the surface of the gel. The gel should not bubble and should have a calm and even surface. At the end of the packing process, shut off the main gas flow.
6. **Inserting the first lower frit** – The lower frit has a diameter of 12mm. lay this in the recession of the insertion device and gently push down to insert the frit into the cartridge. The insertion device is engineered in such a way that a frit is always inserted in the correct insertion depth.
7. **Screwing in the Cartridge** – Next screw in the cartridge with the side of the frit into the cartridge holder.
8. **Packing the column** – Submerge the cartridge into the fluidized gel. Open the vacuum valve on the front side of the cartridge. The cartridge gets filled at once. Leave for about 1 minute and then carefully take out the cartridge from the glass vessel. Remove the adherent gel on the sides and gel projecting beyond the length of the cartridge by using the brushes in the lid.
9. **Inserting the second (upper) frit** – Before inserting the second (upper) frit, use the appropriate knife tool/scrapper knives to present in the black function lid to free the column start of the gel. The scraper knives have a stop that determines the depth. Once this is done, insert the appropriate frit (40mm) into the insertion tool and push down the packed cartridge on the black function lid to secure the frit into its place.
10. **Removing the packed cartridge** – Once the frits have been locked in place, the vacuum valve can be closed and the cartridge can be unscrewed from the cartridge holder. Avoid any jarring or blows to the cartridge as this can damage the column bed which in turn can lead to less than ideal separations. The cartridge is now ready to be used in separating glycolipid samples.

Protocol 6

Buchi separation of glycolipid samples

OBJECTIVE

To separate and purify glycolipid molecules using the Buchi Sepacore system.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

- § Glycolipid samples

Reagents

- § Chloroform 02405E21, SDS, France
- § Methanol 5METHL, ASCC
- § De-ionised water
- § Chloroform: Methanol: Water 80: 20: 1
- § Chloroform: Methanol: Water 70: 25: 4
- § Chloroform: Methanol: Water 65: 35: 8
- § Chloroform: Methanol: Water 40: 40: 12

Equipment

- § Buchi pump controller C-610, Serial # CH9230
- § Buchi pump module C-601, Serial # CH9230

Consumables and Glassware

- § Rotary evaporator flasks

METHOD

1. Connect the screw-ends of a filled cartridge to the Buchi pump controller C-610/ Buchi pump module C-601 and place upright in the clamp provided.
2. Place the suction frit into the solvent bottle containing Chloroform: Methanol: Water 80: 20: 1.
3. Connect the Buchi pump controller C-610/ Buchi pump module C-601 to a power source and turn on.
4. Switch on the green button on the Buchi pump controller C-610.
5. Adjust the flow rate of the solvent to 2-4 ml/minute by rotating the large black knob found on the right-hand side of the Buchi pump module C-601.
6. Place the suction frit into the bottle containing the equilibrating solvent (starting solvent) and a waste-collecting beaker on the other end of the tube
7. Press the green start button on the Buchi pump module C-601 and let the column wet the column. It may take 15-20 minutes for the entire column to be wet evenly.
8. Once the column is wet with the starting solvent, stop the flow rate and adjust to 10ml/min. Allow to pump at this flow rate for about 3-5min to ensure there is no leakage.
9. Make sure that the pump is stopped during the injection procedure.
10. Make a 500 mg/ml sample using the starting solvent as the diluent.
11. Draw up the sample using a gas-tight syringe and needle.
12. Gently remove the needle ensuring that there is no liquid –sample present inside.
13. Inject the glycolipid sample (500mg) into the silica column by gently connecting the Luer-lock on the syringe to the rubber septum on the top end of the column. This is done by gently twisting the syringe clockwise so that its Luer-lock fits/locks in with the rubber injection port.
14. Following this, gently depress the plunger of the syringe so that the glycolipid sample is deposited on top of the column of silica. Ensure that there is no leakage or spillage.
15. Remove the syringe by rotating it anti-clockwise to detach from the rubber septum. Rinse the syringe and the needle (used for drawing the sample) three times with CM2:1 into the tube containing the original sample and dry and store as required. Rinsing can be done once the run is in progress
16. Start the pump and let pump at 10 ml/min for about 10 minutes or until the yellow band (pigmentation due to glycolipids) reaches the bottom end of the silica column. Collect and label this eluent as the pre-collection solvent.
17. Stop the pump and adjust the flow rate to 12.5 ml/min.
18. Start collecting fractions immediately after this using a timer to time the collections.
19. Always stop the pump when changing to a different solvent mix.

Time Program for Buchi System			
Solvent	Number of fractions	Collection time	Flow rate
CMH 80:20:1	1	15	12.5
	1	15	12.5
CMH 70:25:4	1	10	12.5
	1	10	12.5
	1	10	12.5
	1	10	12.5
CMH 65:35:8	1	5	12.5
	1	5	12.5
	1	8	12.5
	1	8	12.5
	1	8	12.5
	1	8	12.5
	1	8	12.5
	1	8	12.5
	1	8	12.5
	1	8	12.5
CMH 40:40:12	1	30	20

Protocol 7

Le^b degradation by acid hydrolysis

OBJECTIVE

To observe the effects of nitric acid on the Lewis b (Le^b) sample.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

§ Purified Le^b sample from human small intestine

Reagents

Concentration

§ Chloroform 02405E21, SDS, France

§ Methanol 5METHL, ASCC

§ Nitric acid 101686E, BDH, England

0.1 M

§ Sodium hydroxide R.06203, Scientific supplies, New Zealand

0.1 M

§ De-ionised (DI) water

§ Chloroform: methanol mix CM 2:1

Equipment

§ Timer

§ Serology tubes

§ Glass Pasteur pipettes

§ Heating block Wealtec Corp HB-2 , Serial # E02W04M0139

§ Thermometer

§ Kimax tubes

§ 2 x 1ml graduated glass pipettes

§ Heating block Wealtec Corp HB-2 , Serial # E02W04M0139

§ Whatman's pH paper, pH range 4-6, 038120, England

§ Whatman's pH paper, pH range 1-4, 318502, England

METHOD

1. Make 5 mg/ml of Le^b sample and draw out 100 µl into the required number of screw top kimax tubes.
2. Add 100 µl of 0.1 M HNO₃ (check pH) with litmus paper to each tube. pH should be less than 2.
3. Screw the cap back on the kimax tube tightly.
4. Put in water bath or heating block set at the required temperature and incubate for the required length of time.
5. Add 100 µl of 0.1 M NaOH to each tube to neutralize the acid once it is taken out at the required time interval.
6. The tubes containing the aliquots then should be dried down using nitrogen gas
7. Once these tubes containing the degraded sample and salt are dry, reconstitute the sample with CM2:1
8. Using glass Pasteur pipettes, draw out the sample into another pre weighed glass serology tube. Continue with two more washings of the dry sample with CM 2:1 and transfer the liquid into the same glass tube for each time interval. This ensures that the entire degraded sample is washed into the serology tube.
9. Add water to the original serology tubes containing the degraded sample (now containing the salt [NaNO₃] residue at the bottom). This would dissolve the salt. Add an equal amount of chloroform to form a two phase system (upper layer would be water and lower layer would be chloroform). Discard the upper layer (containing dissolved salts) using glass Pasteur pipettes. Add more water and swirl gently and then remove the water layer. This should be done at total of 3 times. This washing with water ensures that all the salts are removed and the bottom chloroform layer has the last traces of glycolipids.
10. Wash this chloroform layer, 3 times, into their respective serology tubes and dry under nitrogen gas.
11. Reconstitute this dried sample in about 100µl of CM 2:1
12. Reweigh these tubes and perform an anisaldehyde and immunostain assay.

METHOD VARIATIONS

Variation 1. Le^b versus 0.1 M HNO_3 over 48 hours at 85°C.

