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Synthesis of Sialic Acid Antigens

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Abstract

Synthesis of Sialic Acid Antigens

Ph.D. Thesis

N-Acetylneuraminic acid (NeuAc) is a sialic acid which constitutes terminal positions in glycoproteins and glycolipids. It is part of the antigenic determinant of many forms of cancer (S. Hakomori, Chem. Phys. Lipids. 42, 209 (1986)) and hence a cancer "vaccine" could be made from an appropriate multivalent macromolecular form of NeuAc.

To explore the possibility of inducing anti-NeuAc antibodies, we have synthesized polyvalent conjugates of αNeuAc or NeuAcα2-3Galβ1-4Glc (sialyl2-3lactose) on protein carriers. This was accomplished by reductive amination with 2-oxoethyl α-NeuAc or with the concealed aldehyde of the reducing sugar (glucose) onto the lysine residues of the proteins Bovine Serum Albumin (BSA) and Tetanus Toxoid (TT).

A new procedure involving a Michael-type addition employed the ϵ-amino groups of the lysine residues in a 1,4 nucleophilic addition to the acrylamide functional group in two derivatives of NeuAc; the N-acryloylaminoethylthiopropoyl glycoside, and the N-acryloylsialyl2-3lactosylamine. This technique does not require the use of reagents and was performed under mild basic conditions.

Polyacrylamide copolymers were also synthesized by radical co-polymerization of acrylamide with NeuAc monomers, providing a polymer (Mw≈100 kDa) with pendant NeuAc. Reactive monomers giving different spacer arms between the sugar and the polymer backbone were developed, including the allyl glycoside, a more reactive N-acryloylaminoethylthiopropyl glycoside and several lactose derivatives. The polymers were found to have a better shelf life than the proteins, and had the advantage of sharing only the NeuAc moiety.

Some of the NeuAc/protein conjugates were used to immunize rabbits, and the antibodies formed were screened with polyacrylamide copolymers with pendant NeuAc. The polymer with spacer was found to react better with the antibodies in immunoprecipitation and

enzyme-linked immunosorbent assay (ELISA).

Inhibition of ELISA experiments were done using synthesized derivatives of NeuAc to determine the binding specificity of the antibodies. It was found that the antibodies recognized the glycerol side chain, acid function, spacer, and to a lesser extent, the acetamido function of NeuAc. The same methodology was used to map the binding site of the lectin from *Triticum vulgaris* (called Wheat germ agglutinin, WGA) which binds NeuAc.

Nuclear magnetic resonance (nmr) studies were undertaken to determine the acidity constants (pK_a) and conformation of several NeuAc derivatives. Also studied was the lactone of NeuAcα2-3Galβ1-4Glc which is believed to be responsible for the immunogenicity of certain cancer cells (T. Dohi, G. Nores and S. Hakomori, Cancer Res. 48, 5680 (1988)). This work was complemented by molecular modelling using MM2. A model was proposed for the conformation of the lactone which is consistent with the n.m.r. evidence.

RESUME

Sythèse des antigènes de l'acide sialique

Thèse de doctorat

L'acide N-acétylneuraminique (NeuAc) est un acide sialique présent aux positions terminales des glycoprotéines et des glycolipides. Il fait partie des marqueurs antigéniques dans plusieurs formes de cancers.

Afin de produire des anticorps contre NeuAc, nous avons synthétisé des glycoconjugués du NeuAc. Ceux-ci ont été préparés par amination réductive et par une nouvelle méthode utilisant l'addition de Michael. Les anticorps produits dans des lapins ont été testés par des copolymères d'acrylamide et de NeuAc (avec et sans bras d'espacement). Les copolymères s'entreposent mieux que les protéines et ils ont l'avantage d'avoir seulement le NeuAc en commun. Le copolymère avec le bras d'espacement a mieux réagi avec les anticorps en immunoprécipitation et ELISA.

Des tests d'inhibition d' ELISA ont été effectués avec des dérivés du NeuAc pour determiner la spécificité des anticorps. Nous avons trouvé que les anticorps reconnaissent les groupes glycerol, acide carboxylique, bras d'espacement, et plus ou moins, le groupe fonctionnel acétamido du NeuAc. Cette méthode a aussi été utilisée pour examiner le site de reconnaisance de la lectine de *Triticum vulgaris*.

Des études de RMN ont permis de déterminer les constantes d'acidité (pKa) et les conformations des dérivés du NeuAc. Nous avons étudié la lactone du NeuAc(2-3)lactose qui est responsable de l'immunogénicité de certaines cellules cancéreuses. Un modèle pour la conformation de la lactone basé sur les calculs de programme MM2 est présenté.

Acknowledgements

First of all I would like to thank my supervisor Dr. René Roy. This success of this thesis was in a large part due to his planning and problem solving. Along the way he taught me synthetic organic chemistry and immunochemistry and it is now my responsibility to try to be worthy of his efforts.

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Regarding the preparation of this manuscript, I would like to thank the department for providing the computer and laser printing facilities, and Cynthia Carr for assitance in word

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I am grateful for the support I received from my fellow students, in particular Allan Rey, Robert Pon, Marie Letellier, Frank Tropper and Chantal Grandmaitre. The times we had together will aways be cherished.

To my family, I express the hope that they may appreciate what I have tried to do, and that in spite of the burden I have been as a student, I had something better in mind.

Let us also thank the Lord our God. We have been given life that we may live it fully. This thesis has fulfilled me greatly, and I wish to be able to help provide that opportunity to others.

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List of Abbreviations

A through J	Solvents systems used in t.l.c. are designated letters which may be found in Appendix 1.
ABTS	2,2'azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
BSA	Bovine Serum Albumin
Cer	Ceramide
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
ELISA	enzyme linked immunosorbent assay
ELLA	enzyme linked lectin assay
FAB MS	fast atom bombardment mass spectroscopy
Gal	galactose
GD3	NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer
Glc	glucose
GlcNAc	N-acetylglucosamine
GM3	NeuAcα2-3Galβ1-4Glcβ1-1Cer
H3ax	the hydrogen atom on C3 of NeuAc in the axial position
H3eq	the hydrogen atom on C3 of NeuAc in the equatorial position
HETCOR	hetero-nuclear shift correlated spectroscopy
HOMCOR	homo-nuclear shift correlated spectroscopy
HOMO	highest occupied molecular orbital
h.p.l.c. or HPLC	high pressure liquid chromatography
h.p.t.l.c.	high performance t.l.c.
HRP	Horseradish peroxidase
IR	infrared spectroscopy
LUMO	lowest occupied molecular orbital

MAb	monoclonal antibody
MM2	molecular mechanics (computer program from Allinger 1977)
NeuAc	N-acetylneuraminic acid
NeuGc	N-glycolylneuraminic acid
NCAM	Neural cell adhesion molecule
n.m.r or NMR	nuclear magnetic resonance (spectroscopy)
nOe	nuclear Overhauser effect
PNP	*p*-nitrophenol
Rf	relative flow
Rt	retention time
SOMO	singly occupied molecular orbital
TEMED	tetramethylethylenediamine
t.l.c.	thin layer chromatography
TT	tetanus toxoid
WGA	wheat germ agglutinin (from *Triticum vulgaris*)

Chapter 1

Introduction and Background

1.1 Introduction

Some 25% of deaths in England in 1980 were attributed to cancer (Mould 1983). Methods of fighting cancer have involved surgery, chemotherapy, radiation therapy, and numerous other recipes. These have been compared to killing a mosquito with a hammer. It has become apparent lately that the immune system may be activated against tumors, and the results are promising. Since sialic acid is a major constituent of cell surfaces, and in particular cancer cell surfaces, it is important to understand how it interacts with the immune system.

Making a cancer "vaccine" is a worthy objective, and while such a simplistic idea may seem far-fetched, there are many groups working toward it. A vaccine raises antibodies against diseased cells or disease-causing cells. Antibodies recognize molecules on the diseased cell surface which are not present on healthy cells, and adhere to them. The presence of an antibody on the cell surface activates the immune system against the attached cell.

Several steps must be taken to prepare a chemical vaccine. First the molecule marking a diseased cell molecule must be identified. Then this molecule must be extracted from natural sources or synthesized, and then turned into a vaccine. Finally, methods must be developed to determine the effectiveness of the vaccine in raising useful antibodies.

Sialic acid has been identified as a cancer cell marker (Hakomori 1986). This thesis set out to prepare a chemical vaccine based on sialic acid. Isolation of sialic acid from natural sources is described in Chapter 2. Chapter 3 describes the synthesis of key intermediates and derivatives used in the preparation of the vaccine. Chapter 4 concerns the synthesis of immunogens and antigens used for inoculation and screening of antibodies. In Chapter 5, a model study of these materials is discussed, and Chapter 6 describes the antibodies produced by inoculation of rabbits. Finally, Chapter 7 is a nuclear magnetic resonance (n.m.r.) study of the sialic acid derivatives with computer models.

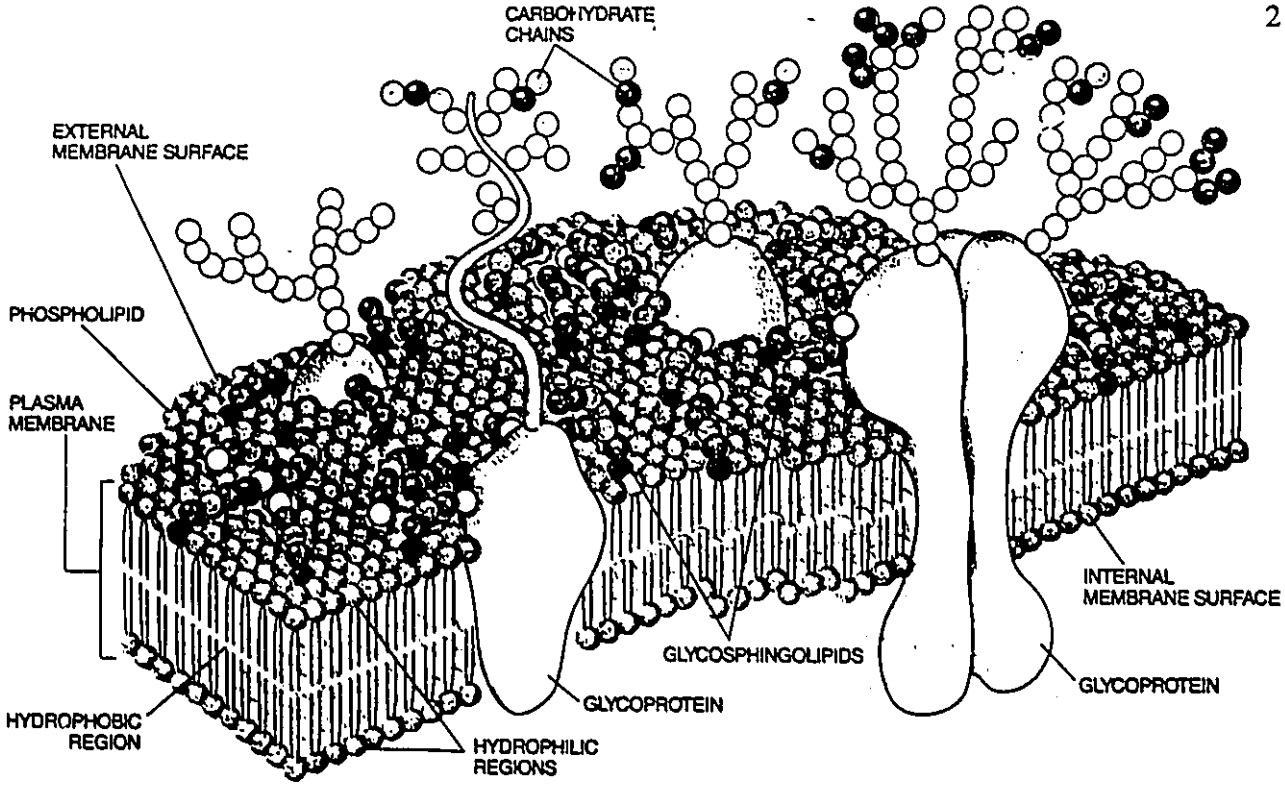
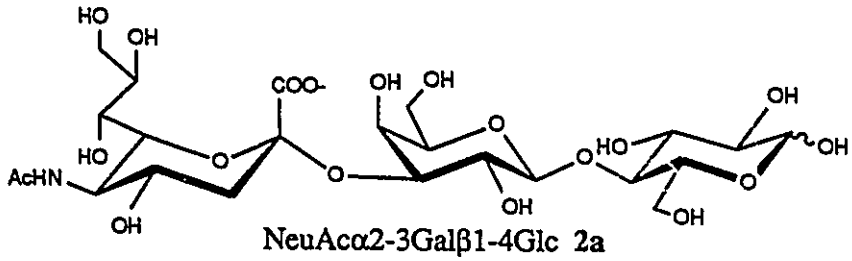
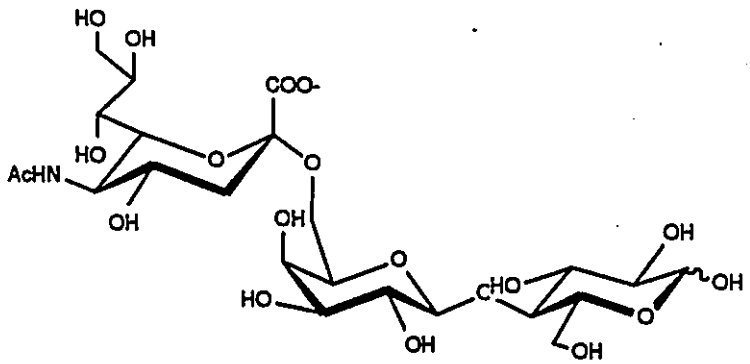


Figure 1-1. Schematic Diagram of the Cell Surface
(Taken from Hakomori 1986b)



NeuAcα2-3Galβ1-4Glc 2a



NeuAcα2-6Galβ1-4Glc 3a

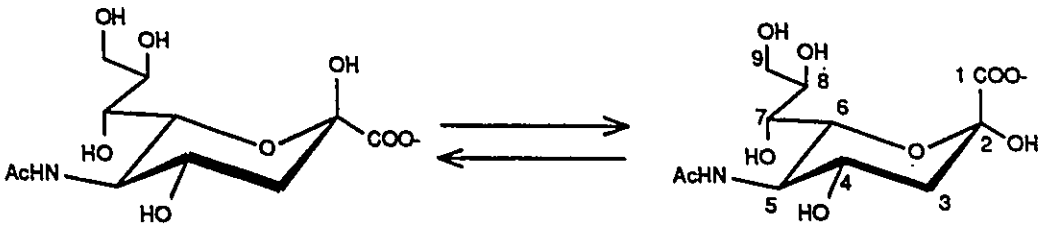
Figure 1-2 Common forms of NeuAc found on the Mammalian Cell Surface

1.2 Background Information

1.2.1 Nomenclature and Derivatives

Sialic acids are a class of acidic carbohydrates with a nine carbon chain. The name originates with the Greek word for saliva (Schauer 1982). Another name is neuraminic acid, reflecting its discovery from brain tissue isolates (Blix 1957).

The most common form in humans, generally found at the terminal positions of a number of glycolipids and glycoproteins, is N-acetylneuraminic acid, or *5-Acetamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosonic acid*, compound **1a**, drawn below. Common abbreviations include NANA and NeuAc.



92% β NeuAc **1a** 8% α

Other common derivatives present in nature include N-glycolylneuraminic acids, with a hydroxyl group on the acetamido, and a wide variety of hydroxyl substituents such as acetyl, lactyl, phosphate, sulfate and methyl groups. Notable examples are the 9-0-acetyl and lactyl derivatives. Table 1-1 gives a listing of the common forms of sialic acid and the animals which express them.

Table 1-1 Representative Sources of Sialic Acids in Nature

Derivative	Animal	Organs
Neu5Ac	human	all
Neu5Gc	starfish	all
Neu5Ac,Neu5,9Ac_2,Neu5,7,9Ac_3	human	mucin
Neu4,5Ac_2	monotreme	milk
Neu5Ac + <10% NeuGc	chicken duck	serum
60% NeuAc + 40 % Neu5Gc	calf	serum
30% Neu5Ac + 70% Neu5Gc	cow	serum
80% Neu5Ac + 20% Neu5Ac9Lt	human	serum

Sialic acid is present in nature at the evolutionary level of worms. Insects, crustaceans

and mollusks do not have sialic acid. On the other hand, a few viruses, bacteria and protozoa do contain sialic acid, and it is likely the result of gene transfer from higher organisms (Schauer 1982). It appears that the first evolutionary forms were N-glycolyl but later the N-acetyl derivative was synthesized directly. Humans do not have the N-glycolyl form of neuraminic acid.

A schematic diagram of a cell surface is presented in figure 1-1. As the diagram illustrates, much of the cell surface contains carbohydrates linked to proteins or lipids. Sialic acid frequently occupies the terminal position of these oligosaccharides, and is always present in the α configuration. In mammals, it exists invariably linked to the 6 or 3 position of galactose or N-acetylgalactosamine, as illustrated in figure 1-2. Lower organisms such as sea urchins and starfish have sialic acid present in "internal positions" such as NeuAcα2-6Glcβ1-8NeuAcα2-6Glc-Cer. Figure 1-3 shows the structure classifications of the polysaccharides found in mammalian cell surface glycoproteins (Boehringer 1987) and glycolipids (Hakomori 1986). These classifications are based upon biosynthesis.

1.2.2 Biochemical Interactions and Functions

As well being on the cell surface, sialic acid is also present in large amounts in mucins, which are excreted in the digestive and respiratory systems, and also in the cervix. Their role is generally considered to be protective, but the ubiquitous presence of sialic acid is an indication of some fundamental fuction, probably imparted by the chemical nature of the molecule.

NeuAc is distinguished from other carbohydrates by the negative charge it imparts. This functionality, while present on proteins, is only present on a few carbohydrates. Hence it is believed that the function is strongly related to this property.

The biological roles of sialic acid have been ennumerated as follows (Schauer,1985):

i) disaggregation of cells by repulsive effects, and Ca^{++} and other positive ion binding,

ii) influence on the conformation of glycoproteins, and protection against proteases,

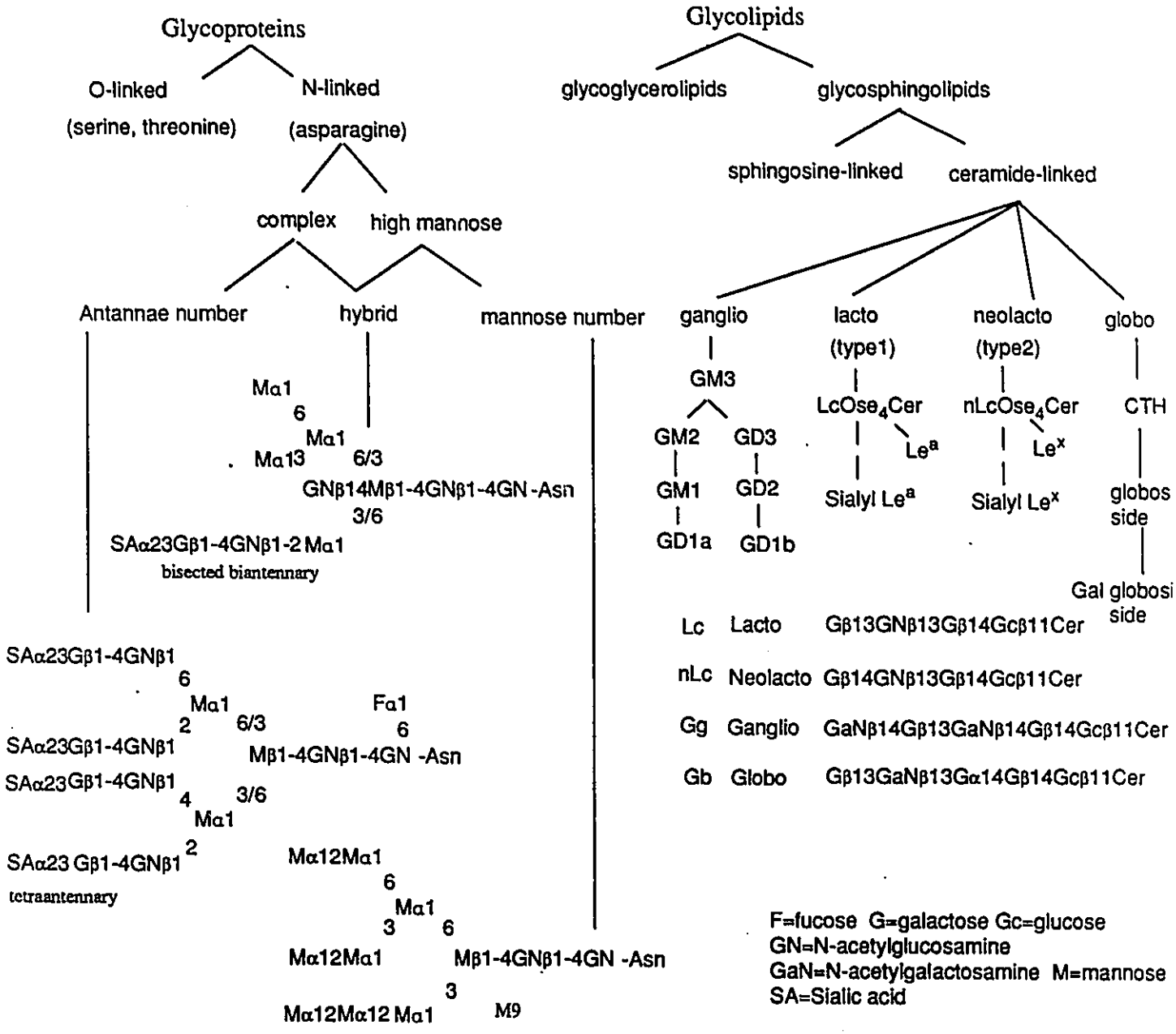
iii) sialic acids are receptors for peptide hormones, viruses and toxins,

iv) sialic acids have been found to prevent recognition of receptors, essentially acting as a mask of antigenic sites,

v) evidence is accumulating that sialic acids can be considered as potential determinants on differentiation amd oncodevelopmental antigens.

This broad range of functions explains the wide occurance of this molecule, and a few interesting examples will be presented in sections 1.2.4.3 and 1.2.4.4.

Figure 1-3
Carbohydrates found on the Cell Surface



1.2.3 Synthesis

1.2.3.1 Enzymatic Synthesis

The biological synthesis of NeuAc involves three enzymes which have been successfully used *in vitro* for the preparation of target compounds (Simon 1988, Thiem 1986, Nilsson 1988). The three steps include 1) synthesis of NeuAc, 2) activation as the cytidylmonophosphate, and 3) transfer of NeuAc to the glycosyl acceptor. These enzymes have been purified from a variety of sources, and are commercially available. A scheme of the biological processes is presented in figure 1-4.

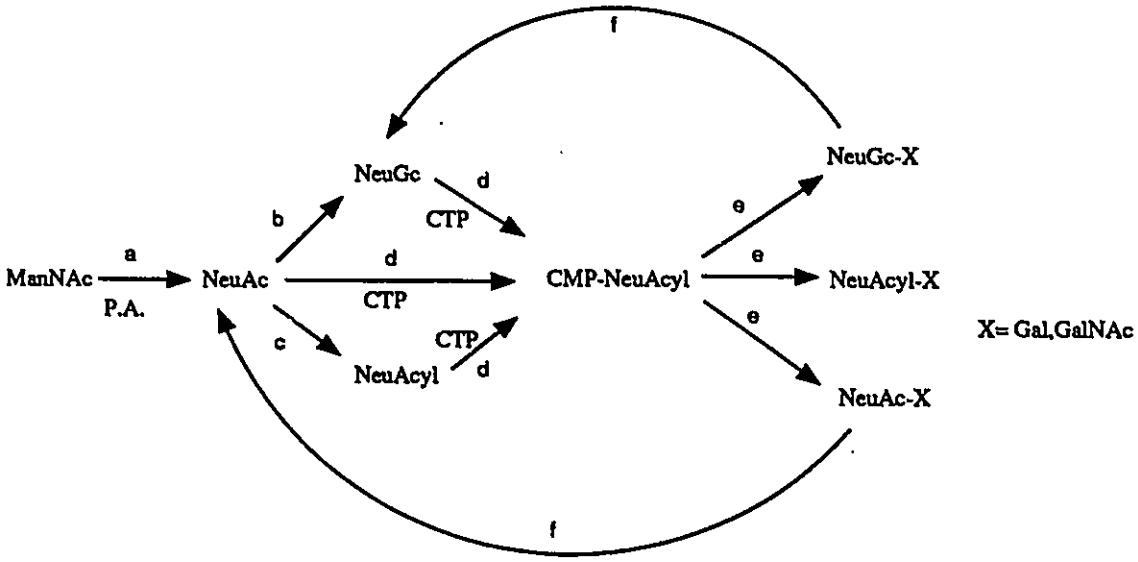


Figure 1-4 Schematic Representation of Metabolism of Sialic Acid

a) N-Acetylneuraminate synthase 4.1.3.20 b) N-Acetylneuraminate monooxygenase 1.14.99.18
c) acylneuraminate O-acetyltransferase 2.3.1.44 d) CMP-acylneuraminate synthase 2.7.7.43
e) sialyltransferase 2.4.99.1 f) sialidase 3.2.1.18

NeuAc itself is synthesized from N-acetylneuraminate pyruvate lyase (EC 4.1.3.3) which catalyses the following equilibria.

$$\text{ManNAc} + \text{pyruvic acid} \rightleftarrows \text{NeuAc} \qquad 1\text{-}1$$

The equilibrium may be shifted to the right by excess pyruvate, and the synthetic potential for this enzyme is being explored through the use of analogues (Augé 1990, Kim 1988).

At this point the N-glycolyl derivative can be prepared by hydroxylation which occurs in the golgi apparatus by the enzyme N-acetylneuraminate monooxygenase (EC 1.14.99.18)

(Schauer 1978, Muchmore 1989).

The next step in glycoside synthesis is the activation via CMP sialate synthase (EC 2.7.7.43) which catalyses the transfer of NeuAc to CMP from CTP. In this type of reaction, usually the sugar is activated as a di-phosphate, but apparently at least two phosphate bonds must be broken to supply the needed energy to form the NeuAc-phosphate bond. This energy is likely stored in the repulsion of the carboxylate group and the negatively charged phosphate. The reaction is shown below.

$$NeuAc + CTP \rightarrow NeuAcCMP + 2P_i \qquad 1\text{-}2$$

As with the first enzyme discussed, the specificity of this enzyme is being researched (Petrie 1989, Christian 1989). It has been observed that the more readily available adenylate kinase is also able to perform the same function (Simon 1988).

The final step in glycosylation requires sialyltransferase which has specificity for the aglycon, commonly either the 6 position of D-galactose (EC 2.4.99.1) or the 3 position of D-galactose (EC 2.4.99.4). These enzymes are commercially available, and are highly regio- and stereo-specific (Sabesan 1986), though the full extent of their substrate specificity with respect to NeuAc-CMP is still being explored (Kadowaki 1989).

1.2.3.2 Chemical Synthesis

The chemical synthesis of NeuAc was recently accomplished by Danishefsky (1988) using a hetero Diels-Alder cyclization followed by extension of the glycerol side chain. Such an approach is potentially useful in the synthesis of a C-glycoside of NeuAc, which has not yet been reported in the literature.

The chemical glycosylation of NeuAc is an interesting story to follow. Normal Koenigs-Knorr glycosylation using the 2-deoxy-2-chloro derivative with silver or mercury catalysts often resulted in low stereoselectivity, especially if the alchohol is secondary (Paulsen 1986). Other methods such as glycosylation with N-iodosuccinimide (Kirchner 1988), or DMTST with the methylthioglycoside (Kanie 1988) and S-glycosyl xanthate (Marra 1990) or with the use of the epoxide (Okamoto 1987) still result in low α selectivity. Progress has been made through the use of a neighboring thiophenyl auxilliary which can be removed by reduction. Yields of up to 60% with high α selectivity at secondary alchohols have been reported (Ogawa 1990).

1.2.3.4 Degradation

Enzymes can be used to remove the carbohydates from glycoproteins (Boehringer

1987) and glycosphingolipids (Li 1989). NeuAc may be hydrolyzed by commercially available enzymes from *Clostridium perfringens* (p 225, Schauer 1982) and *Vibrio cholerae* (Brossmer 1971).

1.2.4 Molecular Recognition and Specificity

The quintessential question facing cell biologists is the manner in which cells recognize and interact with each other. How do cells form tissues and organs? What stops cells from continuing to divide? How does the immune system recognize a "foreign" entity? And specific to this thesis, what is the role of NeuAc?

While a complete discussion of what is known in this field is not relevant to this thesis, certain aspects of the immune system and immunochemistry are of fundamental importance. These are briefly reviewed below followed by a discussion of the molecules of cell-cell adhesion and recognition.

1.2.4.1 Immunochemistry

The study of the immune system has enabled the development of methods to eradicate many diseases. There are variety of cells in the blood, lymph, bone marrow and thymus involved in the immune response. This discussion is limited to antibodies and methods of dectecting and determining the specificity of antibodies.

Antibodies are of the immunoglobulin superclass of proteins excreted by B (bone marrow) cells in response to a foreign molecule in the organism (Roitt 1985). There are four polypeptide chains (two heavy and two light) that join together to form the Y shaped molecule, each with a constant and variable region (see figure 1-5). The variable region is responsible for specific binding with an "epitope" on the foreign molecule. The size of the epitope may be as small as 7 to 10 amino acids. There are two binding sites on antibodies, and hence interaction with polyvalent epitopes will result in cross-linking and associated phenomenon.

In this thesis, the term "antigen" refers to a molecule which binds with an antibody, and "immunogen" is a molecule which elicits an immune response, resulting in antibody production. In both cases, macromolecules are the ideal candidates.

The characteristics of several classes of mammalian antibodies are listed in table 1-2.

IgM molecules are the first to appear in the blood stream during infection, usually due to direct interaction between the immunogen and B cells, where the antibody is part of the cell surface. The B cells may alternately interact with a T (thymus) cell which has processed the immunogen and is "presenting" it on the cell surface in association with MHC (major

histocompatibility complex). The T cell also secretes interleukins which cause a variety of effects such as proliferation and class switching. The B cell population will go on to produce antibody secreting cells and memory cells. These memory cells are the basis of the usefulness of vaccines in that they start secreting specific antibodies much faster than other B cells.

Table 1-2 Mammalian Antibody Classes and Properties (Adapted from Roitt 1985).

Antibody	Mol. Wt.	Serum conc. (mg/ml)	Complement fixation	Binding to: macro-phage	neutro-phils	killer cells
IgM	970,000	1.5	+++	-	-	-
IgG1	146,000	9.	++	+	+	+
IgG2	146,000	3.	+	-	-	+
IgG3	145,000	1.	+++	+	+	+
IgG4	146,000	0.5	-	-	+	-
IgA	160,000	3.	-	-	+	-
IgD	184,000	0.03	-	-	-	-
IgE	188,000	0.00005	-	-	-	-

The complexity of the immune system is still being explored, nevertheless, vaccines have been used for one hundred years as effective ways to prime the immune system against particular diseases. Alternately, antibodies raised in an animal may be used in passive immunization for acute conditions, such as against snake bite venom. In certain cases such as leprosy, an antibody response is undesirable. For these cases, a vaccine must be designed which stimulates a cellular response (Stewart-Tull 1984).

Antibodies have been found useful due to their specific adherance with the antigen. Hence they are diagnostic reagents. AIDS, for example, is diagnosed through the presence of antibodies against the virus HIV glycoproteins. Section 1.2.5 discusses the use of antibodies for cancer diagnosis and treatment.

1.2.4.2 Experimental Methods used in Immunochemistry

In order to assess the immunocompetance or humoral immune response, many experimental methods have been devised (Johnstone 1982). Only methods relevant to this thesis will be discussed here.

i) Double Immunodiffusion (Ouchterlony 1978)

In this experiment, as the name implies, antibodies from serum are allowed to diffuse towards the antigen in a buffered agarose gel. This is done by cutting small wells about 1 cm apart in a thin layer of gel. As the antibody and antigen meet in the gel, precipitation due to binding is easily observed, and can be stained with a dye, usually coomassie blue.

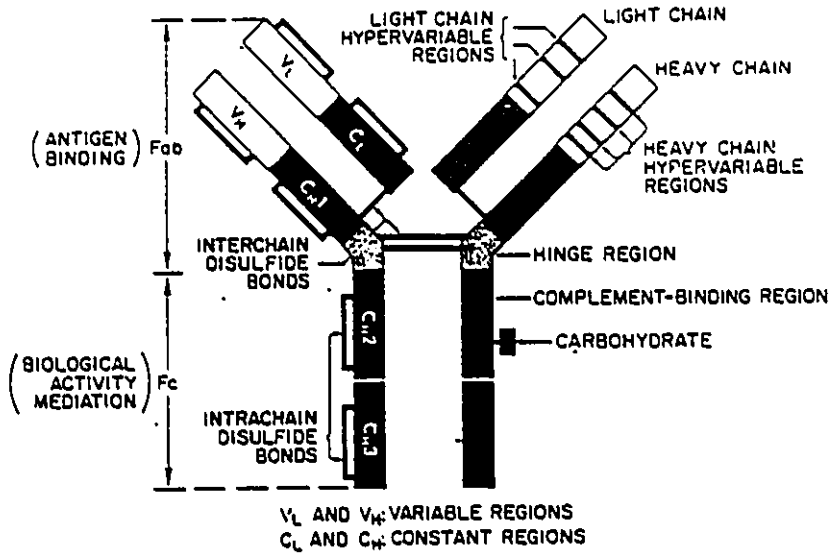


Figure 1-5 Schematic Diagram of the Antibody showing the Light and Heavy Chains (Taken from Kindt 1984)

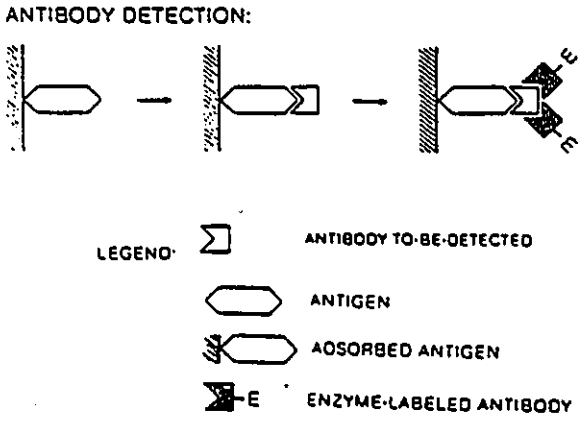


Figure 1-6 Steps in the ELISA Technique
a) coating b) incubation c) detection

ii) Immunoelectrophoresis

This is similar to double immunodiffusion with the inclusion of electrophoretic movement of either the antigen or antibody by electric current. For example, the antigen is placed in a well in buffered agarose, and electric current is applied to the gel. After some time, the current is turned off, and a trough is cut in the gel parallel to the direction of the current. Serum is placed in the trough, and after diffusion, precipitates in the form of arcs will appear.

iii) Immunoprecipitation (Kabat 1961)

Immunoprecipitation involves allowing antibody and antigen to bind in solution, and then centrifuging the precipitate formed. Protein content in the pellet and supernatant may then be measured. In general, immunoprecipitations are done with a constant antibody concentration and increasing antigen concentration. The resulting precipitation curve rises as a quadratic and reaches a maximum called the equivalence point. At this point all of the antibody is precipitated by the antigen. After the equivalence point, there is inhibition of precipitation, and the curve falls slowly.

iv) Enzyme-Linked Immunosorbent Assay (ELISA) (Engvall 1980)

This extremely useful technique is based on the realization that once a plastic surface has had protein adsorbed onto its surface, other proteins will not stick unless there is a specific interaction. Thus, an antigen may be adsorbed onto the surface of a small plastic well, and sera may be screened for antibodies specific to that antigen. The method involves the following five steps:

a) coating: adsorbing the antigen onto the plastic surface,

b) blocking: ensuring the plastic surface is completely covered to prevent further and unspecific adsorption,

c) incubation: addition of the sera, usually in successive dilutions to determine titre,

d) washing: removing unbound antibody,

e) detection: determining the amount of antibody bound to the well.

The last step is most often done by repeating steps c) and d) using anti-antibodies which are conjugated to an enzyme capable of catalyzing a color change. The enzyme is frequently horseradish peroxidase (HRP) and the substrate is 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS). It can also be done using radioactively labelled antibodies in a method called RIA. The different forms of ELISA are shown schematically in figure 1-6.

v) Inhibition of ELISA

Inhibition of ELISA is a useful application which involves a competition between the adsorbed antigen and a soluble form of the antigen. Structural changes in the soluble antigen can be compared with the ability to inhibit binding, and thus the binding site of the antibody can be probed.

Methods i) through v) can also be used with other proteins which bind specifically to carbohydrates. When a lectin (see section 1.2.4.4) is used in the same fashion as ELISA, the method is called "enzyme linked lectin assay" (ELLA). This section will be referenced when these methods are employed in this thesis.

1.2.4.3 Cell-Cell Adhesion

Cell-cell interactions are restricted by glycosphingolipids and glycoproteins (Igarashi 1989, Rutishauser 1989) and sialic acid is particularly involved. The following are a few examples of what is currently believed.

i) Neural Cell Adhesion Molecule (NCAM)

The NCAM glycoprotein is directly implicated in cell developement of the embryo. Two forms of the molecule appear to exist. One form of the molecule has a large quantity of poly α (2-8) linked NeuAc, while the other form has a low quantity (Rutishauser 1989). NeuAc is attached to the protein via a complex type N-asparaginyl carbohydrate (M^{c}Coy 1987). While the amount of NeuAc is believed to attenuate the strength of the NCAM interaction, the molecule is described as a versatile "glue" which is used to initiate cell-cell adhesion (Rutishauser 1989).

ii) Evidence of Carbohydrate-Carbohydrate Binding

More specific interaction between cells is believed to come from glycosphingolipids. Interference with these molecules in the embryo results in failure to develope tissues. It has always been assumed that the carbohydrate portions of the gangliosides interact with endogenous lectins or glycosyltransferases on the cell surface. Recent evidence indicates that there may be binding between specific gangliosides, such as Gg_3 and GM_3, through the carbohydrate portions of the molecules (Kojima 1989). It will be interesting to watch future developments to see if this phenomenon is general.

1.2.4.4 Carbohydrate Binding Proteins: Lectins

Lectins are a diverse class of proteins or glycoproteins which are characterized by their

ability to bind to carbohydrates. There is no enzymatic activity associated with them. An excellent review of the topic is available from Liener *et al.* (1986), and a recent update has been published (Sharon 1989).

Several examples of lectins will be presented as they relate to sialic acid.

i) Aging of Red Blood Cells

Glycophorin A is a major glycoprotein of erythrocyte membrane, and due to the high NeuAc content, contributes to the negative charge of red blood cells and probably prevents agglutination (Schauer 1982). NeuAc content of red blood cells has been associated with the age, and it is believed that NeuAc plays a role in masking galactose (Kelm 1986) residues for which there are receptors (lectins) on Kupffer cells in the liver (Schauer 1985).

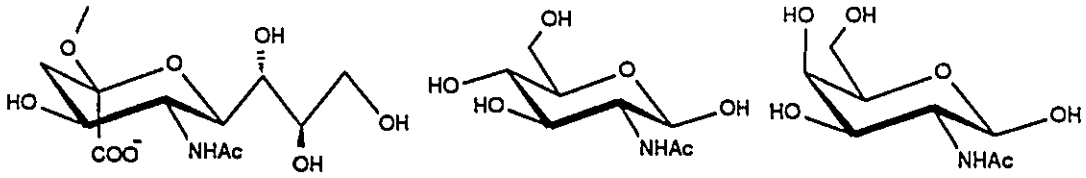
ii) Hemagglutinin from Influenza Virus

The hemagglutinin from influenza virus was demonstrated by X-Ray crystallography (Weis 1988) and n.m.r. immunochemical studies (Sauter 1989) to have specificity for αNeuAc. The binding site of this protein did not contain a positive charge as might have been expected since NeuAc is negatively charged. The N-acetyl moiety of NeuAc was within van der Waals distance of a tryptophan, and n.m.r. studies indicated there was some hydrophobic interaction. There were several possible hydrogen bonding sites, but there appeared to be no obvious interaction of the NeuAc aglycon (lactose), though an amino acid substitution at position 226 resulted in a change of specificity from the 2→6 to the 2→3 isomer (Sharon 1989).

The study of the hemagglutinin molecule was undertaken to help develop pharmaceuticals which would be used to inhibit binding of the virus to its receptor. They (Weis 1988) confirm that a polyvalent form of such an inhibitor would be most effective as proposed by Roy *et al.*(1988d) since the binding constant is low.

iii) Wheat Germ Agglutinin

The lectin from the seed of *Triticum vulgaris* has been known since the 1940's to be able to agglutinate red blood cells (Liener 1986). This lectin is commercially available as a mixture of isolectins. It has specificity for NeuAc and N-acetylglucosamine, and to a lesser extent for N-acetylgalactosamine. These three molecules share the same structure, as illustrated below, required for the binding site: trans di-equatorial N-acetyl and hydroxyl groups (Wright 1980). Though a single carbohydrate can fit in this site, inhibition studies show a pentasaccharide is a better inhibitor than a monosaccharide (Liener 1986). NMR studies indicate there are four binding sites in the protein dimer for NeuAc (Kronis 1985), but X-ray analysis does not concur (Liener 1986).