1. Make 5 mg/ml of Le^b sample and draw out 800 μl into a screw top kimax tube.
2. Add 800 μl of 0.10 M HNO_3 ; check pH in each tube with litmus paper. pH should be less than 2.
3. Put in an 85°C Celsius water bath and draw out 200 μl aliquots into clearly labeled clean serology tubes at each of the time intervals i.e. 1, 2, 4, 8, 14, 24 and 48 hours.
4. Let the tubes cool down to room temperature.
5. Uncap the Kimax tube and add 100 μl of 0.1 M NaOH to each tube to neutralize the acid once it is taken out at the required time interval.
6. Repeat steps 6-12 outlined in the general method section above.

Variation 2. Le^b versus 0.1 M HNO_3 over 90 minutes at 85°C.

1. Make 5 mg/ml of Le^b sample and draw out 100 μl into 7 screw top kimax tubes.
2. Add 100 μl of 0.1 M HNO_3 (check pH) with litmus paper to each tube keeping one tube with the Le^b sample without any acid as the control.
3. Put in an 85°C Celsius water bath and draw out appropriately labeled tubes at 15min, 30 min, 45 min, 60 min, 75 min and 90 min.
4. Remove the tube containing the degraded sample at the required time interval and let it cool down to room temperature.
5. Add 100 μl of 0.1 M NaOH to each tube to neutralize the acid once it is taken out at the required time interval.
6. Repeat steps 6-12 outlined in the general method section above.

Variation 3. Le^b versus varying $[\text{HNO}_3]$ over 90 minutes at 85°C.

1. Make 5 mg/ml of Le^b sample and draw out 100 μl (0.5 mg) into 24 screw top kimax tubes labeled 6 x 0.06 M, 6 x 0.04 M, 6 x 0.02 M and 6 x 0.005 M HNO_3 (each labeled from 15 – 90 minutes for each HNO_3 concentration described below).
2. Add 100 μl of each concentration of HNO_3 and check pH in each tube with litmus paper. pH should be less than 2.
3. Put in an 85°C Celsius water bath and draw out the appropriately labeled tubes at 15min, 30 min, 45 min, 60 min, 75 min and 90 min.
4. Let the tubes cool down to room temperature.
5. Add 60 μl , 40 μl , 20 μl and 5 μl of 0.1 M NaOH to each tube (labeled 0.06 M, 0.04 M, 0.02 M and 0.005 M HNO_3 respectively) to neutralize the acid once it is taken out at the required time interval.
6. Repeat steps 6-12 outlined in the general method section above.

Protocol 8

Preparation of Anhydrous Methanolic Hydrogen Chloride

OBJECTIVE

To prepare anhydrous methanolic hydrogen chloride solution.

SAMPLES, REAGENTS AND EQUIPMENT

Reagents

To prepare anhydrous methanol

- § Magnesium turnings, 2249310 BDH, England
- § Methanol, 8402 J.T Baker, Holland

To prepare anhydrous hydrochloric acid in anhydrous methanol

- § Sodium chloride crystals, 1.06404.0500, Merck, Germany
- § Fused granular calcium chloride, 27586, BDH, England
- § Concentrated sulphuric acid, AC 2067, Sharlau, Spain

Equipment

To prepare anhydrous methanol

- § Reflux glassware-Quickfit range (condenser, round bottom flask [500 ml], modified Soxhlet extractor with side tap)
- § Heating mantle

To prepare anhydrous hydrochloric acid in anhydrous methanol

- § 1 Quickfit double neck round bottom flask (1L)
- § 1 Quickfit 3-neck round bottom flask (500mL)
- § 1 Quickfit dropping funnel (100mL)
- § glass tubing and rubber bungs
- § PTFE thread seal tape BS4375-1968
- § Glass wool

Standardising methanolic hydrogenchloride

- § Anhydrous Sodium Carbonate, S00116, Sharlau, Spain

METHOD

To prepare anhydrous methanol

1. Arrange the glassware as shown in the diagram.
2. Prepare anhydrous methanol by refluxing HPLC grade methanol with magnesium turnings for at least 2 hours. Fit the top of the condenser with a column containing fused granular calcium chloride to ensure that the condensate is as dry as possible. This drying column helps by removing any atmospheric moisture from dissolving into the methanol.
3. Remove the anhydrous methanol produced using the side tap on the modified Soxhlet extractor. Collect the anhydrous methanol in a glass solvent bottle. Flush the headspace with nitrogen gas and cap tightly to prevent any atmospheric water or oxygen from entering.

To prepare anhydrous hydrochloric acid in anhydrous methanol

1. Arrange the glassware as shown in the diagram.
2. Gaseous hydrochloride (HCl) is produced by adding concentrated sulphuric acid in a drop-wise fashion (contained in the dropping funnel) to the solid sodium chloride crystals contained in the double-neck round bottom flask.
3. The gaseous hydrogen chloride passes through the column containing the fused granular calcium chloride before being bubbled into anhydrous methanol contained in another round bottom flask on the other end of the set-up. The purpose of fused granular calcium chloride tube is to dry the HCl gas.
4. Once the bubbling ceases, cap the flask containing methanolic HCl with Quickfit glass stoppers lined with PTFE (PolyTetraFluoroEthylene).

Standardising methanolic hydrogenchloride

1. Pipette 10 ml aliquots of methanolic HCl produced into conical flasks.
2. Titrate against standard 0.1000 M sodium carbonate solution using screened methyl orange as the indicator.

3. Dilute methanolic hydrogen chloride accordingly with anhydrous methanol prepared above.
 4. Calculate the concentration of HCl in methanol using molar ratios
 5. $\text{Na}_2\text{CO}_3 + 2\text{HCl} \rightarrow 2\text{NaCl} + \text{H}_2\text{O} + \text{CO}_2$
-

Protocol 9

Methanolysis of glycolipids

OBJECTIVE

To degrade glycolipids by the methanolysis method.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

§ Purified glycolipids (globoside or Lewis b)

Concentration

Reagents

§ Anhydrous methanolic hydrogen chloride solution	0.1 M
§ Anhydrous methanol under 4Å molecular sieves	
§ 0.1 M Nitric acid 101686E, BDH, England	
§ Sodium hydroxide R.06203, Scientific supplies, New Zealand	0.1 M
§ De-ionised (DI) water	
§ Chloroform 02405E21, SDS, France	
§ Methanol 5METHL, ASCC	
§ Chloroform: methanol mix CM 2:1	
§ Anhydrous methanolic hydrogen chloride solution	0.06 M
§ Anhydrous methanolic hydrogen chloride solution	0.04 M
§ Anhydrous methanolic hydrogen chloride solution	0.02 M
§ Anhydrous methanolic hydrogen chloride solution	0.1 M
§ Anhydrous methanol under 4Å molecular sieves	

Equipment

§ Heating block Wealtec Corp HB-2 , Serial # E02W04M0139

§ Timer

§ Serology tubes

§ Immufuge® II Centrifuge, Serial # B5055-3X

§ Glass Pasteur pipettes

§ pH paper

§ Thermometer

§ 4A Molecular sieves 54005, BDH, England

§ Kimax tubes

METHOD

1. Make 2 mg/ml of glycolipid sample and draw out 0.25 ml (approximately 0.5 mg) each of this solution into a screw cap tube.
2. Add 0.4 ml of methanolic HCl (check pH is approximately 2 using pH paper) to each tube.
3. Flush the head space with nitrogen. This step removes any moisture from the air inside the headspace of the reaction vial.
4. Cap and place in an 85° Celsius heating-block and remove after the required time interval. Let it cool to room temperature.
5. Add 0.4 ml of 0.1 M NaOH to the tube to neutralize the acid.
6. This tube containing the aliquot then should be dried down using nitrogen gas.
7. Once the tube containing the degraded sample and salt are dry, reconstitute the sample with CM2:1
8. Using glass Pasteur pipettes, draw out the sample into another pre weighed glass serology tube. Continue with two more washings of the dry sample with CM 2:1 and transfer the liquid into the same glass tube for each time interval. This ensures that the entire degraded sample is washed into the serology tube.
9. Add water to the original serology tubes containing the degraded sample (now containing the salt [NaNO₃] residue at the bottom). This would dissolve the salt. Add an equal amount of chloroform to form a two phase system (upper layer would be water and lower layer would be chloroform). Discard the upper layer (containing dissolved salts) using glass Pasteur pipettes. Add more water and swirl gently and then remove the water layer. This should be done at a total of 3 times. This washing with water ensures that all the salts formed, upon neutralisation of the acid, are removed and the bottom chloroform layer has the last traces of glycolipids.