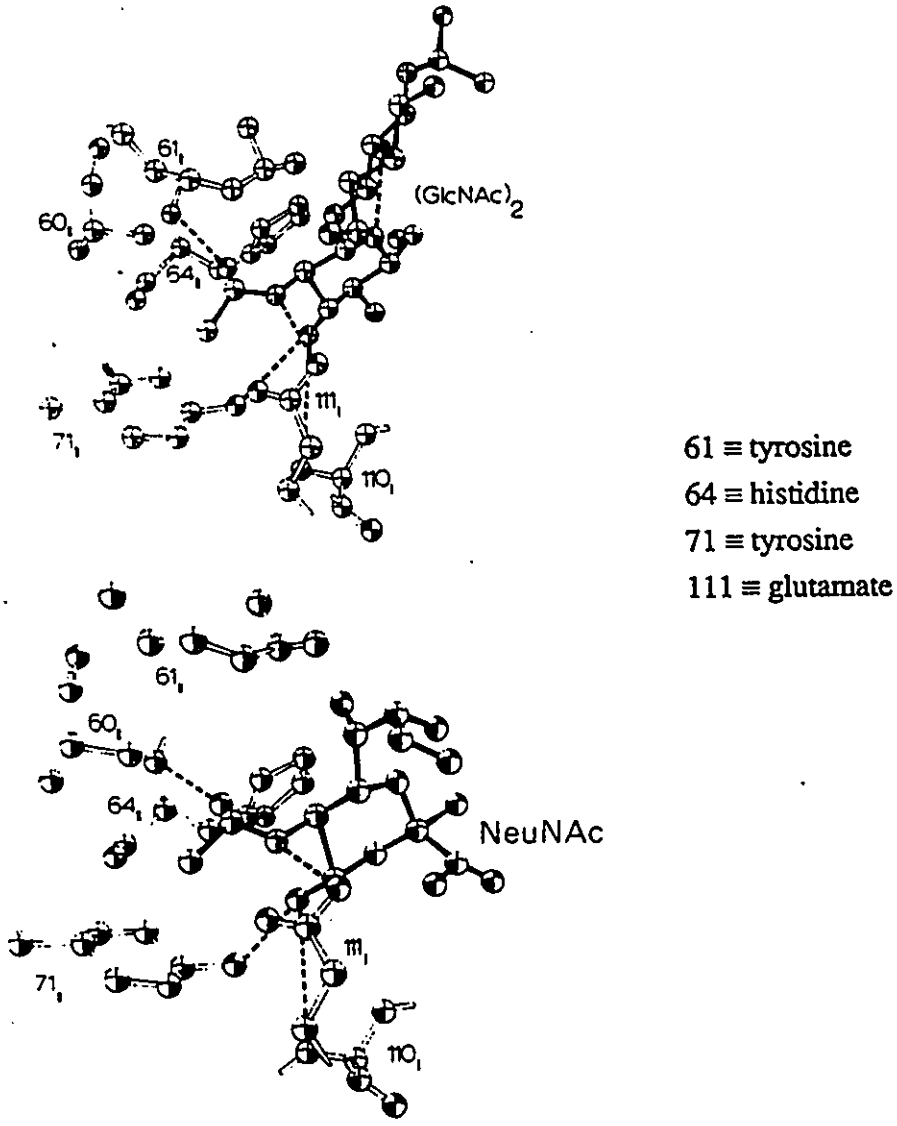


Figure 1-7 Binding Site of Wheat Germ Agglutinin (WGA) as determined by X-Ray Crystallography
(Taken from Wright 1980)

Figure 1-7 shows the binding site based on X-Ray data. There is a hydrophobic interaction of the N-acetyl methyl group with an aromatic, this time a tyrosine, which also serves to hydrogen bond with the trans hydroxyl group. In fact, this interaction appears essential in the binding (Wright 1987).

A commercial colorimetric reagent which detects NeuAc and GlcNAc in solid phase assays is made by the conjugation of WGA to the enzyme HRP (Sigma product L3892).

1.2.5 Tumor Markers

Much of the interest in NeuAc stems from the fact that altered levels of NeuAc are associated with some cancers (Rosenberg 1976, Schauer 1982, Hakomori 1986, Dyaltlovitskaya 1987).

By 1982 it was recognized that protein bound NeuAc levels could be either abnormally high or low in neoplastic cells, and that sialyltransferase activities were also unusual, again with no particular trend. It had been known since 1967 that neuraminidase-treated tumor cells decrease their oncogenicity (Schauer 1982), but no clear explanation was available, and its use as a cancer treatment was at best uncertain. These unrelated facts were drawn together when the advances in immunochemistry were applied to the study of tumor cell antigens.

1.2.5.1 Monoclonal Antibodies (MAbs) to Tumor Cell Antigens

The advent of hybridoma technology for the production of monoclonal antibodies has led to a remarkable increase in knowlege concerning the cell surface antigens of cancer cells. Antibodies raised against tumor cells appear to be directed against glycosphingolipids (Hakomori 1986, Dyaltlovitskaya 1987). Characterization of the antigens using monoclonal antibodies has advanced quickly because glycosphingolipids are relatively simple molecules and appear to be directly related to metastasis. It was recognized by inhibition studies that these MAbs were specific for the carbohydrate portions of the glycosphingolipids.

This kind of detailed immunochemical characterization has shown that tumor cells contain glycosphingolipids which appear on normal cells, but in differing amounts. In some instances new molecules appear. The following events have been proposed (Hakomori 1986) to explain cell surface changes that appear on tumor cells or transformed malignant cells:

i) incomplete sugar chain synthesis due to blocking of glycosyl transferases resulting in an overall simplification of the glycolipid profile,

ii) neosynthesis leading to the appearance on the cell surface of new glycolipids, and

iii) organizational rearrangement of glycolipids on the cell surface.

A detailed analysis of the types of phenomenon which result in simplification of the

glycolipid profile has been discussed by Hakomori (1986). In these cases, there is a build-up of simple gangliosides such as GM_3 and GD_3 which are present on most cell surfaces. In neosynthesis, transferase activity seems to increase, and new carbohydrates result. An example is sialylated Le^X, which is normally lacking in sialic acid. Finally, organization of lipids is apparently affected by the density and crypticity of the antigens in the membrane. For example, monoclonal antibodies against gangliosides GM_3 appear to react specifically against cancer cells, even though this ganglioside is present on most cells.

1.2.5.2 Cancer Specific Antigens

The preparation of monoclonal antibodies with specificity for carbohydrates has recently become quite prominant in the literature (Tai 1987, Tai 1988, Nudelman 1989). There is a great potential for these antibodies to be used as diagnostics (Irie 1985, Hakomori 1989).

Table 1-3 is a compilation of current knowlege of tumor-associated antigens which can be detected using monoclonal antibodies with specificity for carbohydrates. This table is limited to sialic acid-containing antigens, but a more general list can be found in Magnani (1986).

These monoclonal antibodies were generally raised by the immunization of mice with cancer cells, and then hybridomas were prepared and screened for useful MAbs with purified antigen.

In some instances, antibody production in humans against glycosphingolipids causes autoimmune disease. Those involving sialic acid are Hanganutziu-Deicher disease (Mukuria 1986), cold hemagglutinin disease (Pruzanski 1984, Roelke 1980) and peripheral neuropathy (Ilyas 1985).

1.2.5.3 Anti-GD_3 Antibodies

The carbohydrate portion of the ganglioside GD_3 is NeuAcα2-8NeuAcα2-3Galβ1-4Glc (4). It is a ganglioside present in normal cell extracts (Hakomori 1986), but MAbs against this molecule (Magnani 1986) are reactive against a variety of cancers (Furukawa 1989). The conformation (Yu 1986) and lactones (Ando 1989) of this molecule are not yet fully understood, but it is apparent that this structure posesses many different stable conformations. No enzymes have been found to cause lactonization, but these ganglioside lactones are believed to exist *in vivo* (Riboni 1986).

1.2.5.4 Anti-GM_3 Antibodies

Several factors come into play concerning the immunogenicity of gangliosides. The case of GM_3 is particularly interesting. The carbohydrate portion of GM_3 is

NeuAcα2-3Galβ1-4Glc, also known as sialyllactose, compound **2a**. This molecule may form an internal lactone between the NeuAc carboxyl and the C2 hydroxyl group of galactose (Yu 1985). Hakomori's group used B16 melanoma cells to immunize mice which led to monoclonal antibodies which were specific for GM_3, and it is believed that the immunogenicity of GM_3 is due to the lactone form of the NeuAcα2-3Gal moiety (Dohi 1988). MAbs DH_2 (IgG3) and M2590 (IgM) showed specificity to the lactone by ELISA, but also cross reacted with GM_3 depending upon the coating conditions used. Human monoclonal antibodies directed against GM_3 have also been found (Tai 1987, Lloyd 1989), and this was considered surprising since GM_3 is present on a wide variety of cells, but the antibody reacted preferentially against melanoma tumors and other neuroectodermal cancers. These results have been explained by several hypotheses:

i) GM_3 is cryptic in most cells, or

ii) a threshold of antigen density must be surpassed before detection in some assays, or

iii) the lactone is the epitope which may cross react with a similar conformation of GM_3.

Both groups of Hakomori and Lloyd are in agreement over hypothesis ii), and it is clear that antibody functions are density dependent. Hypothesis i) and iii) may be consistent, especially since modeling has predicted an amphoteric surface of GM_3 (Kojima 1989) and the carbohydrate chain may interact with the hydrophilic head of the phospholipids (Hakomori 1986). Three stable conformations of the glycosidic bond of NeuAcα2-3Gal component of GM_3 have been calculated on the basis of n.O.e. studies and molecular modeling using "SUGAR" (Poppe 1989). Is is conceivable that the antibody recognizes a particular conformation of the molecule, and the epitope is hidden in other conformations.

1.2.6 Summary of Background Information

Sialic acids are ubiquitous in higher animals, and it has been postulated that they play an essential role in cell cell recognition, possibly as an attenuator or mask of antigenic sites (Kelm 1986). The role of this molecule in oncogenesis is still being investigated.

1.3 Thesis Objective

In certain papers discussed above, some specificity tests have been made to determine whether NeuAc is in the binding site of the antibody. A small amount of mapping has been done, and it is apparent that the NeuAc part of the gangliosides is involved in binding. "An

Table 1-3 Monoclonal Antibodies used in Diagnosis of Cancer

Cancer (%binding)	Monoclonal Antibody (Type)	Method	Reference	Specificity
Colonic Lung (80%) Adenocarcinomas	FH6 (IgM)	1	Hakomori (1986)	Sialyl Le^x NeuAcα23Galβ14(Fucα13)GlcNacβ13Galβ14Glcβ11Cer
Melanoma Astocytomas	R24 (IgG3)		Magnani (1986)	GD_3 NeuAcα28NeuAcα23Galβ14Glcβ11Cer
Breast Metastatic	TKH2	Immunostaining	Kjeldsen (1988)	Sialyl-Tn NeuAcα26GalNacα1OSerine
Colon (93%) Ovarian	B72-3 (IgG)	Immunostaining	Itzkowitz (1989) Molinolo (1990)	TAG-72 Glycoprotein
Pancreatic (71%)	N-19-9 (IgG1)	ELISA serum protein	Kannagi (1988)	Sialyl Le^a NeuAcα23Galβ13(Fucα14)GlcNacβ13Galβ14Glcβ11Cer
Hepatoma	MSG-15	Immunostaining HPTLC	Taki (1990)	Sialyl paragloboside
Various	FH7 (IgG3)	Immunostaining HPTLC	Hakomori (1989)	Disialyl Le^a
Melanoma	18B81	Immunostaining HPTLC	Dubois (1986)	GT_3 NeuAcα28NeuAcα28NeuAcα23Galβ14Glcβ11Cer

important question that still awaits final resolution is whether NeuAc itself can act as an antigen, or whether it is only active as the specific determinant of a macromolecule." (p 276, Schauer 1982). This question has become the central theme of this thesis.

The preparation of macromolecules with pendant NeuAc could be useful not only as an antigen to detect the presence of antibodies with NeuAc specificity, but also as an immunogen, for use as a cancer vaccine. Such a product must be carefully studied to determine the type and specificity of antibodies produced. Hence this thesis was designed to synthesize immunogenic forms of NeuAc, immunize rabbits, and then detect and study the resulting antibodies.

1.4 Precedents

The underlying theme of this thesis was pursued not without some reference to work that had already been done. Two types of synthetic macromolecules have been described in the literature for immunological studies: Protein conjugates and more recently polyacrylamide copolymers.

1.4.1 Protein Immunogens

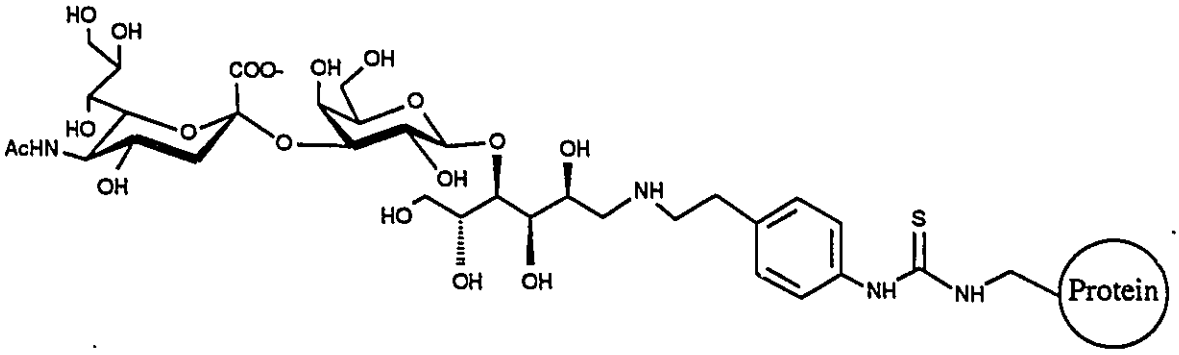
Only larger polysaccharides and proteins are immunogens by themselves. Below a molecular weight of 5000, conjugation (to a protein) increases the immune response (Makela 1987). Serum albumins are usually chosen because they remain soluble after conjugation. While insoluble conjugates can still be used for immunization, they are harder to characterize (Erlanger 1980). Keyhole limpet hemocyanin (KLH) has gained a reputation as a good carrier, being foreign to all mammals, while other groups claim thyroglobulin is superior. It has been demonstrated that synthetic peptides such as poly (L-lysine) or poly (L-glutamic acid) are not effective (Erlanger 1980).

The ratio of hapten to carrier can be as low a 2:1 and still produce an immunogen capable of eliciting antibodies (Erlanger 1980), though a ratio of 15-30:1 is more desirable (Hurn 1980). Landsteiner, in his original work which set the ground rules for immunoconjugates in the early 1900's, suggested that a ratio of 10:1 was optimal (Erlanger 1980).

There are many methods to modify amino acids in proteins (Lundblad 1984). Chemical methods in the preparation of conjugates are reviewed in Erlanger's article (1980). A particular method which conjugates sialyllactose is reviewed here.

Pioneering work in the preparation of anti-NeuAc antibodies was done by Ginsburg

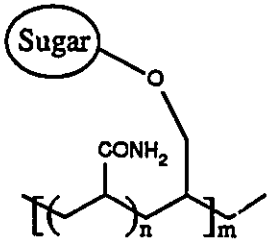
who conjugated NeuAcα2-3Galβ1-4Glc to BSA and keyhole limpet hemocyanin (Smith 1980). The method involved two steps. Reductive amination of the reducing sugar (concealed aldehyde) to a 4-aminophenethylamine spacer with sodium borohydride was followed by coupling of this molecule to a protein via thiophosgene. This procedure gives a product with



the aliphatic amine bonded to the saccharide (Kallin 1986), while reduction with sodium cyanoborohydride give the aromatic amine conjugated. Antibodies raised against the conjugates had a high specificity to sialyllactose, as well as the hydrophobic spacer used for coupling. Nevertheless, this spacer was more recently used to couple the GM_2oligosaccharide to protein for use as a patented cancer vaccine (Irie 1985).

1.4.2 Polyacrylamide Antigens

Soluble polyacrylamide copolymers with pendant carbohydrates were first prepared in 1978 (Horejsi 1978) and used to bind with lectins. The method involved radical copolymerization of acrylamide with allyl glycosides in aqueous solution and resulted in polymers with the carbohydrate only two bonds from the acrylamide backbone. The radical



initation in this example was in the presence of tetramethylethylenediamine (TEMED) and ammonium persulfate.

Many reports have appeared in the literature with copolymers of this type with different pendant oligosacharides. There have been reported improved immunochemical responses with these polymers over the natural oligosaccharides. Examples include: *Salmonella E* in double immunodiffusion (Chernyak 1984), *Streptococcus pneumoniae* in passive hemagglutination

(Chernyak 1985), and enterobacteria in immunoprecipitation (Kosma 1989). They have also be used in ELISA (Kochetkov 1984)

Polyacrylamide conjugates with proteins have also been introduced during the course of this thesis using carbodiimide as a coupling reagent (Nardelli 1989). Mild base hydrolysis of polyacrylamide polymers was used to obtain acid residues, which were then coupled to streptavidin.

There has also been interest in their use as vaccines (Petrov 1985). In this case a synthetic copolymer of acrylic acid and N-vinyl pyrrolidone was coupled to viral particles using carbodiimide. The so called virogate gave titres 200 times stronger than Freund's adjuvant (which has recently been disallowed in animal studies).

In light of these developments, we embarked upon an effort to obtain and study anti-sialic acid antibodies.

Chapter 2
Isolation of Starting Materials from Natural Sources

2.1 Introduction

The isolation of sialic acid from natural sources was necessary to this thesis for one main reason: the high cost of purchase. The current price listed in the 1989 Sigma Catalog for 1 gram of Neu5Ac (**1a**) is $100.00, and NeuAcα2-3Galβ1-4Glc (**2a**) is selling at $100.00 for 5mg.

Natural sialic acids are quite ubiquitous in animals above the evolutionary level of insects. It is also present in a few bacteria including E. coli, N. meningitidis and O serotype Salmonella (Schauer 1982).

For the purpose of isolating free sialic acid by acid hydrolysis of glycoproteins, the natural source must be enriched, readily obtainable and free from interfering compounds. Mucin from the chinese swiftlet (collocalia) is commercially available in the form of bird's nest soup. The sialic acid content is reported to be in the range of 5-6% by weight (Czarniecki 1977).

For the purpose of obtaining sialyllactose, the source must contain the free trisaccharide, thus eliminating the need for partial hydrolysis or enzymatic hydrolysis. Colostrum contains as much as 0.03% sialyllactose by weight (Ginsburg 1978, Schauer 1982) and is readily available from dairies or farms. The major sialyltrisaccharide components of colostrum are the 2,3 and 2,6 isomers (Gronberg 1989), which are compounds **2a** and **3** respectively (see figure 1-3).

2.2 Isolation of NeuAc

The method of isolation involved acid hydrolysis of chinese swiftlet mucin following a modified procedure from Czarniecki (1977). Instead of using sulfuric acid, cation exchange resin (H^+ form) was employed as the source of acid. This technology was used in industrial preparation of NeuAc, but has recently been surpassed by enzymatic synthesis.