10. Wash this chloroform layer, 3 times, into their respective serology tubes and dry under nitrogen gas.
11. Perform an anisaldehyde and relevant immunostain assays.

METHOD VARIATIONS

Variation 1. Le^b versus varying methanolic [HCl] over 40 minute sat 85°C.

1. Make 1 mg/ml of Le^b sample and draw out 0.5 ml (approximately 0.5 mg) each of this solution into 4 screw cap tubes labeled 0.005 M, 0.02 M, 0.04 M and 0.06 M.
 2. Add 1 ml of each concentration of methanolic HCl to each tube.
 3. Flush the head space with nitrogen. This step removes any moisture from the air inside the headspace of the reaction vial.
 4. Cap and place in an 85°C heating-block and remove each tube at 10, 20, 30 and 40 minute intervals.
 5. Draw a small amount of the reaction mixture was into clearly labeled serology tubes at each time interval for each concentration of methanolic HCl. (4 x 4 tubes altogether). Then place the capped tubes back into the heating block after flushing the headspace with nitrogen gas.
 6. It is important to flush the headspace with nitrogen gas every time a reaction vial is opened to prevent any moisture from entering the latter.
 7. Add a small amount of 0.05 M NaOH to each glass serology tube to neutralize the acid. Neutralisation is to be checked with a wide range pH paper.
 8. Repeat steps 6-12 as in the Method section above.
-

Protocol 10

ALe^b degradation by acid hydrolysis

OBJECTIVE

To observe the effects of acid hydrolysis on A Lewis b sample.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

§ Purified ALe^b sample, isolated from human small intestine

Concentration

Reagents

§ Chloroform 02405E21, SDS, France

§ Methanol 5METHL, ASCC

§ Nitric acid 101686E ,BDH, England

0.1 M

§ Sodium hydroxide R.06203, Scientific supplies, New Zealand

0.1 M

§ De-ionised (DI) water

§ Chloroform: methanol mix CM 2:1

§ Nitric acid 101686E ,BDH, England

0.05 M

Equipment and Consumables

§ Timer

§ Serology tubes

§ Glass Pasteur pipettes

§ Heating block Wealtec Corp HB-2 , Serial # E02W04M0139

§ Thermometer

§ Kimax tubes

§ Whatman's pH paper, pH range 4-6, 038120, England

§ Whatman's pH paper, pH range 1-4, 318502, England

§ 2 x 1ml graduated glass pipettes

GENERAL METHOD

1. Make 1 mg/ml ALe^b solution and pipette 0.1 ml (100 ug) into a Kimax tube.
2. Add 100 µl of 0.1 M HNO₃ solution to this tube and cap tightly.
3. Check pH in each tube with litmus paper. pH should be less than 2.
4. Place in an 85°C heating block and start the timer.
5. Remove the tube containing the degraded sample and let it cool down to temperature. Stop the time at the same time.
6. Uncap the Kimax tube and add 100 µl of 0.1 M NaOH to each tube to neutralize the acid once it is taken out at the required time interval.
7. The tube containing the aliquot then should be dried down using nitrogen gas
8. Once the tube containing the degraded sample and salt are dry, reconstitute the sample with CM2:1
9. Using glass Pasteur pipettes, draw out the sample into another pre weighed glass serology tube. Continue with two more washings of the dry sample with CM 2:1 and transfer the liquid into the same glass tube for each time interval. This ensures that the entire degraded sample is washed into the serology tube.
10. Add water to the original serology tubes containing the degraded sample (now containing the salt [NaNO₃] residue at the bottom). This would dissolve the salt. Add an equal amount of chloroform to form a two phase system (upper layer would be water and lower layer would be chloroform). Discard the upper layer (containing dissolved salts) using glass Pasteur pipettes. Add more water and swirl gently and then remove the water layer. This should be done at total of 3 times. This washing with water ensures that all the salts are removed and the bottom chloroform layer has the last traces of glycolipids.
11. Wash this chloroform layer, 3 times, into their respective serology tubes and dry under nitrogen gas.
12. Reconstitute this dried sample in about 100 µl of CM 2:1.
13. Perform an anisaldehyde stain and immunostain assay.

METHOD VARIATIONS

Variation 1. ALe^b versus 0.05 M HNO₃ at 50°C over 120 minutes.

1. Prepare 1 mg/ml of ALe^b sample and draw out 100 µl into 8 screw top Kimax tubes labeled 15, 30, 45, 60, 75, 90, 105 and 120 minutes.
2. Add 100 µl of 0.05 M HNO₃ and check pH in each tube with litmus paper. pH should be less than 2.
3. Cap tightly and place in a 50°C heating block for 120 minutes.
4. Remove the tube containing the degraded sample at the required time interval and let it cool down to room temperature.
5. Uncap the Kimax tube and add 50 µl of 0.1 M NaOH to each tube to neutralize the acid once it has cooled down.
6. Repeat steps 6-11 outlined in the general method section above.
7. Controls for this experiment are
 - native undegraded ALe^b sample
 - ALe^b sample with 0.1 ml of 0.05 M HNO₃ and no heat for 120 minutes.
 - ALe^b sample + heat (50°C) for 120 minutes

Variation 2. ALe^b versus 0.05 M HNO₃ at 60°C over 16 hours.

2. Prepare 1mg/ml of ALe^b sample and draw out 100 µl into 8 screw top Kimax tubes labeled 2,4,6,8,10,12,14 and 16 hours
3. Add 100 µl of 0.05 M HNO₃ and check pH in each tube with litmus paper. pH should be less than 2.
4. Cap tightly and place in a 60°C heating block for 16 hours.
5. Remove the tube containing the degraded sample at the required time interval and let it cool down to room temperature.
6. Uncap the Kimax tube and add 50 µl of 0.1 M NaOH to each tube to neutralize the acid once it has cooled down.
7. Repeat steps 6-11 outlined in the general method section above.
8. Controls for this experiment are
 - native undegraded ALe^b sample
 - ALe^b sample with 0.1 ml of 0.05 M HNO₃ and no heat for 16 hours.
 - ALe^b sample + heat (60°C) for 16 hours

Variation 3. ALe^b versus 0.05 M HNO₃ at 70°C over 16 hours.

1. Prepare 1 mg/ml of ALe^b sample and draw out 100 µl into 8 screw top Kimax tubes labeled 2,4,6,8,10,12,14 and 16 hours
2. Add 100 µl of 0.05 M HNO₃ and check pH in each tube with litmus paper. pH should be less than 2.
3. Cap tightly and place in a 70°C heating block for 16 hours.
4. Remove the tube containing the degraded sample at the required time interval and let it cool down to room temperature.
5. Uncap the Kimax tube and add 50 µl of 0.1 M NaOH to each tube to neutralize the acid once it has cooled down.
6. Repeat steps 6-11 outlined in the general method section above.
7. Controls for this experiment are
 - native undegraded ALe^b sample
 - ALe^b sample with 0.1 ml of 0.05 M HNO₃ for 16 hours

Variation 4. ALe^b versus 0.1 M HNO₃ at 60°C and 70°C over 24h hours.

1. Prepare 1 mg/ml of ALe^b sample and draw out 100 µl into 4 screw top Kimax tubes.
2. Add 100 µl of 0.1 M HNO₃ and check pH in each tube with litmus paper. pH should be less than 2.
3. Cap tightly and place in a 70° and 60°C heating block for 24 hours.
4. Remove the tube containing the degraded sample after 24 hours and let it cool down to room temperature.
5. Uncap the Kimax tube and add 100 µl of 0.1 M NaOH to each tube to neutralize the acid once it is

taken out at the required time interval.

6. Repeat steps 6-11 outlined in the general method section above.
7. Controls for this experiment are
 - native, undegraded ALe^b sample,
 - separate untreated ALe^b samples incubated at 70° and 60°C for 24 hours.

Variation 5. ALe^b versus 0.1 M HNO₃ at 70°C over 120 minutes.

1. Make 1 mg/ml of ALe^b sample and draw out 100 µl into 8 screw top Kimax tubes.
2. Add 100 µl of 0.1 M HNO₃ and check pH in each tube with litmus paper. pH should be less than 2.
3. Place in an 70° Celsius heating block and draw out appropriately labeled tubes at 15min, 30 min, 45 min, 60 min, 75 min ,90 min, 105 min, 120 min. Start the timer at the same time.
4. Remove the tube containing the degraded sample at the required time interval and let it cool down to room temperature.
5. Uncap the Kimax tube and add 100 µl of 0.1 M NaOH to each tube to neutralize the acid once it is taken out at the required time interval.
6. Repeat steps 6-11 outlined in the general method section above.
7. Control for this experiment is native, undegraded ALe^b sample

Variation 6. ALe^b versus 0.1 M HNO₃ at 85°C over 120 minutes.

1. Make 1 mg/ml of ALe^b sample and draw out 100 µl into 8 screw top Kimax tubes.
2. Add 100 µl of 0.1 M HNO₃ and check pH in each tube with litmus paper. pH should be less than 2.
3. place in an 85° Celsius heating block and draw out appropriately labeled tubes at 15min, 30 min, 45 min, 60 min, 75 min ,90 min, 105 min, 120 min. Start the timer at the same time.
4. Remove the tube containing the degraded sample at the required time interval and let it cool down to room temperature.
5. Uncap the Kimax tube and add 100 µl of 0.1 M NaOH to each tube to neutralize the acid once it is taken out at the required time interval.
6. Repeat steps 6-11 outlined in the general method section above.
7. Control for this experiment is native, undegraded ALe^b sample

Variation 7. ALe^b versus 0.1 M HNO₃ at 85°C over 96 hours.

1. Prepare 1 mg/ml of ALe^b sample and draw out 100 µl into 5 screw top Kimax tubes labeled 8,24,48, 72, 96
2. Add 100 µl of 0.1 M HNO₃ and check pH in each tube with litmus paper. pH should be less than 2.
3. Cap tightly and place in an 85° C heating block for 96 hours.
4. Remove the tube containing the degraded sample at the required time interval and let it cool down to room temperature.
5. Uncap the Kimax tube and add 100 µl of 0.1 M NaOH to each tube to neutralize the acid once it has cooled down.
6. Repeat steps 6-11 outlined in the general method section above.
7. Controls for this experiment are
 - native undegraded ALe^b sample
 - ALe^b sample with 0.1 ml of 0.1 M HNO₃ and no heat for 96 hours.
 - ALe^b sample + heat (85° C) for 96 hours

Protocol 11

Degradation scores of the globoside and ceramide molecules

OBJECTIVE

To assign degradation scores to globoside breakdown reactions.

SAMPLES, REAGENTS AND EQUIPMENT

Materials

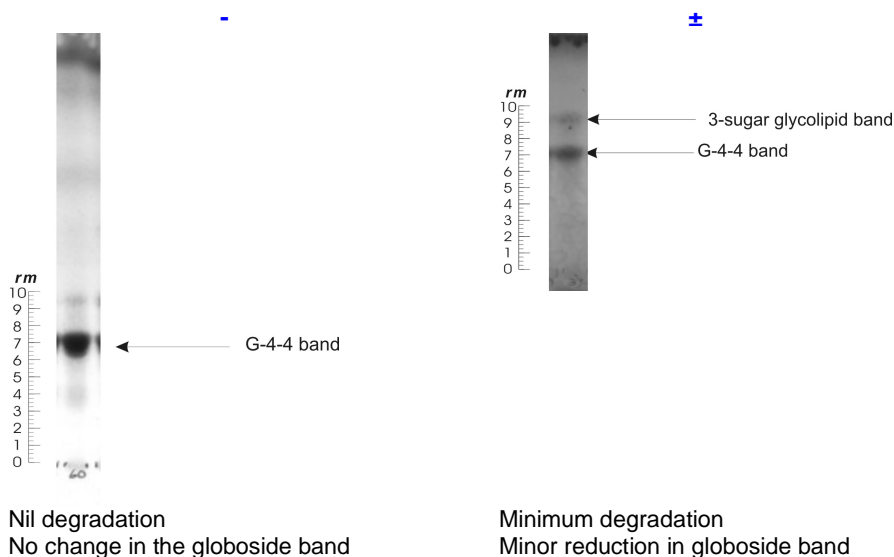
§ Anisaldehyde stained TLC plates

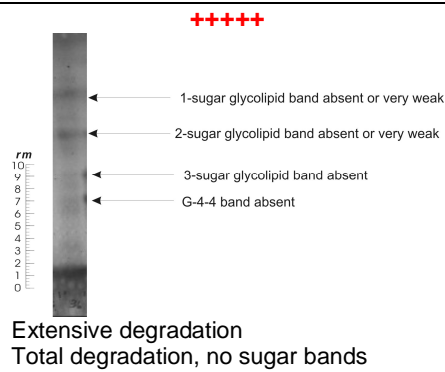
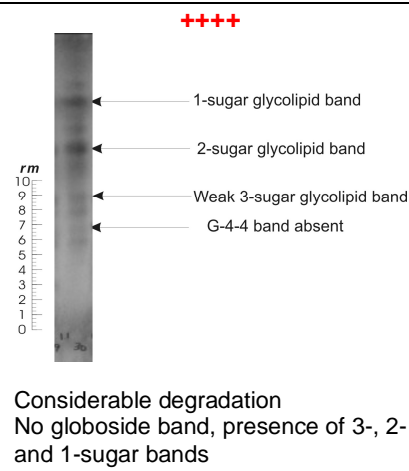
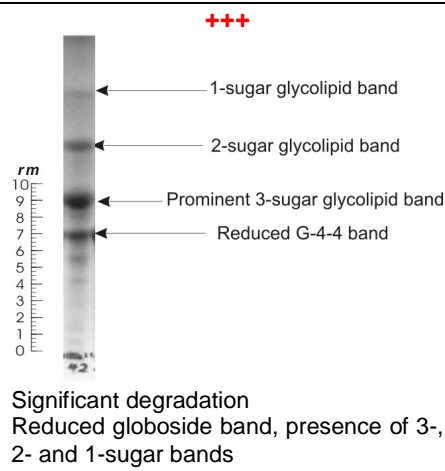
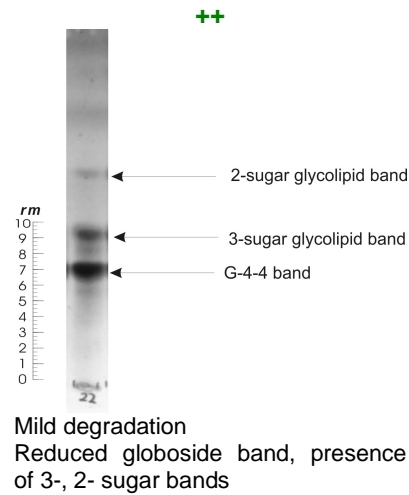
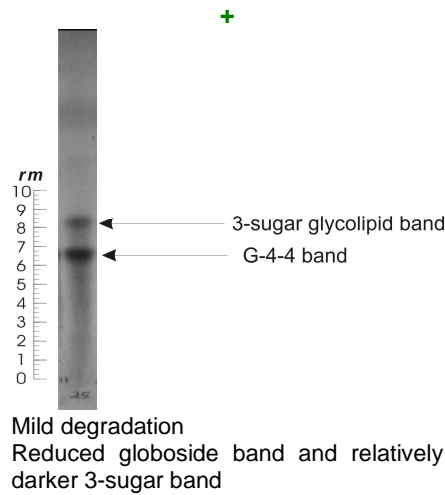
METHOD

1. Colour code for globoside degradation
2. Globoside upon incubation with acid or certain metallic salt solutions can have a range of degradation intensities. The following is a guideline of scoring these intensities.