The relative rates of hydrolysis were compared by following the reaction using the Aminof test (see Appendix 1), which is specific for free NeuAc. The results are reported in figure 2-1. Later work, not reported here, indicated a reduction in NeuAc content after several hours at higher temperatures (Roy 1990). This is likely due to destruction of NeuAc by

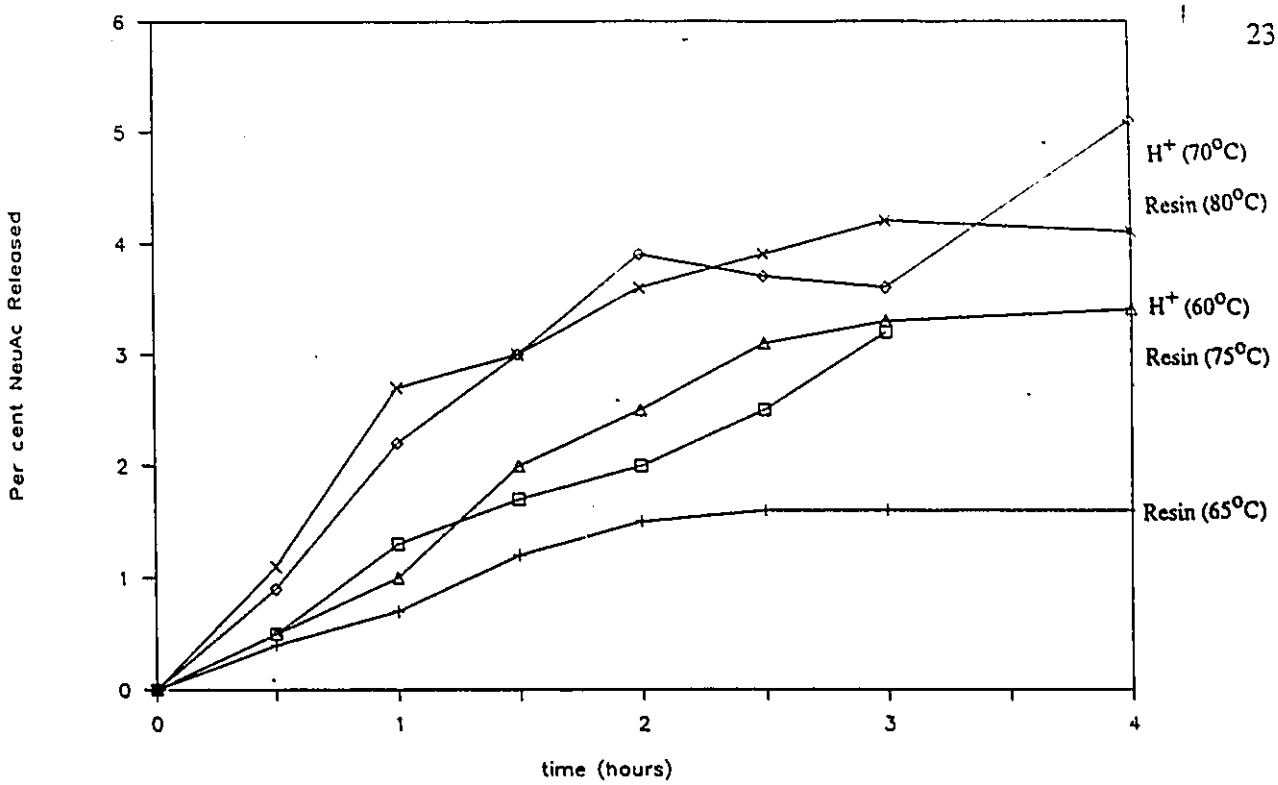


Figure 2-1 Rate of Hydrolysis of Sialic Acid from Chinese Swiftlet Mucin
Resin = 1:2 H^+resin/Mucin H^+ = 0.0125 N H_2SO_4

dehydration and rearrangement (Saito 1989). The optimal condition for hydrolysis was determined to be 75°C for 6 hours.

The advantage to using resin as the acid source is that it can be removed by simple filtration, as compared to the lengthy removal of sulfuric acid by precipitation with barium hydroxide.

Purification of NeuAc was then done using published procedures. The crude solution was loaded directly on an anion exchange resin column, and eluted with a linear 0 to 2 M formic acid gradient. The undesired dehydration product elutes at higher concentrations and may be avoided by taking fractions. The product was then lyophilized.

The identity of NeuAc **1a** was confirmed by comparison of the physical and spectroscopic data with those of the literature.

2.3 Isolation of Sialyllactose

The procedure of sialyllactose isolation from colostrum described in the literature (Veh 1981) was found to be extremely lengthy, sometimes taking as much as a month if the column suddenly slowed down due to clogging from residual lipids. Hence a more direct method was developed.

2.3.1 Isolation of Sialyloligosaccharides from Bovine Colostrum

The anionic nature of sialic acids makes their isolation quite simple using anion exchange resins, but lipids must first be removed to prevent fouling. In the literature method referred to above (Veh 1981), lipids were removed from colostrum by chloroform extraction followed by extrusion chromatography on Sephadex G-25. We attempted several alternatives such as dialysis and reverse osmosis, but finally chloroform extraction of the lipids followed by vacuum evaporation of the aqueous phase proved to give a clear solution which reduced anion exchange column fouling. A crude mixture of sialyloligosaccharides could then be obtained by elution with 100 mM pyridinium acetate buffer. Elution with 200 mM pyridinium acetate resulted in a crude mixture of disialyloligosaccharides.

2.3.2 Separation of the Sialyllactose Isomers

Separation of the silayllactose isomers as described by Veh (1981) was found to require the 4°C temperature for the column to obtain resolution. The method was sometimes difficult to reproduce. A quick isolation of NeuAcα2-3Galβ1-4Glc (**2a**) was envisioned using the internal lactone which was known to form in the corresponding ganglioside GM3 (Yu 1985). The first evidence that the oligosaccharide also forms a lactone when treated with glacial acetic acid came from t.l.c. analysis which showed the appearance of a new compound at a higher Rf. An infrared (IR) spectrum of the material isolated after lyophilization of the acetic acid revealed the characteristic band for a lactone at 1750 cm^{-1}. The NeuAcα2-6β1-4Glc **3a** isomer did not show any change upon similar treatment with glacial acetic acid. Since the 2,3 isomer was the only isomer forming the lactone, it would pass through an anion exchange column and could be isolated directly.

Isolation of NeuAcα2-3Galβ1-4Glc lactone (**2b**) using this method was also difficult to reproduce, and it was finally determined that the quality of the crude mixture of isomers played an important role. Large amounts of protein caused interference, and hence the fact that sialyllactose is soluble in methanol was used to improve the quality of the crude mixture. An outline of the method is shown in figure 2-2. Once the 2,3 isomer was isolated, the column was eluted with buffer to obtain the 2,6 isomers.

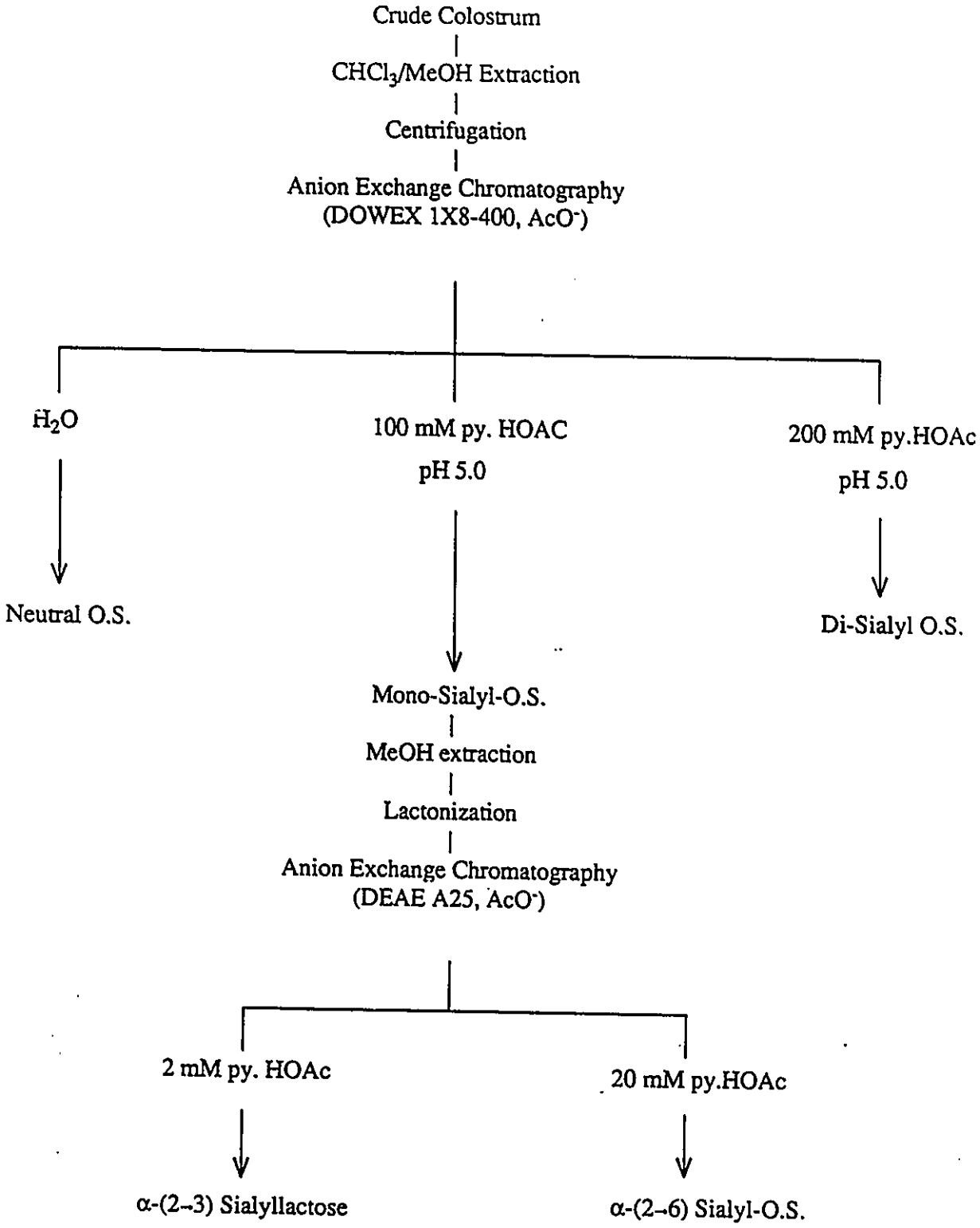


Figure 2-2 Outline of the Isolation of Sialyllactose from Bovine Colostrum

The purity of NeuAcα2-3Galβ1-4Glc 2a obtained via the lactone was verified by high performance liquid chromatography (h.p.l.c.) on an Aminex A-28 column (Shukla 1982). Figure 2-3 shows the elution profile for the crude mixture and the purified **2a** from a typical run. There is a substantial decrease in the quantity of 2,6 isomers as noted by the loss of those peaks. A small peak was observed to follow elution of **2a**, and we suspected there would be a small quantity of the corresponding N-glycolylneuraminic acid form of **2a** (Gronberg 1989) which would also lactonize, and hence be present in the product. Due to the lack of an authentic sample, we could not confirm the identity of the small peak.

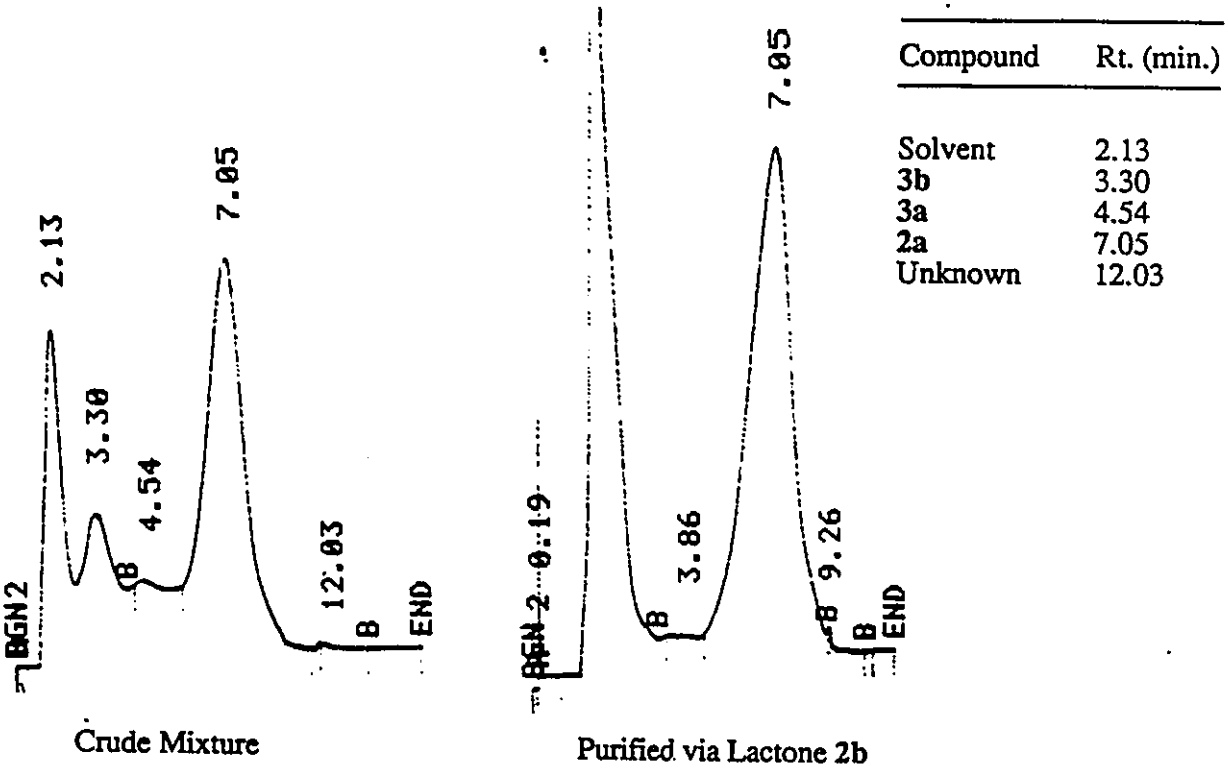


Figure 2-3. HPLC Profile of Crude and Purified Sialylactose
(0.45X15 cm Ammi nex A-28, 1.0 mL/min. 0.75 mM Na_2SO_4)

The ^1H n.m.r. resonances of **2b** were identified by correlated spectroscopy (COSY) and this is described in detail in section 7.6.1 which considers the lactone configuration and conformation. The identity of the sialyllactose isomers were confirmed by mass spectroscopy

and by ^{13}C and ^{1}H n.m.r. data which matched the literature. For example, the 2,3 isomers could be clearly identified by the ^{1}H n.m.r. chemical shift of Gal H3 which appears uniquely at 4.115 p.p.m. in D_2O (Sabesan 1986). Similarly, the 2,6 isomers were identified by comparison of the ^{13}C n.m.r. data in table 2-1 with the data reported by Berman (1984).

2.3.3 Separation of Disialyllactose Isomers

Crude disialyl isomers were also processed by the method employed by Veh (1981). Three separate peaks were eluted from the anion exchange column using 200 mM pyridinium acetate in accord with their results. FAB MS was used to try and identify the compounds. The first peak did not move on TLC and was ninhydrin positive, and hence was assumed to be a glycoprotein. The second peak did not give a clear FAB MS, and ^{1}H nmr showed the compound to be free NeuAc. It is possible that th original product hydrolyzed. The last peak was identified as NeuAcα2-8NeuAcα2-3Galβ1-4Glc (4). Although the ^{1}H nmr does not match exactly with the literature (Dorland 1986), this could be due to the pH of the D_2O solution. The spectrum clearly shows two N-acetyl groups as well as two NeuAc $H3_{eq}$ resonances at 2.63 and 2.82 ppm. There is also a quartet at 4.10 ppm which is characteristic of glycosidation at the C3 hydroxyl position of galactose. In a second isolation, the FAB MS indicated a monoacetylated disialyllactose. HPLC analysis using reverse phase indicated a difference in the retention time of two separate preparations. ^{1}H nmr revealed the presence of approximately 10 mole% of free NeuAc impurity in the material, and HPLC showed the preparations to be approximately 66% pure.

Table 2-1 ^{13}C NMR Data for Sialyllactose Isomers in D_2O at 20°C. See Berman 1984

Atom	2a	2b (DMSO-d6)	3a	3b
NeuAc				
C1	174.9	164.7	175.0	174.2
C2	101.0	96.5	101.5	100.9
C3	40.9	40.8	41.4	41.1
C4	69.6	66.0	69.8	69.8
C5	53.0	51.9	53.2	53.2
C6	74.2	72.9	73.8	74.1
C7	69.4	68.0	69.8	69.8
C8	73.0	69.8	73.2	72.8
C9	63.9	63.2	64.1	64.2
C=O	176.3	171.7	176.6	176.6
Ac	23.3	22.2	23.4	23.3
Gal				
C1	103.9	99.8	104.7	105.0
C2	70.7	73.8	72.2	72.1
C3	76.4	75.1	74.0	74.0
C4	68.8	65.1	69.9	69.4
C5	76.8	75.5	75.1	75.1
C6	62.3	19.8	65.0	64.8
αGlc				
C1	93.1	91.6	93.3	92.1
C2	72.5	71.6	72.5	54.8
C3	72.7	70.8	73.1	70.8
C4	79.6	74.3	81.2	82.4
C5	71.4	69.0	71.4	71.4
C6	61.4	59.7	61.5	61.6
βGlc				
C1	97.0	96.2	97.1	96.2
C2	75.1	74.1	75.1	57.3
C3	75.6	74.4	76.1	73.8
C4	79.5	79.9	81.1	82.2
C5	76.1		76.1	76.0
C6	61.3	59.6	61.7	61.7
				176.2 (NAc)
				23.6 (NAc)

2.4 Experimental Methods

Please see Appendix 1 for general experimental methods and codes used for the t.l.c. solvent systems.

5-Acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranos onic acid, **1a**

Kwong Cheong Swallow's Nest (200g) was added to 1600 mL water and allowed to soak overnight. Then 100 g acid washed Rexyn 101 cation exchange resin was added, and the mixture was stirred mechanically at 75°C for 6 hours. The mixture was cooled and filtered over celite, and loaded on a 3.5 cm X 40 cm Dowex 1-X8 (20-50 mesh, formate form) anion exchange column. The column was eluted with 2 Litres of 0 to 2 M formic acid linear gradient. Ten mL fractions were taken and those testing positive with resorcinol reagent for sialic acid were pooled and lyophilized. Yield: 5.0 g, 2.5 % w/w.

mp 177-183°C (lit. 184-186°C Czarniecki 1977);$[\alpha]_D$=-30.8° (c 1 H_2O) (lit. -32.1° Czarniecki 1977); Rf.=0.47 solvent A; FAB MS for $C_{11}H_{19}O_9N$ 309.28: (M-1) 308.

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-3)β-D-galactopyranosyl(1-4)α,β-D-glucopyranose, **2a**

Crude bovine colostrum (1 L) was extracted with a mixture of chloroform-methanol (3:1 v/v, 1 L) which, after a single centrifugation step at 5000 r.p.m. for 15 minutes at 4° and phase separation, gave a clear solution. The aqueous phase was then concentrated to 500 mL on a rotary evaporator. The solution was passed onto a Dowex 1X8-400 ion exchange column (3.0 x 60 cm, acetate form). The neutral oligosaccharide fraction was eluted with water (5 L) and the crude monosialyloligosaccharide fraction was eluted with pyridinium acetate buffer (100 mM, pH 5.0, 1.0 L). The disialyloligosaccharide fraction was then eluted with a 200 mM concentration of the same buffer (1.0 L). The individual fractions were lyophilized. At this stage, they could be processed according to published procedures to obtain the well separated oligosaccharides (Veh 1981, Parkkinen 1987).

The crude powder containing the monosialyloligosaccharides (0.8-1.0 g) was extracted three times with methanol (6.0 mL) to remove residual ninhydrin-positive material. The methanol extracts were evaporated under vacuum. The lactonization of **2a** was allowed to proceed in glacial acetic acid (50 mL) for 3 days at room temperature, then the reaction mixture was lyophilized. The extent of lactonization was followed by TLC in n-propanol-water (8:2 v/v). The following compounds and their respective R_F were observed as follows: **2a**, R_F 0.21; **2b** (lactone), R_F 0.34; and α-D-Neup5Ac-(2-6)-β-D-Galp-(1-4)-D-Glcp

3a (II^{6}Neup5Ac-Lac), R_F 0.18. The corresponding α-D-Neup5Ac-(2-6)-β-D-Galp-(1-4)-D-GlcpNAc, **3b**, although present, could not be well differentiated from **2a** in this system.

The lyophilized reaction mixture was dissolved in pyridinium acetate buffer (2 mM, pH 5.0, 100 mL) and loaded onto a Sephadex-DEAE A-25 column (2.0 x 20 cm, acetate form). The neutral lactone **2b** was not retained and was eluted with an additional 100 mL of the same buffer, and lyophilized to a white powder. Yield of pure **2b**: 180 mg/ L colostrum.

$[\alpha]_D$ + 10.5° (c 1.0, 0.1 M pyridinium acetate); FAB MS for $C_{23}H_{37}NO_{18}$ 615.54: (M-1) 615; IR ν_{max} (KBr) 1750 cm^{-1} ; ^{13}C data appear in table 2-1, and ^{1}H n.m.r (DMSO-d6) data appear in table 7-5. ^{1}H n.m.r. (D_2O) δ: 5.21 (d, 1H, $J_{1,2}$=3.2 Hz, αGlc-H1), 4.75 (d, 1H, $J_{1,2}$=8.0, Gal-H1), 4.64 (d, 1H, $J_{1,2}$=7.3, βGlc-H1), 4.30 (m, 1H, NeuAc-H4), 4.21 (dd, 1H, $J_{3,2}$=10.6, $J_{3,4}$=2.5, Gal-H3), 3.89 (NeuAc-H5), 3.68 (NeuAc-H8), 3.59 (NeuAc-H6), 3.56 (NeuAc-H7), 3.29 (t, 1H, $J_{2,3}$=$J_{2,1}$=8.3, βGlc-H2), 2.65 (dd, 1H, $J_{3a,3e}$=13.1, $J_{3e,4}$=5.2, NeuAc-H3eq), 2.033 (s, 3H, NAc), 1.77 (t, 1H, $J_{3a,3e}$=$J_{3a,4}$=11.9, NeuAc-H3ax)

The lactone **2b** was hydrolyzed to **2a** with 0.1 M NaOH, then neutralized with H^+ resin, and lyophilized to give a white powder. Yield: 180 mg/L colostrum.