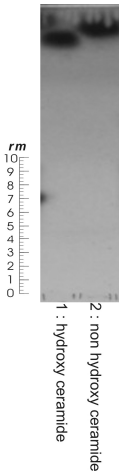

§ +++++ = total degradation, no sugar bands = extensive degradation
 § ++++ = no globoside band, presence of 3-, 2- and 1-sugar bands = considerable degradation
 § +++ = reduced globoside band, presence of 3-, 2- and 1-sugar bands = significant degradation
 § ++ = reduced globoside band, presence of 3-, 2- sugar bands = mild degradation
 § + = reduced globoside band and relatively darker 3-sugar band = mild degradation
 § ± = minor reduction in globoside band = minimum degradation
 § - = no change in the globoside band = nil degradation

The degradation scores are represented diagrammatically as follows;





- Colour code for ceramide degradation
- 1. û = ceramide degradation
 - 2. û = no ceramide degradation

Ceramide Degradation		
Score	Example	Description
û		Nil degradation
û		Extensive degradation

Protocol 12

Thin Layer Chromatography (TLC) of glycolipids

OBJECTIVE

To separate glycolipid molecules according to their approximate number of sugar moieties and to visualize them with the anisaldehyde chemical stain.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

- § Pure glycolipid samples
- § Glycolipid controls
- § TLC reference
- § Degraded glycolipid samples

Reagents

- § Anisaldehyde A0519, Sigma, Germany
- § Glacial acetic acid, 1.00063.2500, Merck, Germany
- § Concentrated sulphuric acid 30743, Riedel de-Haen, Germany
- § Chloroform 02405E21, SDS, France
- § Methanol 5METHL, ASCC
- § Developing solvent CMH 60:35:8
- § Anisaldehyde stain
- § Diluent CM 2:1

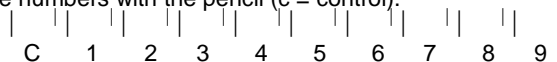
Equipment

- § Oven set at 200°C, Sunbeam BT-5300
- § Camag TLC developing chamber
- § TLC plates 818133, Macherey Nagel, Germany
- § Developing wicks
- § Electronic timer
- § Regulated compressed air
- § Microsyringe, SGE, D07-B1223
- § Soft pencil

METHOD

LOADING TLC PLATES

1. Prepare a TLC worksheet (appended) and then determine the size of TLC plate required. Generally allow for 15mm margins, 4-5mm lanes and 2mm gaps between lanes. All plates must start (and end) with a TLC control lane.
2. Aluminium plates come as 20 X 20 cm. Cut in half to make two 10 X 20 cm plates.
3. Take new plate and using a ruler score a stopping line into the top of the plate 1cm from the top of the plate (the side which was previously cut). It is important that the pencil cuts through the silica so that the solvent cannot migrate past this line.
4. In the upper region of the plate, i.e. above the stopping line record the full experimental number for the plate.
5. Using the soft pencil and the loading apparatus carefully mark the lanes for loading and the required spacing for the lanes. Do this gently taking care not to damage the plate surface. Generally the left mark is long and the right mark is short (see diagram below). Label lightly the lane numbers with the pencil (c = control).



6. Now cut the plate to size and return to TLC loading apparatus.
7. Arrange samples in rack exactly in the order of loading and check order off TLC worksheet.
8. Wash the micro-syringe thrice using the first wash (always discarding the contents into the discard container) then thrice using the second wash.
9. Uncap the TLC reference and load 2 µl into the micro-syringe, recap the control and return to the rack, then carefully load into the first control lane labelled "C". There is often a control in the last lane but load that last.

10. Wash the micro-syringe as above (step i).
11. Uncap the first sample and load 2 μ l into the micro-syringe, recap the sample and return to the rack, then carefully load into first sample lane labelled "1",
12. Repeat the process steps k-l until all the samples are loaded.
13. Allow the plate to be completely dry before proceeding with developing (5 mins).

DEVELOPING CHROMATOGRAMS

1. Turn on the oven to preheat the oven to the required temperature (200°C or 120°C). It must be in a fume cupboard.
2. Clear a space in a fume cupboard and set up the TLC developing draft shield (this reduces drafts which cause differences in migration at the ends of the tank).
3. Place a clean dry wick in one side of the TLC chamber (Camag twin trough). Fold the wick so that it curves into the bottom of the chamber – this prevents the wick from falling over.
4. Pour a measured amount of developing solvent (eg. CMH60:35:8) over the wick and into one side of the chamber only (15 ml for 10 cm and 25 ml of 20 cm chambers).
5. Place the loaded TLC plate into the other side of the chamber, with the silica surface facing in (or the aluminium surface facing the glass of the chamber). Push the foot of the plate as far out as possible (to prevent it tipping over).
6. Place the lid on chamber and allow the TLC atmosphere to equilibrate for 10 minutes.
7. Pick up the chamber and carefully tip it towards yourself with the TLC plate closest to you. Allow about 50% of the solvent to pass over into the second chamber. This takes practice and skill to prevent the TLC plate from falling over. If the plate does fall over, leave it resting on the wick – it will not affect the run.
8. Place the developing tank in developing air shield at allow to develop.
9. Allow the development to go to the stopping line. Do not stop until both ends are even. It is a good idea to set a timer for about 25 minutes as it is not good to leave the reaction "stopped" too long as it is still developing via solvent evaporation from the plate.
10. Remove the plate from the TLC chamber and either allow to dry, or place on top of the oven (but only for a few minutes) to assist in the drying process.

ANISALDEHYDE STAINING

1. Ensure the plate is dry before spraying – it should not be cool to touch.
2. Check that the oven is at 200°C.
3. Set up the anisaldehyde spraying apparatus and the spray shield in the fume cupboard.
4. Clamp the TLC plate on to the spray grid in the spray shield.
5. Using compressed gas gently spray the plate until wet – but without runs. Do not under-spray
6. Immediately transfer the plate to the 200°C oven, standing against the rack.
7. Observe until appropriate colour has developed (1-3 minutes) – either hard or soft baked depending on information required.
8. When ready and using tongs remove the plate from the oven. Take care of the fumes escaping the oven.
9. When the plate is cool either take a photo or scan it– do this within a few hours as the information fades. You can sometimes leave the plate for a little while to fade the background.
10. Wrap the plate in tin foil and keep for a few days.

INTERPRETATION OF THE ANISALDEHYDE STAINING

1. Use the TLC Reference to interpret migratory differences and to ensure the plate has run correctly. If the chamber previously contained salt or was not dry the TLC reference will have an abnormal run pattern – check previous runs.

Glycolipids – green or blue green

Sphingomyelin and ceramide – blue

Glycerophospholipids – grey or violet

Degraded glycerophospholipids – intense red/violet

Protocol 13

Reinvestigation of catalytic effect

OBJECTIVE

To reinvestigate the catalytic effect of metal ions on globoside.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

§ globoside

Reagents

§ 0.005 M FeCl_3
 § 0.005 M CuCl_2
 § 0.005 M HCl
 § 0.005 M NaOH
 § 0.005 M Fe_2O_3
 § 0.005 M $\text{Cu}(\text{NO}_3)_2$
 § Chloroform
 § De-ionised water

Equipment

§ Timer
 § Serology tubes
 § Glass Pasteur pipettes
 § Heating block Wealtec Corp HB-2 , Serial # E02W04M0139
 § Whatman's pH paper, pH range 4-6, 038120, England
 § Whatman's pH paper, pH range 1-4, 318502, England
 § Thermometer
 § Nitrogen gas for drying

METHOD

Short Term Experiment

1. Suspend 20 mg (i.e. 1 ml of 0.02 M globoside) into 2 ml of metal ion solution (1 g of globoside/100 ml of metal ion solution)
2. Take pH of the solution and place on a heating block set at 80 degrees centigrade.
3. If the pH is greater than 6 then add known amounts of 0.005 molar NaOH so that the pH is about 6
4. Sample at 8, 24h... intervals.

Chloride addition

5. 2 ml of 0.005 M FeCl_3 contains 3 times more chlorine per Fe atom; therefore 6 ml of 0.005 M HCl can balance off the Cl^- ions in the solution not containing any Fe^{3+} .
6. 2 ml of 0.005 M CuCl_2 contains 2 times more chlorine per Cu atom; therefore 4 ml of 0.005 M HCl can balance off the Cl^- ions in the solution not containing any Cu^{2+} .

Long Term Experiment

7. Suspend 10 mg (i.e. 0.5 ml of 0.02 M globoside) into 1 ml of metal ion solution (1 g of globoside/100 ml of metal ion solution)
8. Take pH of the solution and place on a heating block set at 80 degrees centigrade
9. If the pH is greater than 6 then add 0.005 molar NaOH to bring the pH up to about 6.
10. Stop reaction on the 28th day.

Chloride addition

11. 1 ml of 0.005 M FeCl_3 contains 3 times more chlorine per Fe atom; therefore 3 ml of 0.005 M HCl can balance off the Cl^- ions in the solution not containing any Fe^{3+} .
12. 1 ml of 0.005 M CuCl_2 contains 2 times more chlorine per Cu atom; therefore 2 ml of 0.005 M HCl can balance off the Cl^- ions in the solution not containing any Cu^{2+} .

13. Take the pH of the solution of the solution. If the pH is less than 6 then add known amounts of 0.005 M NaOH solution so that the pH is about 6.

Sample treatment

14. Take out samples at each time interval into glass serology tubes and record their pH. If the measured pH is below 7 then add a known amount of 0.005 M NaOH to neutralize the acid (pH 7).
 15. Dry down this solution under nitrogen gas.
 16. Once the sample is dry, add a small amount of CM2:1 to each tube using glass Pasteur pipettes. Draw out the sample into another glass serology tube. Repeat with two more washings of the dry sample with CM 2:1 and transfer this into the same glass tube for each time interval. This ensures that the entire degraded sample is washed into the serology tube.
 17. Then add water to the original serology tubes containing the degraded sample (now containing the salt residue at the bottom). This would dissolve the salt. Add an equal amount of chloroform to form a two phase system (upper layer would be water and lower layer would be chloroform). Discard the upper layer (containing dissolved salts) using glass Pasteur pipettes. Add more water, swirl gently and then remove the water layer. Repeat this at a total of 3 times using glass Pasteur pipettes. This washing with water ensures that all the salts are removed and the bottom chloroform layer had the last traces of globoside (and degraded globoside).
 18. Then wash the remaining chloroform layer three times into their respective serology tubes and dry under nitrogen gas.
 19. Reconstitute the dried sample with about 2 μ l of CM 2:1 and load onto the TLC plates to perform the anisaldehyde staining.
-

Protocol 14

Immunostaining

OBJECTIVE

To immunostain Lewis blood group glycolipids using monoclonal antibodies.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

Immunostain control samples

- § Le^a control, c536/0063, Le^a from O Le(a+b-) small intestine
- § Le^a control, c076/0037, Extended Lewis structures from Le(a+b+) small intestine
- § Le^b control, c578/0067, Le^b from O Le(a-b+) small intestine
- § Le^c control, Extended non-Lewis structures from O Le(a-b-)NS small intestine
- § A control, m49/0055, Type 1 structures from group A Le(a+b+) meconium
- § ALe^b control, c408/0057, ALe^b from A₁ Le(a-b+) small intestine
- § H control, H type 1 from OLe(a-b-) plasma
- § Lewis blood group glycolipid samples
- § TLC control

Reagents

Dilution

- § Bovine serum albumin (BSA) Frct V IgG free, 30063.572, Gibco, New Zealand
- § Anti-mouse Ig (alkaline phosphatase labeled), AP326A, Chemicon, Australia 2.5 µl/1ml
- § Anti-human immunoglobulin (sheep), AB7108, Chemicon, Australia 2.5 µl/1ml
- § NBT/BCIP substrate, 1681451, Roche, Germany 20 µl/1ml
- § n-Hexane, 34484, Riedel de-Haen, Germany
- § Di-ethyl ether, 100921.5000, Merck, Germany
- § Chloroform 02405E21, SDS, France
- § Methanol 5METHL, ASCC
- § 1x PBS (phosphate buffered saline)
- § 2% BSA in PBS 2%
- § Substrate buffer (0.1 M Tris, 0.05 M MgCl₂, 0.1 M NaCl – pH corrected to 9.5)
- § Plasticiser solution 0.5%
- § Developing solvent CMH 60:35:8
- § Anisaldehyde stain
- § Diluent CM 2:1

Monoclonal antibodies – primary antibodies

- § Anti-Le^{ab}, Ortho-Clinical Diagnostics, clone 7A5A9 1 ml/4 ml
- § Anti-Le^{ac}, Ortho-Clinical Diagnostics, clone 17A5G8 1 ml/4 ml
- § Anti-H type 1 (SIGNET BG-4 CLONE 17-206) 1 ml/4 ml
- § Anti-A type-1 AH-21, Gift from H. Clausen 1 ml/4 ml
- § Anti-A ALBA clone, Diagnostic Scotland, Edinburgh 1 ml/4 ml
- § Anti-ALe^b - HH3, Gift from H. Clausen 1 ml/4 ml
- § Anti Le^b/H Glasgow and West of SBTS, clone LM 137/264.3 1 ml/4 ml

Equipment

- § Oven set at 200°C, Sunbeam-BT-5300
- § Camag TLC developing chamber
- § TLC plates 818133, Macherey Nagel, Germany
- § Developing wicks
- § Electronic timer
- § Regulated compressed air
- § Microsyringe, SGE, D07-B1223
- § Soft pencil
- § Tongs
- § Tall glass beaker

METHOD

Plasticising

1. Load TLC plates as outlined in the TLC protocol. However, load the TLC control on either side of the plate by leaving a 1cm gap between the control and the samples.
2. Develop as normal. Air dry and then carefully cut out the lanes loaded with the TLC control with 0.5cm spacing on either side of the strip.
3. Spray the strip with the anisaldehyde stain and develop in the oven until the bands develop.
4. Then carefully align these strips on either side of the unstained middle section of the TLC plate (containing the samples to be immunostained) and cut out the top section above the blue sphingomyelin band as all the Lewis blood group glycolipids migrate below this band.
5. Pour the plasticiser solution into a tall, narrow beaker. Pour enough plasticiser so that it would cover the entire surface of the unstained TLC plate.
6. Drop the TLC plate at once and start the timer for 1 minute.
7. Remove the plate from the plasticiser solution using a pair of tongs and stand it against a wall and let dry overnight. This should be carried out in the fume cupboard.

Immunostaining

§ Blocking plates

1. Spray 2% BSA in PBS solution over the plasticised TLC plates and then flood the plates with this solution. This step is calling the blocking stage where BSA binds to all the unspecific ligands to prevent or reduce excessive background staining. Leave the plates covered with 2% BSA in PBS for at least an hour.

§ Primary antibody

1. Make a 1 ml/4 ml dilution of the primary antibody in 2% BSA in PBS.
2. Remove the 2% BSA in PBS from the TLC plate and flood it with the primary antibody solution.
3. Leave for at least 90 minutes -3 hours

§ Conjugate antibody

1. Make a 2.5µl /1 ml dilution of the conjugate antibody using 2% BSA in PBS solution as the diluent.
2. Remove the primary antibody solution from the plasticised TLC plate by washing several times in 1xPBS.
3. Flood the plate with the conjugate antibody and leave for an hour.

§ Developing the Immunostain with the substrate

1. Make a 20 µl/1 ml dilution of the substrate using the substrate buffer as the diluent.
2. Remove the conjugate antibody solution from the plasticised TLC plate by washing several times in 1xPBS.
3. Then wash the plate several times with the substrate buffer.
4. Flood the plate with the substrate and leave until the bands have developed with minimal background staining. This process takes about 15-20 minutes.

§ Washing plates

1. Once the bands have developed, remove the substrate by washing the plate under a gentle stream of de-ionised water. Place the plate on a tissue and pat dry. Leave to dry overnight.