$[\alpha]_D$ + 20.6° (C 0.7 0.1 M pyridinium acetate, lit. +16.8° Schneir 1966); Rf= 0.21 C; FAB MS for $C_{23}H_{39}NO_{19}$ 633.56 : (M-1) 632., (M+1) 634.; ^{13}C data appear in table 2-1, and ^{1}H n.m.r (DMSO-d6) data appear in table 7-5. ^{1}H n.m.r. (D_2O) δ: 5.22 (d, 1H, $J_{1,2}$=3.8 Hz, αGlc-H1), 4.53 (d, 1H, $J_{1,2}$=7.9, Gal-H1), 4.66 (d, 1H, $J_{1,2}$=7.9, βGlc-H1), 4.12 (dd, 1H, $J_{3,2}$=9.9, $J_{3,4}$=3.2, Gal-H3), 3.75-3.56 (≈12H), 3.28 (t, 1H, $J_{2,3}$=$J_{2,1}$=7.9, βGlc-H2), 2.76 (dd, 1H, $J_{3a,3e}$=12.31, $J_{3e,4}$=4.5, NeuAc-H3eq), 2.030 (s, 3H, NAc), 1.80 (t, 1H, $J_{3a,3e}$=$J_{3a,4}$=12.3, NeuAc-H3ax).

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-6)β-D-galactopyranosyl(1-4)α,β-D-glucopyranose, **3a**

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-6)β-D-galactopyranosyl(1-4)α,β-D-2-acetamido-2-deoxyglucopyranose, **3b**

Compounds **3a** and **3b** were obtained by eluting the anion exchange column described above with 20 mM pyridinium acetate buffer pH 5.0 at 4°C and collecting fractions.

Compound **3b** eluted after 50 mL buffer had passed through the column. Yield: 23 mg/L colostrum.

$[\alpha]_D$= 0. (C 0.6 H_2O); Rf= 0.21 C; FAB MS for $C_{25}H_{42}O_{19}N_2$ 674.61: (M-1) 673; ^{13}C data appear in table 2-1, ^{1}H n.m.r. (D_2O) δ: 5.22 (d, 1H, $J_{1,2}$=3.7 Hz, αGlc-H1), 4.43 (d, 1H, $J_{1,2}$=7.7, Gal-H1), 4.67 (d, 1H, $J_{1,2}$=7.9, βGlc-H1), 3.75-3.56 (≈12H), 3.31 (t, 1H,

$J_{2,3}$=$J_{2,1}$=7.8, βGlc-H2), 2.71 (dd, 1H, $J_{3a,3e}$=12.3, $J_{3e,4}$=4.9, NeuAc-H3eq), 2.030 (s, 3H, NAc), 1.76 (t, 1H, $J_{3a,3e}$=$J_{3a,4}$=12.3, NeuAc-H3ax).

Compound **3a** eluted after 75 mL buffer had passed through the column. Yield: 18.2 mg/L colostrum.

$[\alpha]_D$= +6.95 (c 0.5 H_20, lit. +27.9 Schneir 1966); Rf= 0.18 C; FAB MS for $C_{23}H_{39}O_{19}N$ 633.56: (M-1) 632; ^{13}C data appear in table 2-1, ^1H n.m.r. (D_2O) δ: 5.20 (d, 1H, $J_{1,2}$=1.7 Hz, αGlc-H1), 4.45 (d, 1H, $J_{1,2}$=7.6, Gal-H1), 3.75-3.56 (≈12H), 2.67 (dd, 1H, $J_{3a,3e}$=11.6, $J_{3e,4}$=4.5, NeuAc-H3eq), 2.03 (s, 3H, NeuAc-5NAc), 2.07 (s, 3H, GlcNAc-2NAc) 1.76 (t, 1H, $J_{3a,3e}$=$J_{3a,4}$=12.0, NeuAc-H3ax).

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-8)5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-3)β-D-galactopyranosyl(1-4)α,β-D-glucopyranose, **4**

A crude mixture of sialobiosyl oligosaccharides was collected from three successive 1L batches of colostrum processed as described above. The material (appoximately 1 g) was dissolved in pyridinium acetate (2 mM, pH 5.0, 100 mL) and loaded on a 3.0 dia cm X 60 cm column of fresh Dowex 1X8-400 anion exchange resin, acetate form. The column was rinsed with water (2 L), then eluted with pyridinium acetate (20 mM, pH 5.0, 6°C, 1 L) to remove monosialyloligosaccharides, then with a higher concentration of the same buffer (200 mM pyridinium acetate, pH 5.0, 6°C,2L). Three peaks were observed, fractionated and lyophilized. The last peak at 710 mL buffer was identified as **4.** It was lyophilized to an oil, and the quality of the material was improved by desalting on 1.5 dia X 60 cm Sephadex G10 eluted with water. A fluffly white powder was obtained after lyophilization. Yield: 69 mg/ 3L

Rf 0.15 C, 0.37 F; HPLC Rt.= 3.7 min. (0.5 cm dia. X 25 cm RP C18 column, 1.5 mL/min. 10 mM triethylamine/acetic acid pH 4.8); FAB MS for $C_{34}H_{56}O_{27}N_2$ 924.82: (M-1) 923.5, 923.1, 922.6 (three different runs); ^1H n.m.r. (D_2O) δ: 5.2 (d, 1H, αGlc-H1), 4.52 (d, 1H, Gal-H1), 4.66 (d, 1H, βGlc-H1), 4.11 (dd, 1H, Gal-H3), 3.27 (t, 1H, βGlc-H2), 2.63 (dd, 1H, NeuAc"-H3eq), 2.81 (dd, 1H, NeuAc'-H3eq), 2.008 (s, 3H, NeuAc"- NAc), 2.024 (s, 3H, NeuAc'-NAc), 1.92 (t, 1H, NeuAc"-H3ax), 1.89 (t, 1H, NeuAc'-H3ax).

Chapter 3

Key Intermediates and Derivatives of Sialic Acid

3.1 Introduction

The study of the binding receptors of NeuAc requires two fundamental forms of sialic acid; polyvalent forms of sialic acid which can be used to amplify weak interactions, and derivatives which may or may not inhibit these interactions. The preparation of polyvalent forms of sialic acid will be discussed in chapter 4. This chapter deals with the synthesis of the key intermediates used in the preparation of the polyvalent macromolecules, and derivatives used to study the binding specificity.

Since sialic acid is present in nature only as the α anomer when linked as an oligosaccharide (Schauer 1982), it was necessary to invert the chirality at the anomeric carbon because the hemiketal exists mainly in the β configuration. This was accomplished starting with the glycosyl chloride which also exists in the β configuration, and then inverting using Koenigs-Knorr conditions. Other inversions were made using phase transfer catalysis or palladium as outlined in figure 3-1.

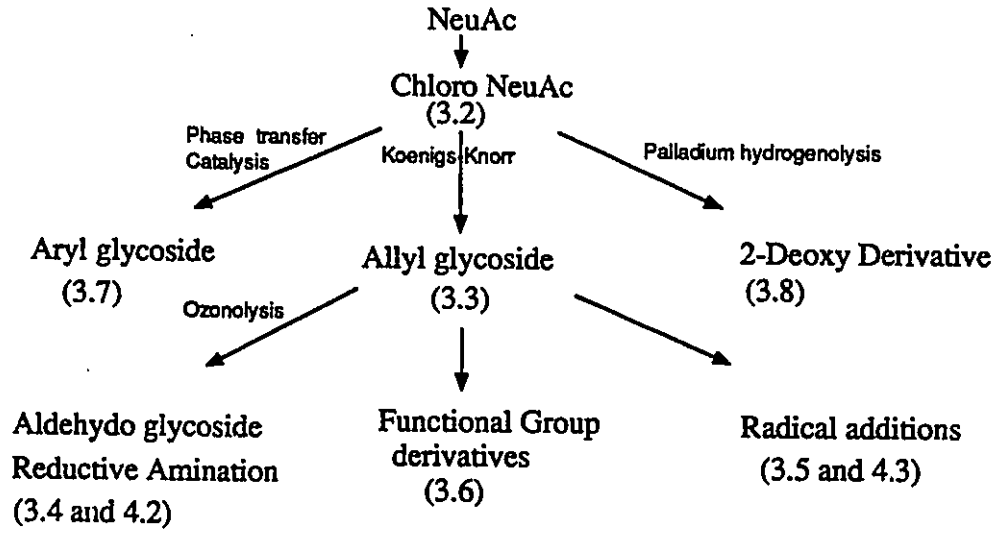


Figure 3-1 General Outline of Synthesis. Brackets refer to Sections.

The allyl glycoside **7** was a key synthetic intermediate used in the preparation of many

derivatives and polymers. Another key intermediate was the 2-oxoethyl (aldehyde) glycoside **10** which was modified via the aldehyde.

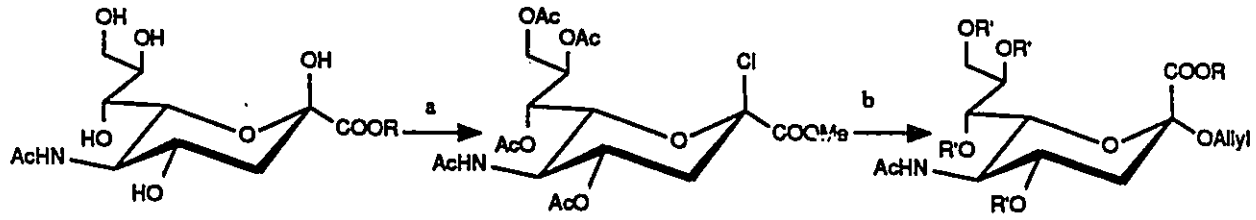
3.2 Acetochloroneuraminic Acid (5)

The methyl ester of NeuAc (**1b**) was prepared with H^+ resin catalyst as previously done by Kuhn (1966) and Yu (1969). It was found that NeuAc was insoluble in methanol until complete esterification occurred, and hence the presence of solid was an indication of the extent of reaction. The methyl ester crystallized readily and was used in the next step. All the physical data (m.p., $[\alpha]_D$, ^1H and ^{13}C n.m.r. and mass spectroscopy) agreed with the literature values.

The preparation of the glycosyl chloride of sialic acid was accomplished using acetyl chloride with acetic acid as a co-solvent. Previous syntheses have involved addition of HCl gas to the preformed penta-O-acetyl derivative (Eschenfelder 1980), but this was found unnecessary because HCl is produced *in situ* when the pentol **1b** was used. The purity of the acetochloroneuraminic acid **5** was determined to be greater than 93% by comparison of the ^1H n.m.r. in C_6D_6 and $CDCl_3$ with published spectra (Sharma 1984, Paulsen 1984). Compound **5** was used without further purification.

3.3 Allyl Glycoside (6)

Koenigs-Knorr glycosylation was accomplished using silver salicylate as the "catalyst" (Vleugel 1982). This catalyst was chosen over other available catalysts (Schauer 1982) because it gave improved yields and in this case gave exclusively α selectivity. It is less toxic than the mercury catalysts used in more demanding situations (Paulsen 1985). The reaction scheme is presented in figure 3-2. The allyl and methyl α-NeuAc glycosides (**6** and **8c**



1a R=H
1b R=Me

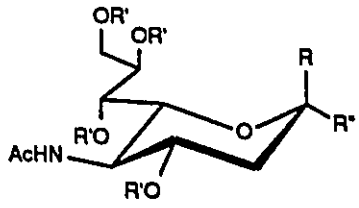
5

6 R=Me R'=Ac
7a R=Me R'=H
7b R=H R'=H

Figure 3-2 Koenigs-Knorr Glycosylation of NeuAc
a) AcCl/HOAc b) Allyl alcohol, Silver salicylate

respectively) were prepared with 90% yield in this manner, and the compounds were identical to those previously reported in other syntheses (Eschenfelder 1980).

Zemplén de-O-acetylation in methanol with sodium methoxide provided crystalline **7a** which was a key intermediate in the preparation of many of the compounds used in this work. For a comparison, the β methyl glycoside **9a** was prepared using Fischer glycosylation (p62 Schauer 1982) by refluxing **1** in methanol in the presence of H^+ resin (Yu 1969). The anomeric



8a	R= COOMe	R'=H	R"=OMe
8b	R= C00Na	R'=H	R"=OMe
8c	R=COOMe	R'=Ac	R"=OMe
9a	R=OMe	R'=H	R"=COOMe
9b	R=OMe	R'=H	R"=COONa

configurations of these compounds were confirmed by gated decoupled ^{13}C n.m.r. spectroscopy, discussed in more detail in section 7.2.1.

In many cases the methyl ester was maintained as protecting group and removed after derivatization; however, in certain compounds it was found necessary to remove the ester first to avoid strongly basic conditions with sensitive functional groups. This was true of aldehydes and acryloyl derivatives. The ester was hydrolyzed with 0.1 N aqueous sodium hydroxide, which was verified by the loss of the ester signals in both ^1H and ^{13}C n.m.r. spectroscopy.

3.4 Ozonolysis and Reductive Amination

A key intermediate was made by changing the alkene into the highly reactive 2-oxoethyl-NeuAc **10**. This compound was used to make derivatives as shown in figure 3-3 and also to couple with proteins, as described in section 4.2.

3.4.1 Ozonolysis

Insertion of ozone into the carbon-carbon double bond to form the ozonide was found to occur quickly at -78°C. The reaction was found to be complete within 5 minutes once the solution became blue with excess ozone. The reactivity of the allyl group with ozone is in contrast to its reactivity with monoperoxylphthalic acid (Brougham 1987). Though epoxidation proceeded with allyl-GalNAc (Nashed 1983), preliminary studies with allyl-NeuAc **7** were not successful.

Reductive work-up of the ozonide with dimethylsulfide under N_2 ensured the reaction

produced the aldehyde **10** and not the carboxylic acid (Pappas 1966). The aldehyde is apparently hydrated in D_2O according to the ^1H n.m.r. which shows the aldehydo proton at 5.119 ppm, and the ^{13}C CHO at 89.6 ppm; however, the FAB MS and elemental analysis of the solid are more consistent with the aldehyde.

3.4.2 Reductive Amination

This procedure involves reduction of the imine formed when an amine reacts with an aldehyde or ketone. The amine may be primary or secondary (March 1979) and may also be present as the ϵ amino group of lysine in proteins (Lundblad 1984).

The reducing reagent may be sodium borohydride or cyanoborohydride. Sodium borohydride will cause reduction to the alchohol, as shown for the synthesis of compound **11** in figure 3-3, whereas sodium cyanoborohydride is more stable in aqueous solution and is less reactive towards aldehydes (Borch 1969).

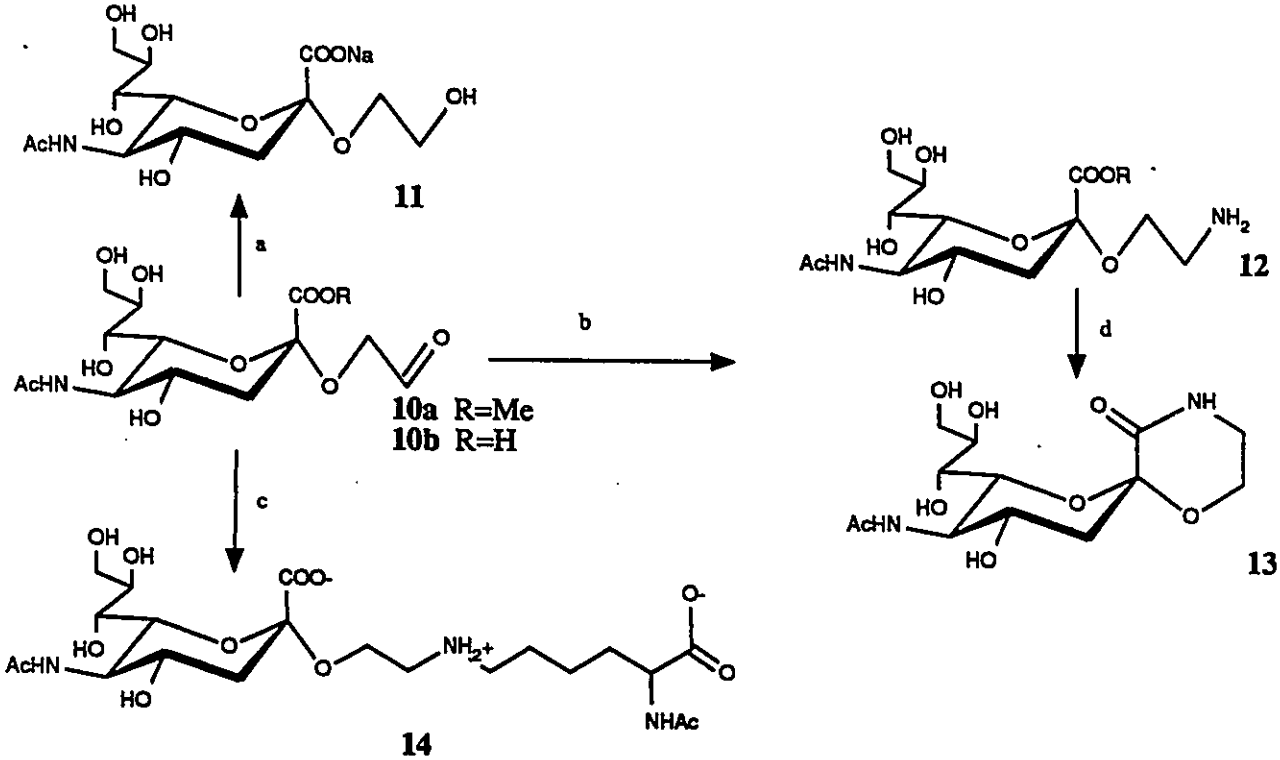


Figure 3-3 Derivatives Made from Aldehydo NeuAc by Reductive Amination

a) $NaBH_4$ b) $NH_4HOAc/NaCNBH_3$ c)N^{α}-acetyl-L-lysine/buffer/$NaCNBH_3$ d) EDC

Imine formation is promoted in non-aqueous solvents, but the solubility of the amine or aldehyde may be a limiting factor (Lee 1980).

Reductive amination of **10b** in the presence of ammonium acetate yielded compound

12 in 51% yield. The $[\alpha]_D$ of **12** (-18.5°) agreed well with the value (-17°) obtained in an alternate synthesis by another group (Eschenfelder 1980). When the ester **10a** was used in the same reaction instead of the acid, a side product was isolated by preparative t.l.c., which was tentatively assigned the lactam structure **13**. This was verified when treating compound **12** with a carbodiimide (EDC) resulted in the same product as characterized by t.l.c. and FAB MS.

This raised the concern that reductive amination with lysine using the ester **10a** would result in a similar cyclic amide. This has particular signifigance in section 4.2 when considering reductive amination to proteins. However, it was found that reductive amination to the ϵ-amino group of lysine resulted only in a single product which was identified as **14** as discussed below. It is interesting to note that the methyl ester hydrolyzed during the reaction giving the free acid. This fact is referred to again in section 4.2.1.2 when considering reductive amination to lysine in proteins. Reductive amination of **10b** with lysine was used to prepare **14** on a larger scale, and the large increase of molecular weight of the product allowed a simple isolation by extrusion chromatography on Sephadex G-10. The proof of conjugation was confirmed by mass spectroscopy and ^{1}H n.m.r. which showed the loss of the aldehydic proton at 5.12 ppm and the appearance of the corresponding lysine protons.

3.5 N-Acryloylaminoethylthiopropyl Glycoside (16)

The allyl group is particularly susceptible to radical reactions, and section 4.4 considers radical copolymerization with acrylamide. It was found that the polymers formed were not particularly useful in immunological studies possibly due to the close proximity of the sugar to the polymer backbone. To overcome this, an aminoethanethiol (cysteamine) spacer was added, as shown in figure 3-4. The amine could then be acryloylated to provide the necessary functionality for radical polymerization.

Indeed, addition of 2-aminoethanethiol hydrochloride (cysteamine) to the allyl glycoside **7b** in neutral aqueous solution gave the 3-(2-aminoethylthio)propyl glycoside **15b** in 84% yield after purification on Sephadex G-10. The ninhydrin spay reagent on t.l.c. (see appendix 1) confirmed the presence of a primary amine, and n.m.r. showed the loss of the allyl function. The reaction worked equally well (77% yield) with the methyl ester of allyl NeuAc **7a**, and did not result in signifigant hydrolysis of the ester, as apparent from the ^{1}H n.m.r.