§ Analysing plates

1. The next day, align the photocopied section of the strips stained with anisaldehyde with the immunostained plate and secure with cello tape on an A4 sheet specifying the details of the samples loaded in each lane.
2. The bands can be identified by their relative migration and staining with specific antibodies by comparing with the migration of the standard glycolipids.
3. Laminate and store in a folder.

6 Appendix

Purification of Lewis Glycolipids by HPLC and Buchi Sepacore Chromatography

HPLC separation of Lewis b (Le^b) sample

The Lewis blood group structures underwent HPLC separation programme in order to obtain pure samples of Le^b and ALe^b samples. These structures needed to be purified to ensure that there would not be any interference of contaminants in the acid hydrolysis process. This section will describe the process by which Le^b and ALe^b structures were purified using the Buchi and High Performance Liquid Chromatography (HPLC) programmes.

The separation of a total Lewis b (Le^b) pool (intestinal origin) by High Performance Liquid Chromatography (HPLC) is shown in Figure 50 . The HPLC programme was able to separate the Le^b structures from other components based on polarity. The separation of the components of the Le^b mixture was based on their miscibility with the mobile phase and their interaction with the polar hydroxyl groups on the silica in a normal phase separation system. In a normal phase separation, the stationary phase is usually polar such as un-bonded silica while the mobile phase consists of non polar solvents such as chloroform. The crude Le^b glycolipid sample contained compounds with a wide range of polarities hence a binary gradient elution programme or a programme where two solvents which differ in polarity was employed. The two solvent mixtures used were chloroform: methanol: water (C:M:H) 60:25:4 and C:M:H 40:40:12. The ratio of these solvents was programmed to vary continuously over time to enhance the separation process. Further the composition of the mobile phase gradually changed from non polar to a polar mixture over time i.e. at the start of the programme, 100% C:M:H 60:25:4 was pumped through the silica column and gradually changed to 100% C:M:H 40:40:12 over a period of 180 minutes (refer to Protocol 4). There were 63 fractions collected of which fractions 51-63 contained structures of interest i.e. Le^a, Le^b and Le^c. Fractions 0-50 showed negative staining with anisaldehyde stain and are therefore not shown. The immunostains against various antibodies, Figure 50, plates II – V, had a vital role in deciding which fractions had pure Le^b molecules. This will be discussed in detail below.

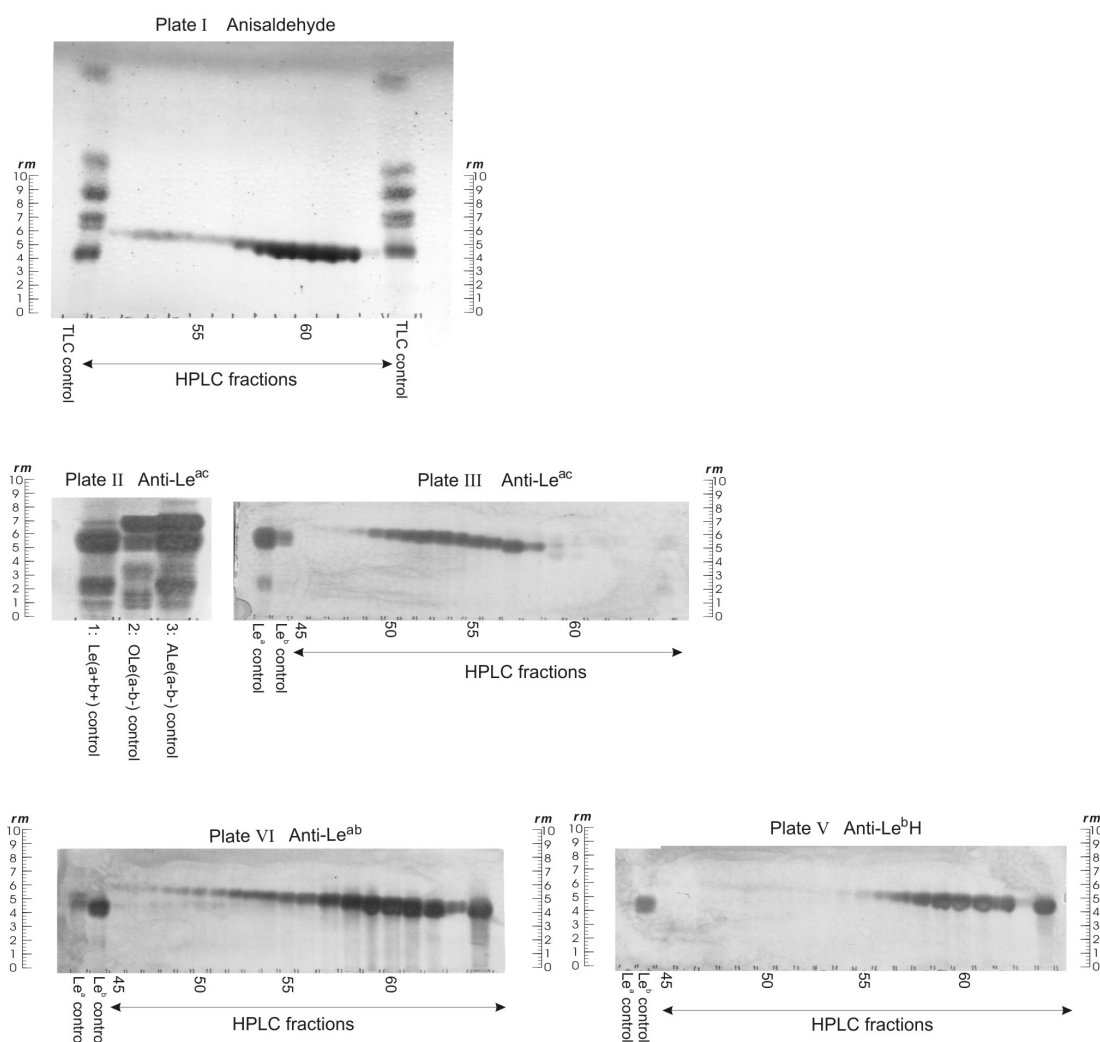


Figure 50: HPLC separation of impure Le^b sample.

Plate I is an anisaldehyde stain and plates II – V are immunostains with various antibodies. Note that the Le^c and Le^b controls have small amounts of Le^a molecules (plate II and III) and the Le^a control has a small quantity of Le^b structures (plate VI).

From the immunostain in Figure 50, plate V, it can be seen that the Le^b structures were in fractions 55-63. These fractions were pooled together to be used in degradation experiments in a later part of the project for degradation experiments (section 10).

HPLC separation of A Lewis b (ALe^b) sample

The purification of ALe^b molecules by the same binary gradient HPLC separation described above is shown in Figure 51, plate I. The molecules of interest were present between fractions 18-26. The Le^b molecules eluted in fractions 10-20. The ALe^b molecules were roughly present in fractions 21-26. The immunostains against various antibodies

were vital in separating the different components of the ALe^b mixture in order to obtain pure ALe^b structures for use in degradation experiments in a later part of the project.

Immunostaining the fractions with the anti-ALe^b reagent was solely able to identify the ALe^b structures as shown in fractions 20-25 (Figure 51, plate V). These fractions were pooled together and kept for use in degradation experiments in a later part of the project (Section 11).

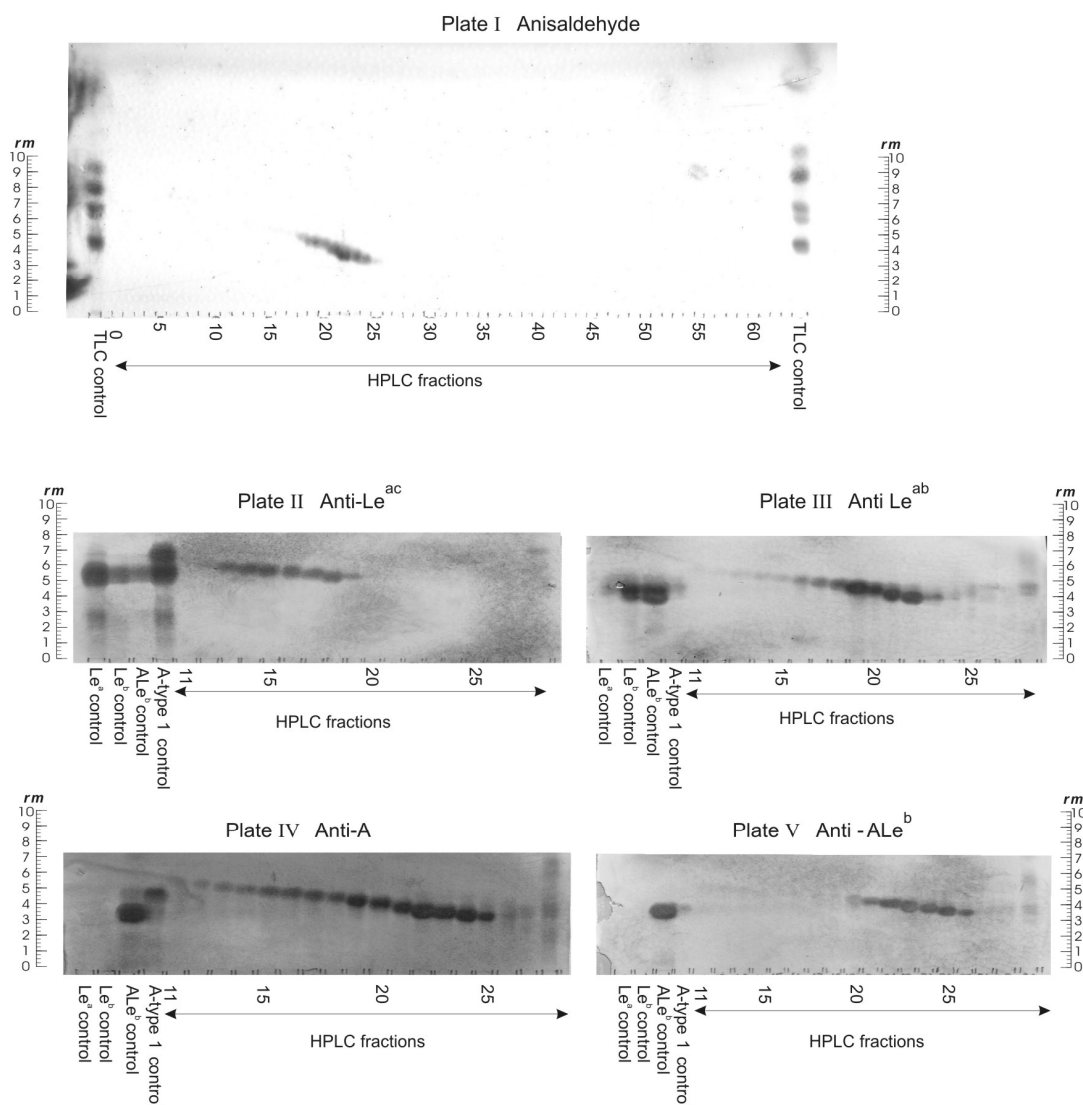


Figure 51: Anisaldehyde and immunostains of ALe^b fractions separated by HPLC. Plate I is an anisaldehyde stain and plates II –V are immunostains with various antibodies. Note that the Le^b control has trace levels of Le^a structures (plate II), the ALe^b control as small amounts of Le^b structures (plate III), the Le^a control has small amounts of Le^b molecules (plate III) and the A-type 1 control has ALe^b structures (plate V).

Buchi Sepacore separation of ALe^b

A Buchi Sepacore system was employed to carry out similar separations/purifications of glycolipids but having the flexibility of injecting larger volumes of crude sample and using the common and inexpensive laboratory grade solvents. A new method for glycolipid separation was developed but which was very similar to that of the gradient HPLC program. Figure 52, plates I – III show the different stages of development of the method. The method is outlined in Protocol 5 and Protocol 6.

Summary

The Buchi Sepacore system is a cheaper and more economical alternative to HPLC separations. The principal of separation is the same as that in HPLC. Larger sample volumes (or sample mass i.e. up to 500mg) can be injected into the Buchi Sepacore column compared to HPLC.

These methods were employed to purify the glycolipids, namely Le^b and ALe^b from the other contaminants. These purified glycolipids were then consumed in acid hydrolysis process and the main aim of the latter would be to determine the breakdown of structures into molecules of interest.

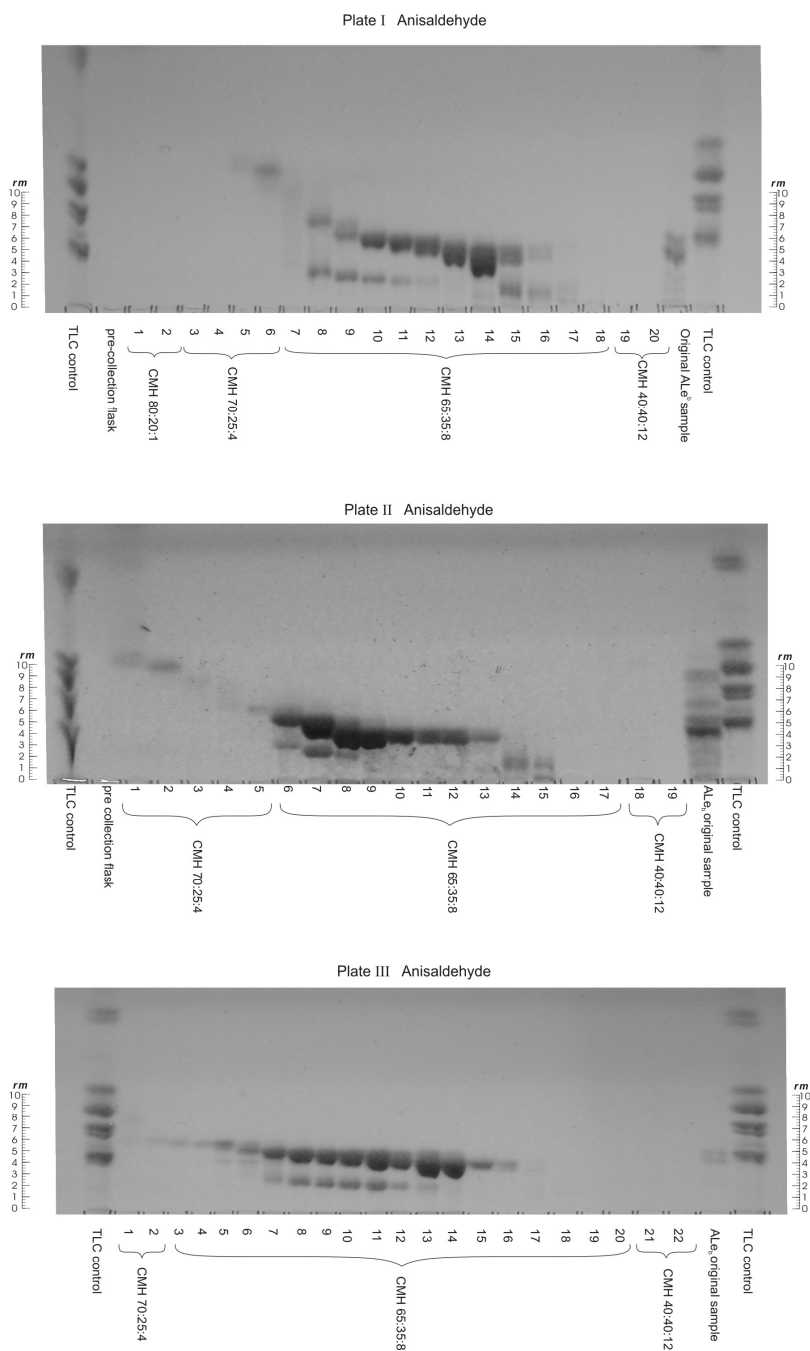


Figure 52: Separation of ALe^b sample using the Buchi Sepacore system. Each of the plates shows a slightly different elution programme.

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