In the original work (Lee 1974), it was speculated that the reaction with cysteamine was a Michael-type addition; however, we have noted that pH has little effect on the reaction, whereas UV light (254 nm) increased the rate. Furthermore, there was no detectable methyl

group in the ^1H n.m.r. and hence the addition was in anti-Markovnikov fashion. We have concluded the reaction is radical in nature.

The addition took somewhat longer than in the corresponding neutral allyl α- or β-2-acetamido-2-deoxyglucopyranosides, which had reacted within three hours (Roy 1988b). It is possible that steric hindrance from the carboxyl reduced the reactivity.

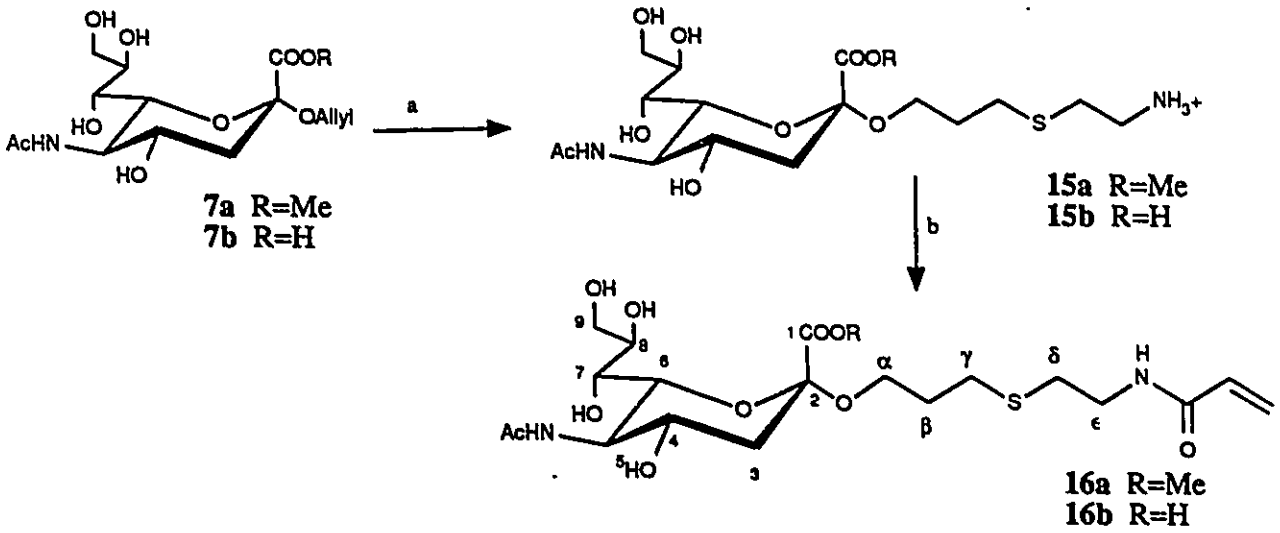


Figure 3-4 Addition of a Cysteamine Spacer to Allyl NeuAc
a) Cysteamine, hv b) Acryloyl chloride, base

N-Acryloylation of the cysteamine adduct **15** was straight-forward; however, yields were somewhat low when the ester was used. This is due to the use of anion exchange resin as the base, which exchanges any acid **16b** formed by hydrolysis. The N-acryloylaminoethylthiopropyl aglycon was confirmed by ^1H n.m.r., which showed the highly characteristic acryloyl group (see section 7.3.3). Elemental analysis for this molecule also proved satisfactory.

3.6 Functional Group Modification

Modified functional groups of the parent compound **7** are useful in inhibition studies to determine which parts of the sialic acid molecule are involved in potential antibody binding sites. Other reasons for preparing derivatives stem from the different physiological properties of the compounds, especially compounds such as the N-glycolylneuraminic acid (Mukuria 1986) and the 9-O-acetate (Lindahl 1987, Schauer 1987).

3.6.1 Acid Derivatives

Modification of the acid group was done as shown in figure 3-5. Sodium borohydride is

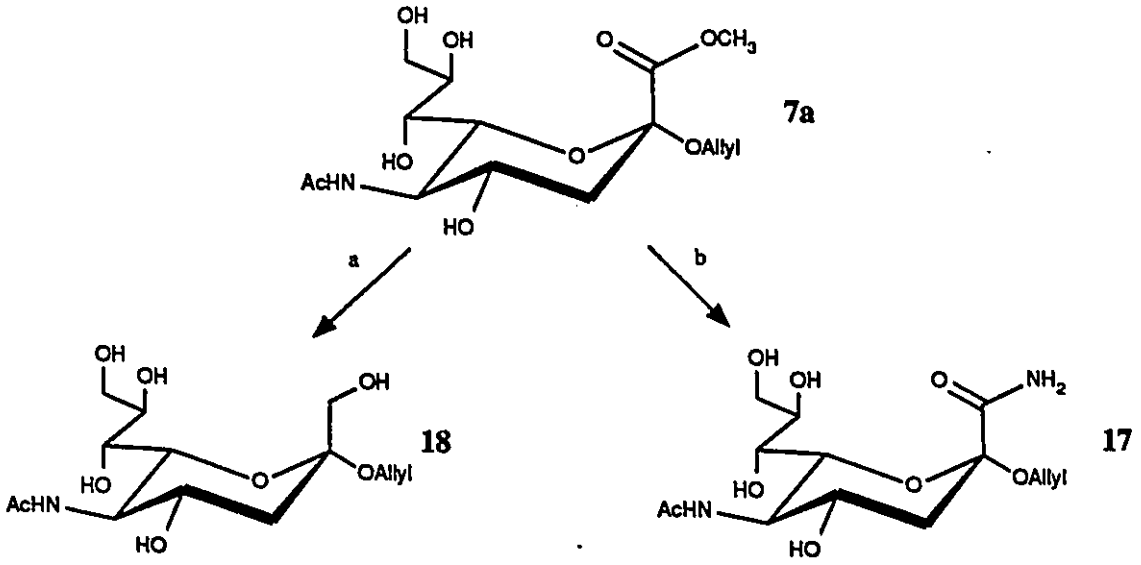


Figure 3-5 Modification of the Carboxyl Group

a) $LiBH_4$/MeOH/THF b) NH_3/MeOH

not usually regarded as being capable of reducing esters to alcohols (March 1979), but NeuAc ester was known to react as early as 1971 (Brossmer). This reaction was done in water, while a more recent method reported in the literature used methanol as solvent (Ogura 1986). In our hands we found this reaction difficult to reproduce. A modification involved co-solvating the allyl glycoside **7a** with water in THF followed by reduction with lithium borohydride. It was effective, but resulted in a small amount of hydrolysis to **7b**. When methanol was used as co-solvent the reaction was clean and essentially quantitative giving compound **18**. ^{13}C n.m.r. spectroscopy showed the loss of one of the carbonyl resonances, and a new resonance appeared at 61.3 ppm when compared to the starting material. IR spectroscopy also showed the loss of a carbonyl band at 1750 cm^{-1}. Mass spectroscopy showed a mass loss consistent with the reduction of the ester to a hydroxyl.

Aminolysis was also high-yielding, though in one instance the reaction was slow, requiring 48 hours, and it did not go to completion. The amide product **17** did not show a large difference in the ^{13}C or ^{1}H n.m.r., but the IR did show a shift in the carbonyl from 1750 to 1690 cm^{-1}. The Svennerholm (1957) resorcinol test (see appendix 1) was also effected. There was a reduction in the absorbance at 580 nm to 46% on a mole basis.

3.6.2 Amide Derivatives

Chemical modification of the acetamido function of NeuAc has been given much attention (Faillard 1965, Schauer 1978, Shirai 1988, Sonnino 1988, Nores 1988).

We studied the hydrolysis of the acetamido group in the parent compound **7** under a variety of conditions such as microwave heating, reducing the temperature of reflux with methanol, and different bases. No improvement could be made on the conditions reported in the literature (Sonnino 1985) using tetramethylammonium hydroxide. Selective hydrolysis of the N-acetyl of the GM3 ganglioside was reported with potassium hydroxide in *n*-butanol (Nores 1988), but we did not require these conditions. Using the aqueous solvent allowed the work-up of the reaction to be easily performed on a desalting column, and the de-N-acetylated compound **19** was obtained in high purity in 84% yield. The ^{1}H n.m.r. showed the loss of the acetyl group at 2.033 ppm and a concomitant upfield shift of H5 to 3.24 ppm. The ^{13}C n.m.r. showed a downfield shift at C5 from 53.1 ppm in **7b** to 53.9 ppm in **19**. C4 was also strongly effect, and moved upfield by 2.0 ppm. The ninhydrin spray reagent (see appendix 1) indicated the presence of a primary amine.

Subsequent N-glycolylation was at first done with 1,3-dioxolane-2,4-dione (Sonnino 1988), prepared from glycolic acid and triphosgene (Daly 1988); however, the dione was hard to crystallize, and was never obtained in high purity. N-glycolylation yields were poor and the presence of residual acid caused hydrolysis of the glycoside, and required column chromatography on silica gel. The reagent acetoxyacetyl chloride was made commercially available during this work (Aldrich). It was easier to handle and gave N-glycolylneuraminic acid **20a** cleanly. Hydrolysis of the O-acetyl group did not occur completely under the conditions used for acetylation (triethylamine base), but could be removed later with a slightly stronger base treatment to yield **20b**. The ^{1}H n.m.r. showed a signal at 4.121 ppm which is characteristic of the methylene of the hydroxyacetamido (p134 Schauer 1982).

Bromoacetylation was also performed, though the product **21b** was somewhat unstable and characterization was difficult. The ^{13}C n.m.r. showed the bromoacetamido methylene carbon at 29.0 ppm which is consistent with observations made on the corresponding homopolymer of NeuAc (Roy 1988f).

An attempt was made to produce N-glycolylneuraminic acid **20** by nucleophilic substitution of the bromo group of **21**, but as shown in figure 3-6, the main product of the reaction was the partially characterized di-acid **22**. This was confirmed when treatment of **19** with bromoacetic acid produced (slowly) the same material as confirmed by t.l.c. and mass spectroscopy.

It was found that careful addition of slight amounts of base would produce the desired

N-glycolylneuraminic acid **20**, but signifigant amounts of the di-acid (5% or more) make this method undesirable. Other nucleophiles were found to substitute, and hence the N-bromoacetylneuraminic acid **21** could be useful for conjugation to proteins through the amide.

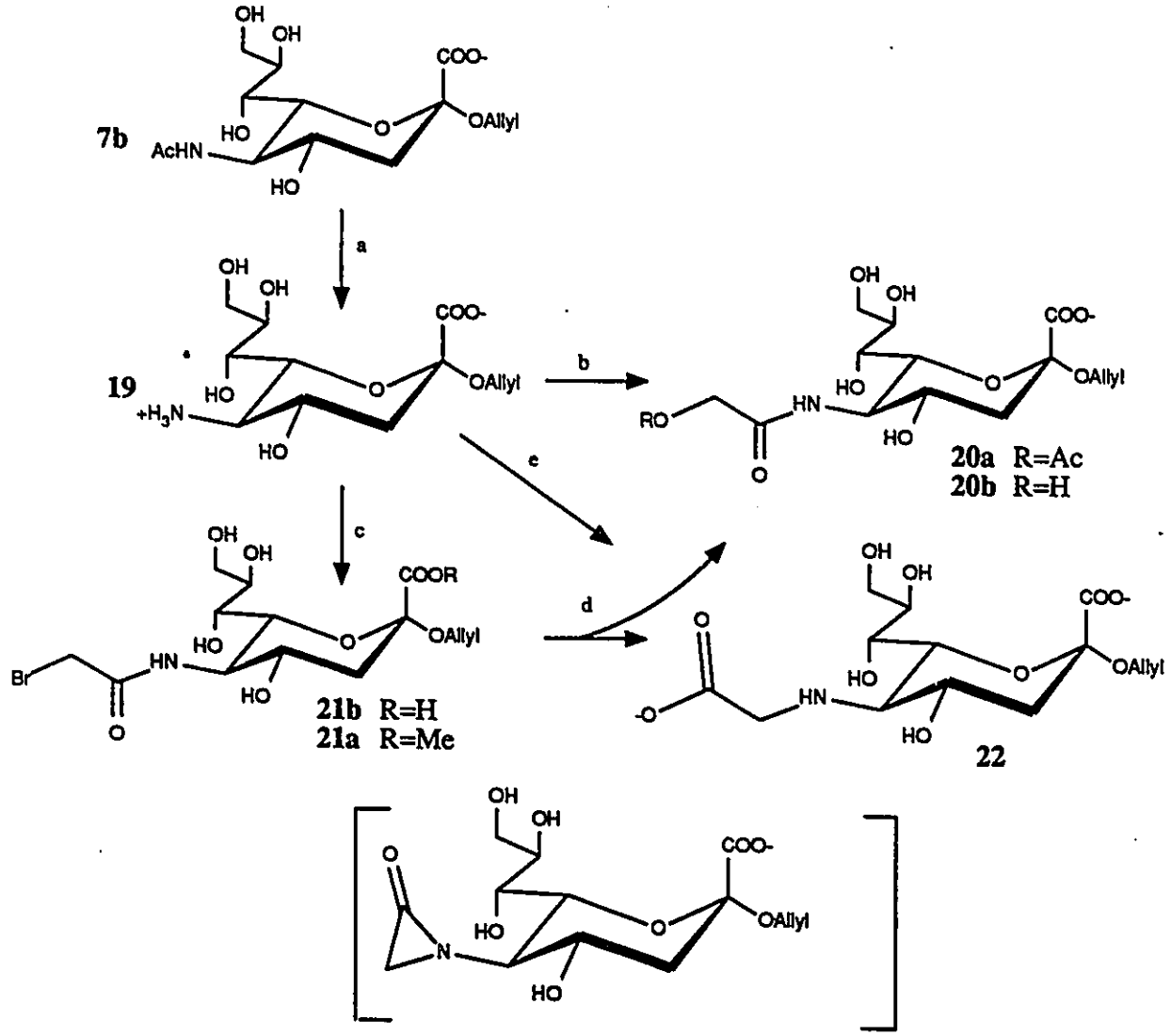


Figure 3-6 Derivatives of the Acetamido Function

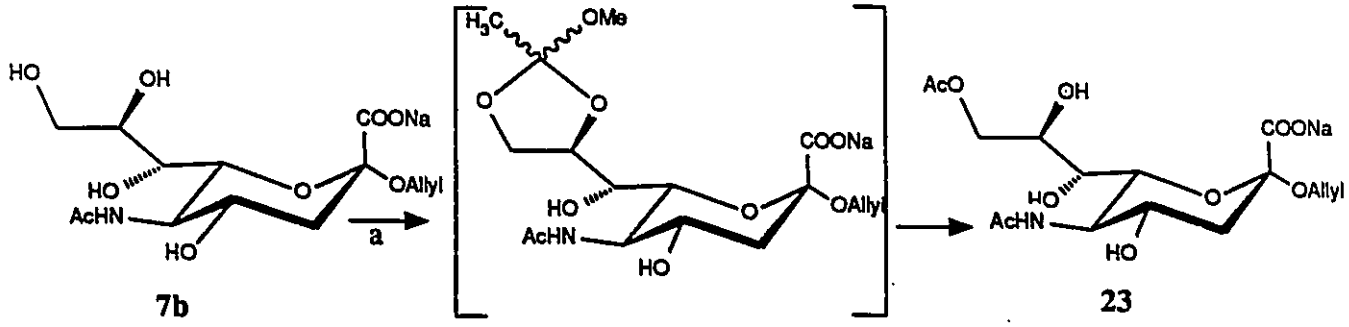
a) Tetramethylammonium hydroxide, reflux, 5 hrs. b) Acetoxyacetyl chloride/TEA
c) Bromoacetyl chloride/TEA d) pH 8 aqueous e) $BrCH_2CO_2^-$/base

The tentative mechanism for the production of **22** is via an α-lactam in a Favorskii-like rearrangment (p971 March 1985). A ^1H n.m.r. spectrum of the reaction mixture showed a compound which resembled **20**, but no signal was observed for the hypothetical α-lactam.

3.6.3 Glycerol Side Chain Derivatives

Two derivatives were prepared. The 9-O-acetyl-NeuAc has been prepared chemoenzymatically using pancreas lipase in pyridine (Forstner 1989), and the biochemical synthesis is being elucidated (Diaz 1989, Higa 1989). We followed a facile chemical method which is quite versatile (Ogura 1987), and gave the 9-O-acetyl-α-allyl-NeuAc **23** in modest yields. While the procedure worked very well, an excess amount of trimethylorthoacetate had to be added, and the result was two compounds. The major product was the desired compound **23** identified by the additional acetate signal in the ^{1}H n.m.r. The regioselective acetylation at the C9 hydroxyl was confirmed by the downfield shift of H9 from 3.87 to 4.41 ppm, and the ^{13}C n.m.r. downfield shift at C9 from 63.8 ppm to 66.8 ppm.

The second product, isolated by preparative t.l.c. and present in 5%, was identified as



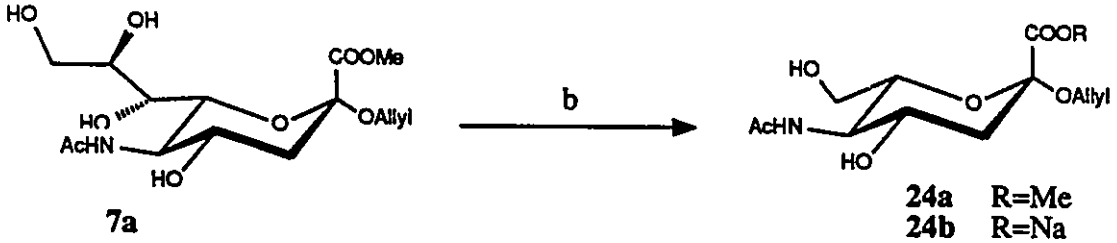


Figure 3-7 Derivatives of the Glycerol Side Chain

a) Trimethylorthoacetate/H^{+} b) Periodate/Borohydride Supported on Anion Exchange Resin

the di-O-acetyl derivative by FAB MS and ^{1}H nmr. The mechanism of regioselective acetylation depends on orthoester formation on a vicinal diol, and an inductive effect for regioselective hydrolysis to a primary O-acetate, as illustrated in figure 3-7. It is conceivable that the hydroxyls on C7 and C8 were also able to form the internal orthoester which rearranged to form a second acetate. Not enough of the di-O-acetate was isolated for complete characterization of the position of the second acetylation.

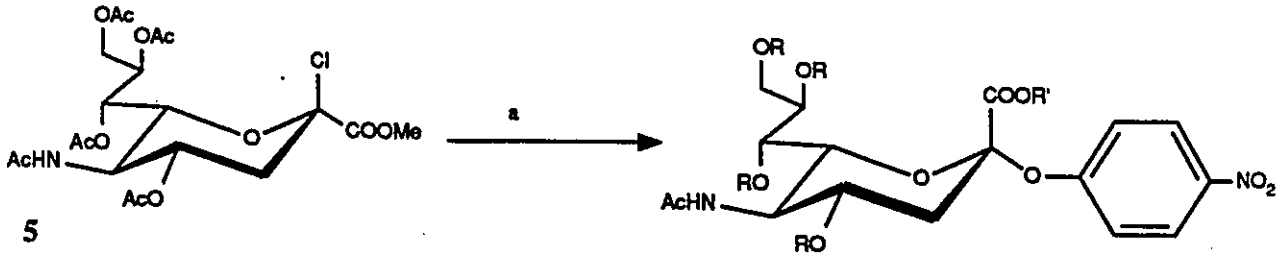
Modification of the glycerol side side chain by periodate cleavage is popular for a

variety of analytical uses (Yu 1969, Bayer 1988, Murray 1989). For example, 1 mM periodate will selectively cleave the glycerol side chain of NeuAc and leave other carbohydrate diols intact.

The vicinal diol array in the glycerol side chain of NeuAc was simultaneously cleaved and reduced in a single pot using periodate and borohydride anions supported on exchange resins as described for other diols (Bessodes 1985, Harrison 1982). The yield of the heptuloside **24a** (illustrated in figure 3-7) was somewhat low (59%), and this can be attributed to the partial hydrolysis of the methyl ester under the reactive conditions generating the acid **24b** which remained on the resin. Treatment of the recuperated resin with acetic acid released some of **24b** which was identified by t.l.c. The identity of **24a** was confirmed by the loss of the C8 and C9 signals in the ^{13}C n.m.r., and by mass spectroscopy.

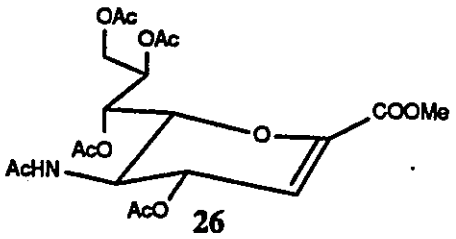
3.7 Aryl Glycoside by Phase Transfer Catalysis

The aryl glycosides have been particularly useful as chromogenic substrates to detect sialidase activity (Eschenfelder 1987, Baumberger 1986). These methods involve reacting the sodium salt of the phenol with acetochloroneuraminic acid (**5**) in DMF. A new method reported at the Sialic Acids Conference in 1988 involved a refluxing phase transfer catalyzed synthesis (Rothermel 1990), and we employed it to make the *p*-nitrophenyl α-glycoside of NeuAc, **25**. A second component was found, and after isolation it was characterized as the well known dehydro compound methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-2,6-anhydro-3,5-dideoxy-D-*glycero*-D- *galacto*-non-2-enonate, **26** (Okamoto, 1987).



5

25a R=Ac R'=Me
25b R=H R'=Me
25c R=H R'=Na



26

Figure 3-8 Phase Transfer Synthesis of NeuAc-PNP

a) CH_2Cl_2/1 N NaOH/4-nitrophenol/$EtPh_3P^+Br^-$

We modified the procedure to use a phosphorus phase transfer catalyst, as illustrated in figure 3-8, and we found the reaction was complete within 10 minutes without the need to reflux. The method proved superior and resulted in higher yields and less elimination to give the product **25a** with specific rotation, melting point and n.m.r spectra identical to the known compound (Eschenfelder 1987).

3.8 Hydrogenolysis with Pd on Charcoal

Following two recent reports in the literature (Kim 1988, Schmid 1988), it was found that the hydrogenolysis of acetochloroneuraminic acid (**5**) was easily performed with 10% Pd on charcoal to yield with high stereoselectively the 2-deoxy compound **27**. A proposed mechanism is shown in figure 3-9, and some interest was taken in the possibility of using palladium to prepare other compounds with the same kind of chiral inversion. It was found that

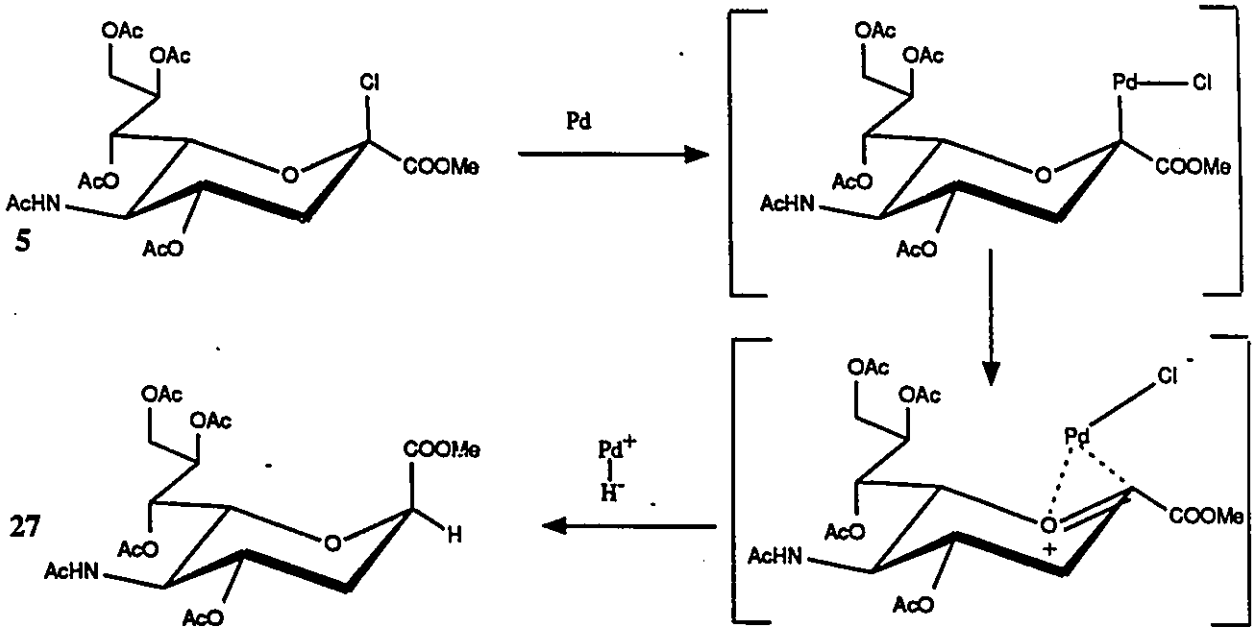


Figure 3-9 Mechanism Of Palladium Hydrogenolysis

tetrakis(methyldiphenylphosphine)palladium was also capable of causing the reduction, though several other side products resulted, one of which was the dehydro compound **26**. Using methanol, the methyl glycoside **8c** could be prepared, but with cyanide no detectable C glycoside was obtained.

Palladium on charcoal was also used to prepare the propyl glycoside of NeuAc (**28**) by hydrogenation of the double bond in **7a**. The product crystallized readily from methanol and was characterized by ^1H n.m.r. spectroscopy which showed the loss of the allyl group.

3.9 Sialyllactose Derivatives

The trisaccharides **2a** and **3** isolated from colostrum were considered ideal candidates for immunological studies, but it was required to prepare polyvalent forms of these molecules.

Direct reductive amination of the reducing sugar to the lysine residues of protein is possible, and described in section 4.2.2. A similar conjugation to spacer molecules is considered below. This method suffers from the fact that the reducing sugar is modified to a non-cyclic form as shown in figure 3-10. An alternate approach which maintains glucose as a pyranose involves the glycosylamine shown in figure 3-11.

3.9.1 Reductive Amination

The reducing ends of saccharides are concealed aldehydes, and as such can form Schiff bases with an amine. Reduction of the imine to an amine with cyanoborohydride is preferred over borohydride (see section 3.4.2). This type of reaction has been done with NeuAcα2-3Galβ1-4Glc **2a** on several occasions previously (Wiegandt 1974, Smith 1980).

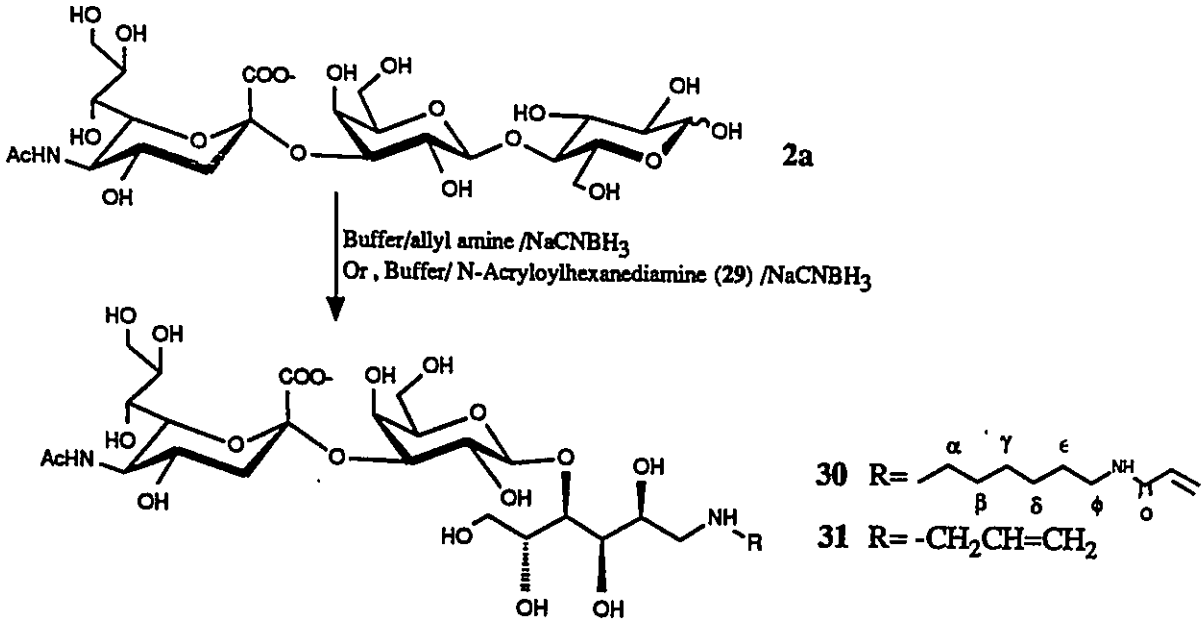


Figure 3-10 Derivatives of Sialyllactose via Reductive Amination

The compound **2a** was reductively aminated to allylamine which was readily available,

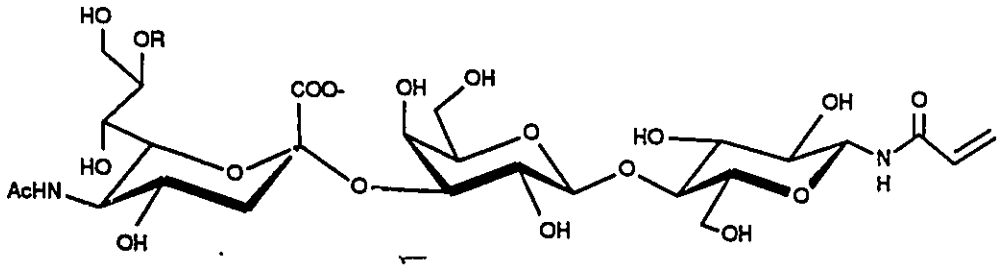
and also to N-acryloyldiaminohexane (**29**) (Kojima 1966, Stahl 1978) because we anticipated better reactivity of the acrylamide function in the polymerization reaction.

Reductive amination went smoothly in borate buffer (Roy 1984) to give the desired products **30** and **31**. Purification was done by trituration using acetone, followed by desalting. The reductively aminated products were expected to have shorter retention volumes due to the increased molecular weight, but were in fact retained somewhat over the starting material. We suspected this was due to the slightly negative nature of Sephadex, and the fact that the products became amines.

The identities of the products were confirmed by the highly characteristic acryloyl and allyl functions in the ^{1}H n.m.r in compounds **30** and **31** respectively. The ^{13}C n.m.r., reported in table 3-3, match very well with the starting material **2a** except for the resonances associated with the glucose moiety. This demonstrates that the glucose has been modified to an aminoglucitol which cannot form ring structures.

3.9.2 Acryloylated Glycosylamines

Glycosylamines have been made by reduction of the glycosyl azide (Kunz 1987); however, a recent publication shows the glycosylamine may be prepared by equilibration with ammonium bicarbonate in aqueous solution (Kallin 1989). Subsequent N-acryloylation stabilizes the glycosylamine by delocalizing the nitrogen electrons into the carbonyl, thus preventing hydrolysis.



32 R=H
33 R=NeuAcα2→

Figure 3-11 Structure of N-Acryloylglycosylamines

Preliminary efforts to obtain the glycosylamine from NeuAcα2-3Galβ1-4Glc **2a** as reported by Kallin (1989) resulted in only 20% yield after isolation using a cation exchange resin column. Since t.l.c. showed that approximately 75% of the starting material had reacted, the low yield was due to the inefficiency of the column or hydrolysis of the glycosylamine. Hence we opted for direct acryloylation of the glycosylamine using Schotten-Baumann

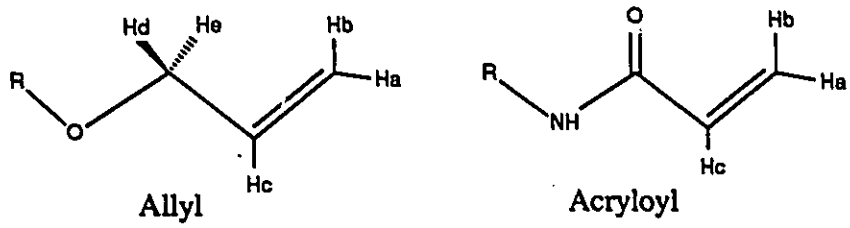
procedure (March 1979) after removing the excess ammonium bicarbonate by lyophilization. To increase the equilibrium concentration of the glycosylamine and speed up the reaction rate, the temperature was increased to 37°C. The yields of **32** were still modest (≈ 50%), but unreacted **2a** could be recovered after reverse phase h.p.l.c purification (Lindh 1988). ^{1}H n.m.r. spectroscopy confirmed the presence of the acryloyl group, and also revealed that the glucopryranose moiety was greater than 95% in the β configuration as determined by the doublet ($J_{1,2}$=9.1 Hz) at 5.10 ppm which was identified as the glucose H1 proton.

The reaction was also done on disialyllactose **4**, again resulting in modest yields after considering the purity of the starting material isolated from colostrum. Baseline separation of product **33** with the additional hydrophobic acryloyl group from the starting material **4** was accomplished readily using reverse phase h.p.l.c. This material displayed the two N-acetyl groups (2.03 and 2.07 ppm) and two characteristic NeuAc $H3_{eq}$ resonances (2.79 and 2.68 ppm) in the ^{1}H n.m.r., indicating the presence of the NeuAcα2-8NeuAc moiety. As with **32**, **33** had the glucose in better than 95% β configuration, as no detectable α isomer could be observed by ^{1}H n.m.r. The ^{13}C n.m.r. spectrum (data in table 3-3) was assigned based on a comparison with the α 2,8 linked NeuAc trisaccharide (Michon 1987).

3.10 Experimental Methods

General Methods are found in Appendix 1. Identity of the t.l.c. solvent code letters may also be found in Appendix 1.

^{1}H and ^{13}C nmr data have been tabulated for ease in reading, and can be found in tables 3-1, 3-2 and 3-3 at the end of this section. The following system has been used to label the protons in allyl and acryloyl compounds.



The coupling patterns associated with these functional groups are shown in figure 7-1.

The allyl protons generally showed the same multiplicity pattern throughout and only the chemical shift varied. The following is a first order approximation: H_a, dd, $J_{a,b}$=1.5 Hz, $J_{a,c}$= 10.5 Hz; H_b, dd, $J_{a,b}$=1.5 Hz, $J_{b,c}$= 17.0 Hz; H_c, m; H_d and H_e ddd, $J_{d,e}$= 12.5 Hz, $J_{c,d}$=$J_{c,e}$=5.5Hz.

The acryloyl compounds had the same multiplicity except for the N-acryloylglycosylamines which showed a ABX pattern at 300 MHz. In general: H_a, dd, $J_{a,b}$=1.9 Hz, $J_{a,c}$=9.7; H_b, dd, $J_{b,a}$=1.9 Hz, $J_{b,c}$=17.2 Hz; H_c, dd, $J_{c,a}$=9.7 Hz, $J_{c,b}$=17.2 Hz.

Methyl 5-acetamido-3,5,dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonate **1b**

Compound **1a** (2.99g, 9.7 mmole), 2.0 g Amberlite IR-20 (H^+) resin and 50 mL methanol were stirred 4 to 6 hours at room temperature until the solution became clear. The solution was filtered and vacuum evaporated to a volume of ~5 mL. Ether was added and **1b** crystallized as a white solid. Yield: 3.18 g, 9.8 mmol, quantitative.

mp 193.5-197.4°C ; $[\alpha]_D$=-28° (c 3.5 H_20)(lit. mp 179-180°C $[\alpha]_D$=-28°, Kuhn 1966); Rf.=0.55 solvent A; For ^{1}H and ^{13}C n.m.r, see tables 3-1 and 3-2.

[Methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-2-chloro-3,5-dideoxy-D-*glycero* -β-D-*galacto*-2-nonulopyranosyl)onate] chloride **5**

Compound **1b** (0.47 g, 1.5 mmole) was added to 5 mL acetic acid and acetyl chloride (10 mL, freshly distilled) was added at 0°C with stirring. The flask was sealed and stirred 24 to 48 hours at room temperature. The extent of the reaction was monitored by t.l.c. When complete, the reaction mixture was vacuum evaporated, and then co-evaporated four times

with dry toluene to produce **5** as a white foam.

Yield: 0.75 g, 1.5 mmole, quantitative.

$[\alpha]_D$ = -61° (lit. -63° Kuhn 1966); Rf.= 0.32 EtOAc; ^{1}H n.m.r. ($CDCl_3$) δ: 5.45 (dd, 1H, $J_{7,8}$=6.8, $J_{6,7}$=2.1 Hz, H_7), 5.40 (m, 1H, H_4), 5.33 (d, 1H, $J_{5,NH}$≈10 Hz, NH), 5.15 (m, 1H, H_8), 4.38 (dd, 1H, $J_{9,9'}$= 12.5, $J_{8,9'}$= 5.8 Hz, H9'), 4.31 (dd, 1H, $J_{5,6}$=10.4, $J_{6,7}$=2.1, H_6), 4.21 (dd, 1H, $J_{4,5}$=$J_{5,6}$=10.4, H_5), 4.04 (dd, 1H, $J_{8,9}$=2.5, $J_{9,9'}$=12.5, H9'), 3.86 (s, 3H, Methyl ester), 2.77 (dd, 1H, $J_{3ax,3eq}$=12.0, $J_{3eq,4}$=4.8 Hz, H_{3eq}), 1.90, 2.03, 2.11, 2.05, 2.06 (5xs, 15H, N, O-Ac), 2.29 (dd, 1H,$J_{3ax,3eq}$≈$J_{3ax,4}$≈12 hz, $H3_{ax}$); ^{13}C n.m.r. ($CDCl_3$) δ: 171.2, 170.9, 170.5, 170.2 (N, O-Ac), 165.8 (CO_2Me), 96.6 (C-2), 73.9 (C-6), 69.9 (C-8), 66.8 (C-7), 68.7 (C-4), 62.0 (C-9), 48.7 (C-5), 40.6 (C-3), 23.0 (N-Ac), 20.6, 20.7, 20.8 (O-Ac); Anal. calc'd for $C_{20}H_{28}O_{12}NCl$: C 47.06, H 5.54, N 2.75 Found: C 47.38, H 5.75, N 2.78

Methyl (allyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate, **6**

Compound **5** (0.75 g, 1.4 mmol) was dissolved in dry allyl alchohol (5.0 mL) and silver salicylate (0.35 g, 1.4 mmol) was added with 4A molecular sieves (0.5 g). The reaction was stirred for 2 hours at room temperature in the dark. The slurry was filtered over Celite and rinsed with CH_2Cl_2. The resulting clear solution was vacuum evaporated to dryness and dissolved in 25 mL CH_2Cl_2. This was washed with cold 5% aqueous sodium bicarbonate (50 mL, twice), 5% aqueous sodium thiosulphate (50 mL, twice) and water (50 mL, twice). The organic layer was dried over sodium sulphate, then vacuum evaporated to give a clear oil which was homogeneous by tlc (90% yield). Compound **6** was crystallized from methanol-ether. Yield: 0.59g, 1.1 mmole, 73%.

mp 153.6-155.7 °C (lit.154-156 °C, Eschenfelder 1980), $[\alpha]_D$ = -14.2° (c 1. $CHCl_3$) (lit. -13°, Eschenfelder 1980), Rf.= 0.30 EtOAc, ^{1}H n.m.r. ($CDCl_3$) δ: 5.83 (4xd, 1H, $J_{b,c}$=17.0, $J_{a,c}$=10.5, $J_{c,d}$≈$J_{c,e}$≈5 Hz, H_c), 5.35 (m, 1H, H_8), 5.30 (dd, 1H, $J_{7,8}$=8.5, $J_{6,7}$=1.8 Hz, H_7), 5.26 (dd, 1H, $J_{b,c}$= 17.0, $J_{a,b}$≈$J_{b,d}$≈$J_{b,e}$≈1.5 Hz, H_b),5.14 (d, 1H, $J_{5,NH}$≈10 Hz, NH), 5.13 (dd, 1H, $J_{a,c}$= 10.5, $J_{a,b}$≈$J_{a,d}$≈$J_{a,e}$≈1.5 Hz, H_a), 4.83 (m, 1H, H_4), 4.27 (dd, 1H, $J_{9,9'}$= 12.5, $J_{8,9'}$= 5.8 Hz, H9'), 3.76 (s, 3H, Methyl ester), 2.59 (dd, 1H, $J_{3ax,3eq}$=12.0, $J_{3eq,4}$=4.8 Hz, H_{3eq}), 1.86, 2.00, 2.02, 2.11, 2.13 (5xs, 15H, N, O-Ac), 1.96 (dd, 1H,$J_{3ax,3eq}$≈$J_{3ax,4}$≈12 hz, $H3_{ax}$); ^{13}C n.m.r. ($CDCl_3$) δ: 171.2, 170.9, 170.4, 170.3 (N, O-Ac), 168.5 (CO_2Me), 133.6, 117.4 (allyl), 98.5 (C-2), 72.5 (C-6), 69.0 (C-8), 68.5 (C-7), 67.3 (CH_2-allyl), 66.0 (C-4), 62.3 (C-9), 49.3 (C-5), 38.0 (C-3), 23.1 (N-Ac), 21.0, 20.7, 20.6 (O-Ac); Anal. calcd for $C_{23}H_{33}O_{13}N$: C 51.97, H 6.26, N 2.64 Found: C 52.22, H 6.46, N 2.82.

Methyl (allyl 5-acetamido-3,5-dideoxy-D-*glycero*- α-D-*galacto*-2-nonulopyranosid)onate 7a

Compound **6** (1.00 g, 1.9 mmole) was de-O-acetylated in 20 mL dry methanol (20 mL) containing several drops of 1M sodium methoxide (pH≈10 with wet pH paper). The reaction followed by tlc was complete in 2 hours at room temperature. The mixture was neutralized with Amberlite 120 (H^+) resin, filtered and vacuum evaporated to ≈5 mL, and **7a** was crystallized by addition of ether. Yield: 0.64 g, 1.8 mmole, 93 %.

mp 143-144°C; $[\alpha]_D$= -10.1° (c 1.0 MeOH); Rf.= 0.49 solvent B; FAB MS for $C_{15}H_{25}O_9N$ 363.37: (M+1) 364; IR (KBr) ν_{max} 3380, 2944, 1750, 1650, 1570, 1440, 1040 cm^{-1}; Anal. Calculated: C 49.58, H 6.94, N 3.86 Found: C 49.47, H 7.10, N 3.98; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Allyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*- 2-nonulopyranosidonic acid **7b** (NeuAc allyl glycoside)

Compound **7a** (0.28 g, 0.77 mmole) was dissolved in aqueous NaOH (0.1M, 5 mL). TLC showed the deesterification to be instantaneous. The mixture was cooled to 0°C, and neutralized with Rexyn 101 (H^+) resin, then filtered. The pH was adjusted to 6. with 0.1N NaOH, then the solution was lyophilized to give a white powder **7b**. Yield: 0.25 g, 0.72 mmole, 93%.

mp 245-250°C (dec); $[\alpha]_D$= -9.1° (c 0.7 H_2O); Rf.= 0.57 solvent C; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2; FAB MS for $C_{14}H_{23}O_9N$ 349.33: (M-1) 348; Anal. Calcd for $C_{14}H_{22}O_9NNa$: C 45.28, H 6.24, N 3.77 Found: C 46.43, H 6.02, N 5.06.

Methyl (methyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*- 2-nonulopyranosid)onate **8a**

Compound **5** (0.29g, 0.51 mmole) was dissolved in methanol (10 mL) and silver salicylate (0.15 g, 0.6 mmol) was added, and the reaction was allowed to proceed for two hours in the dark. Then the solution was treated as with compound **6**. Compound **8c** was crystallized from methanol/ether as clear prisms. Yield: 0.21 g, 0.42 mmol, 74%. $[\alpha]_D$= -13.8 (c 1.0 $CHCl_3$)(lit. -19° Eschenfelder 1980); mp 94°C (lit. 135-137°C); R.f.= 0.29 EtOAc. Compound **8c** (0.233g, 0.46 mmol) was was de-O-acetylated as above. It was dissolved in methanol (10 mL) and sodium methoxide was added. After one hour, the solution was neutralized with Rexyn 101 (H^+) resin, and vacuum evaporated. **8a** was crystallized from methanol/ether. Yield: 0.134g, 0.39 mmole, 87%.

$[\alpha]_D$= -5.3° (c 0.3 MeOH); mp 168.9-171.8°C; Rf= 0.51 B; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Methyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*- 2-nonulopyranosidonic acid **8b**

Compound **8a** (0.076 g, 0.22 mmole) was dissolved in aqueous NaOH (0.1 N, 5 mL). After one hour the mixture was cooled to 0°C, and neutralized with Rexyn 101 (H^+) resin, then filtered. The pH was adjusted to 6. with 0.1N NaOH, then the solution was lyophilized to give **8b** as a white powder. Yield: 0.079 g, 0.24 mmole, quantitative.

$[\alpha]_D$= -3.5° (c 0.8 H_2O); Rf= 0.39 C; FAB MS for $C_{12}H_{21}O_9N$ 323.30: (M-1) 322; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Methyl (methyl 5-acetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*- 2-nonulopyranosid)onate **9a**

Compound **1a** (56.7 mg, 0.18 mmole) was dissolved in methanol (20 mL) with Amberlite IR20 (H^+) cation exchange resin (1 g). The solution was refluxed for 24 hours. Flat white crystals were obtained from methanol/ether. Yield: 35.6 mg, 0.11 mmole, 59%.

mp 85-90°C (lit. 111-115°C); $[\alpha]_D$= -37.8° (c 0.3 MeOH)(lit. -45° Yu 1969); Rf.= 0.46 D; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Methyl 5-acetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*- 2-nonulopyranosidonic acid **9b** (βNeuAc methyl glycoside)

Compound **9a** (0.076 g, 0.22 mmole) was dissolved in aqueous NaOH (0.1 M, 5 mL). After one hour the mixture was cooled to 0°C, and neutralized with Rexyn 101 (H^+) resin, then filtered. The pH was adjusted to 6. with 0.1N NaOH, then the solution was lyophilized to give **9b** as a white powder. Yield: 0.079 g, 0.24 mmole, quantitiative.

$[\alpha]_D$= -24.7° (c 0.8 H_2O); Rf= 0.22 C; FAB MS for $C_{12}H_{21}O_9N$ 323.30: (M-1) 322; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Methyl (2'-oxoethyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate **10a**

Compound **7a** (68 mg, 0.19 mmole) was dissolved in 15 mL methanol and cooled to -78°C over N_2. Ozone was then bubbled through the solution until a blue color could be observed. The ozone was purged with N_2 until the solution was colorless, then 0.5 mL dimethylsulfide was added and the solution was allowed to warm to room temperature over a one hour period. The solution was vacuum evaporated to give **10a** as a colorless oil. Yield: 66 mg, 0.18 mmole, 95%.

$[\alpha]_D$= -10.3° (c 1.0 H_20 pH 5.0); Rf= 0.35 D, 0.61 C; FAB MS for $C_{14}H_{23}O_{10}N$ 365.34: (M+1) 366; IR (KBr) ν_{max} 3410, 2940, 1740, 1650, 1560, 1040 cm^{-1}; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2; Anal. Calculated: C 46.07, H 6.35, N 3.83 Found: C 45.65, H 6.39, N 3.79

2'-Oxoethyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*- 2-nonulopyranosidonic acid **10b**

The above procedure for **10a** was repeated using compound **7b** (1.0g, 2.70 mmole) in methanol (50 mL) to give **10b** as an oil. Yield: 1.0 g, 2.70 mmol, quantitative.

$[\alpha]_D$= -1.8° (c 1.0 H_2O pH 5.0); Rf= 0.27 D; FAB MS for $C_{13}H_{21}O_{10}N$ 351.31: (M-1) 350; IR (KBr) ν_{max} 3390, 2940, 1620, 1560, 1060 cm^{-1}; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2; Anal. Calculated for $NaC_{13}H_{20}O_{10}N$: C 41.83, H 5.36, N 3.75 Found: C 41.96, H 6.60, N 4.05

2'-Hydroxyethyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*- 2-nonulopyranosidonic acid **11**

Compound **10b** (27.1 mg, 0.077 mmole) was dissolved in of methanol (2 mL) and sodium borohydride (10 mg, 0.29 mmole) was added. After two hours acetic acid was added to neutralize the excess borohydride, and the solution was vacuum evaporated to dryness. The residue was dissolved in water and desalted on a 1.5 cm X 60 cm. column of Sephadex G10 eluted with water. The first fractions containing resorcinol reactive material were pooled and lyophilized to give **11** as a white powder. Yield: 18.1 mg, 0.051 mmol, 67%.

$[\alpha]_D$= -7.6° (c 0.3 H_2O); Rf 0.39 C; FAB MS for $C_{13}H_{23}O_{10}N$ 353.33: (M-1) 352; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

2'-Aminoethyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **12**

Compound **10b** (18 mg, 0.051 mmole) was dissolved in methanol (2 mL) saturated with ammonium acetate. Sodium cyanoborohydride (10 mg) was added and the solution was refluxed. After 24 hours the solution was evaporated several times with methanol then dissolved in 1 mL water and desalted on a 1.5 cm X 60 cm. column of Sephadex G10 eluted with water. The combined ninhydrin reactive fractions were lyophilized to give **12** as a white powder. Yield: 9.2 mg, 0.026 mmol, 51%.

$[\alpha]_D$= -18.5 (c 1.0 H_2O) (Lit.= -17° Eschenfelder 1980); Rf. 0.17 C, 0.21 G; FAB MS for $C_{13}H_{25}O_9N_2$ 353.34: (M-1) 352; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

(2-Aminoethyl-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulo pyranosid)onolactam **13** (bicyclic lactam)

Compound **12** (7.0 mg, 0.020 mmole) was dissolved in MeOH (3 mL), and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (3 mg, 0.015 mmol, Aldrich) was added. After several hours, and additional 3 mg EDC was added. After 24 hours t.l.c. showed the reaction was complete, and the material was purified on a silica gel column eluted with ethanol, followed by methanol which gave compound **12** as an oil. Yield 1.7 mg , 25%.

Rf. 0.35 C; IR (KBr) ν_{max} 3420, 1650, 1560, 1350, 1040 cm^{-1}; FAB MS for $C_{13}H_{22}O_8N_2$ 334.33: (M+1) 335; For ^{13}C n.m.r, see table 3-2; ^1H n.m.r. (D_2O) δ: 2.44 (dd, 1H, $J_{3a,3e}$=13.0, $J_{3e,4}$=4.7, H3eq), 2.03 (s, 3H, NAc), 1.63 (t, 1H, $J_{3a,3e}$=13.4, $J_{3a,4}$=11.6, H3ax)

2'-[N^{ϵ}-(N^{α}-Acetyl-(L)-lysyl)]ethyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **14**

Compound **10b** (34.1 mg, 0.097 mmole) was dissolved in phosphate buffer (0.2 M, pH 7.0, 1 mL) containing N^{α}-acetyl-(L)-lysine (30 mg, 0.15 mmol, Sigma). Sodium cyanoborohydride (12.9 mg, 0.22 mmol) was added and the reaction was allowed to proceed for 48 hours at room temperature. Then acetic acid (0.25 mL) was added to the reaction and stirred for 3 hours to quench the excess borohydride, and the solution was loaded directly on a 3.0 cm X 60 cm column of Sephadex G10 eluted with 0.03 M ammonium bicarbonate in water. The product **14** was identified and lyophilized to give a white powder. Yield: 39.9 mg, 0.076 mmole, 78%.

$[\alpha]_D$= -6.5° (c 0.9 H_2O); Rf. 0.31 G; FAB MS for $C_{21}H_{37}O_{13}N_3$ 523.54: (M-1) 522, (M+1) 524; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2. ^1H n.m.r. (D_2O) δ (aglycon): 4.16 dd, 1H, $J_{\alpha,\beta}$=8.2 and 4.9 Hz, H_{α}), 3.87 (m, 1H, $O\underline{CH_2}CH_2N$), 3.25 (t, 2H, J=4.7 Hz, OCH_2-$\underline{CH_2}N$), 3.08 (t, 2H, J=7.8 Hz, H_{ϵ}), 2.04 (s, 3H, N^{α}-Ac), 1.69-1.82 (m, 5H, H_{3ax}+$4H_{\beta,\gamma}$), 1.42 (q, 2H, H_{γ}); ^{13}C n.m.r. (D_2O) δ (aglycon): 22.8, 23.2, 26.0, 31.8, 47.9, 48.1, 55.6, 60.0, 174.4, 179.2; Analysis Calculated for $C_{21}H_{37}O_{13}N_3.NH_4HCO_3$: C 43.91, H 7.03, N 9.30 Found: C 43.30, H 7.53, N 9.07

Methyl (3'-(2'-aminoethylthio)propyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate **15a**

Compound **7a** (75.3 mg, 0.21 mmol) was dissolved in water (2 mL). Cysteamine hydrochloride (0.0352 g, 0.31 mmol, Sigma) was added, and the reaction was stirred under a UV lamp for three days. The solution was loaded on a 1.5 cm X 60 cm. column of Sephadex G10 and eluted with water. The product **15a** was identified and lyophilized to a white powder.

Yield: 71.1 mg, 0.16 mmol, 77%

$[\alpha]_D$= -9.2° (c 1.0 H_2O); Rf 0.37 G; IR (KBr) ν_{max} 3420, 1795, 1642, 1410, 1040 cm^{-1}; FAB MS for $C_{17}H_{32}O_9N_2S$ 440.49: (M+1) 441; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2. ^1H n.m.r. (D_2O) δ (aglycon): 3.213 (t, 2H, $J_{\epsilon,\delta}$=6.6 Hz, H_ϵ), 2.842 (t, 2H, $J_{\delta,\epsilon}$=6.6, H_δ) 2.650 (t, 2H, $J_{\gamma,\beta}$= 7.1, H_γ) 1.857 (m, 2H, H_β); ^{13}C n.m.r. (D_2O) δ (aglycon): 64.0, 29.5, 29.1, 28.2, 39.3

3'-(2'-Aminoethylthio)propyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **15b**

Compound **7b** (0.148 g, 0.425 mmol) was dissolved in water (2 mL) and cysteamine hydrochloride (0.104 g, 0.912 mmol) was added and the solution was placed under vacuum for 2 minutes, then purged with Nitrogen. The solution was was stirred for 18 hours under a U.V. lamp, then loaded directly on a 3 cm X 60 cm column of Sephadex G10 eluted with water. Compound **15b** was obtained as a white powder after lyophilization. Yield: 0.152 g, 0.357 mmole, 84%.

$[\alpha]_D$= -8.3° (c 1.0 pH 5.0); Rf 0.15 G; IR (KBr) ν_{max} 3390, 1630, 1430, 1120, 1100 cm^{-1}; FAB MS for $C_{16}H_{30}O_9N_2S$ 426.48:(M+1) 427; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2. ^1H n.m.r. (D_2O) δ (aglycon): 3.224 (t, 2H, $J_{\epsilon,\delta}$=6.5 Hz, H_ϵ), 2.857 (t, 2H, $J_{\delta,\epsilon}$=6.5, H_δ) 2.687 (t, 2H, $J_{\gamma,\beta}$= 7.0, H_γ), 1.853 (m, 2H, H_β); ^{13}C n.m.r. (D_2O) δ (aglycon): 64.1, 29.9, 29.3, 28.3, 39.4

Methyl (3'-(N-acryloyl-2'-aminoethylthio)propyl 5-acetamido-3,5-dideoxy-D-*glycero* -α-D-*galacto*-2-nonulopyranosid)onate **16a**

Compound **15a** (120 mg, 0.28 mmole) was dissolved in methanol (100 mL). Amberlite IRA 400 anion exchange resin (OH^- form) (1 g) was stirred in the solution at 0°C and then 10% acryloyl chloride in dioxane was added dropwise until ninhydrin reagent showed no presence of amine (approximately 1 mL). The solution was filtered and vacuum evaporated. Compound **16a** was obtained as an oil after silica gel chromatography with 8:2 chloroform : methanol. Yield: 89 mg, 0.18 mmole, 64%.

$[\alpha]_D$= -11.3° (c 1.4 pH 5.0); Rf.= 0.54 G, 0.41 H; IR (KBr) ν_{max} 3410, 1740, 1650, 1560, 1130, 1040 cm^{-1}; FAB MS for $C_{20}H_{34}O_{10}N_2S$ 494.56: (M-1) 493 (weak); Analysis Calculated: C 48.57, H 6.93, N 5.66, S 6.47. Found: C 48.83, H 7.16, N 5.48, S 6.42; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.^1H n.m.r. (D_2O) δ(aglycon): 6.282 (dd, 2H, $J_{c,b}$=17.0, $J_{c,a}$=9.6, H_c) 6.183 (dd, 2H, $J_{b,a}$=2.0, $J_{b,c}$=17.0, H_b), 5.769 (dd, 2H, $J_{a,b}$=2.0, $J_{a,c}$=9.5, H_a) 3.481 (t, 2H, $J_{\epsilon,\delta}$=6.6 Hz, H_ϵ), 2.734 (t, 2H, $J_{\delta,\epsilon}$=6.4, H_δ) 2.641 (t, 2H, $J_{\gamma,\beta}$= 7.1, H_γ), 1.841 (m, 2H, H_β);^{13}C n.m.r. (D_2O) δ (aglycon): 63.9, 31.2, 29.6, 28.4, 39.6, 169.2, 130.6, 128.1

3'-(N-Acryoloyl-2'-aminoethylthio)propyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **16b**

Compound **15b** (0.102 g, 0.24 mmole) was dissolved in water (1 mL) and Na_2CO_3 (0.2 g) was added and dissolved, followed by methanol (1 mL). The slurry was cooled to 0°C and acryloyl chloride in dioxane (20% v/v, 0.5 mL) was added with stirring over a three minute period. Thin layer chromatography showed the reaction was complete, and the solution was rotovaped to dryness, dissolved in water (1 mL) and desalted on a 3cm . X 60 cm. column of Sephadex G10 eluted with water. The product **16b** was obtained as an oil after lyophilization. Yield: 0.108g, 0.22 mmole, 93%.

$[\alpha]_D$= -11.° (c 1.0 pH 5.0); Rf 0.36 G; IR (KBr) ν_{max} 3400, 1620, 1560, 1400, 1110, 1100 cm^{-1}; FAB MS for $C_{19}H_{32}O_{10}N_2S$ 480.51: (M+1) 481 (weak); For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.^1H n.m.r. (D_2O) δ(aglycon): 6.282 (dd, 2H, $J_{c,b}$=17.0, $J_{c,a}$=9.6, H_c) 6.183 (dd, 2H, $J_{b,a}$=2.0, $J_{b,c}$=17.0, H_b), 5.766 (dd, 2H, $J_{a,b}$=2.0, $J_{a,c}$=9.6, H_a) 3.483 (t, 2H, $J_{\epsilon,\delta}$=6.6 Hz, H_ϵ), 2.744 (t, 2H, $J_{\delta,\epsilon}$=6.6, H_δ) 2.642 (t, 2H, $J_{\gamma,\beta}$= 7.1, H_γ), 1.836 (m, 2H, H_β); ^{13}C n.m.r. (D_2O) δ (aglycon): 64.3, 30.2, 31.5, 28.7, 39.9, 169.4, 130.9, 128.4

Allyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonamide, **17**

Compound **7a** (40.0 mg, 0.110 mmole) was dissolved in methanol (5 mL). Methanol (5 mL) saturated with NH_3 gas (Lindane) was added and solution was allowed to react at room temperature for one hour. The solution was vacuum evaporated and **17** crystallized upon standing. Yield: 32.0 mg, 0.092 mmole, 84%

mp 82-87°C; $[\alpha]_D$= -3.6° (c 2.0 H_2O); Rf. 0.63 D; IR (KBr) ν_{max} 3400, 1690, 1600, 1385, 1040 cm^{-1}; FAB MS for $C_{14}H_{24}O_8N_2$ 348.35: (M+1) 349; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Allyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranoside **18**

Compound **7a** (21.2 mg, 0.058 mmole) was dissolved in methanol (0.5 mL), then diluted with THF (5 mL). Lithium borohydride (1 mg) was added to the solution and the reaction was allowed to proceed for 1 hour at room temperature. The solution was neutralized with H^+ resin, filtered and vacuum evaporated several times with methanol to give **18** as an oil. Crystallization could not be induced. Yield: 19.4 mg, 0.058 mmole, quantitative.

$[\alpha]_D$= -15.4° (c 1.8 H_2O); Rf. 0.50 D; IR (KBr) ν_{max} 3400, 1640, 1560, 1040 cm^{-1}; FAB MS for $C_{14}H_{25}O_8N$ 335.35: (M+1) 336; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Allyl 5-amino-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **19**

Compound **7a** (95 mg, 0.26mmole) was dissolved in water (1 mL). Then tetramethylammonium hydroxide (1 mL, 20% w/w in methanol, Aldrich) was added and the solution was refluxed for 5 hours and monitored by tlc. When the reaction was complete, the solution was neutralized with acetic acid, vacuum evaporated to dryness, dissolved in 1 mL water and desalted on a 3 cm X 60 cm column of Sephadex G10 eluted with water. The product was identified with resorcinol and ninhydrin reagents (see Appendix 1), and fractions containing the product **19** were pooled and lyophilized. Yield: 64 mg, 0.21 mmole, 80%.

$[\alpha]_D$= -18.0° (c 0.9 H_2O); Rf 0.26 D, 0.33 E; IR (KBr) ν_{max} 3400, 1610, 1400, 1130 cm^{-1}; FAB MS for $C_{12}H_{21}O_8N$ 307.30: (M+1) 308, (M-1) 306; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Allyl 3,5-dideoxy-5-hydroxyacetamido-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **20b**

Compound **19** (34 mg, 0.11 mmol) was dissolved in methanol (2 mL) with triethylamine (0.5 mL) and cooled to -78°C. Then acetoxyacetyl chloride (50 μL, Aldrich) was added slowly with stirring. The reaction was followed by tlc and was almost instantaneous. The solution was vacuum evaporated to dryness, dissolved in water (2 mL) and desalted on a 3 cm X 60 cm column of Sephadex G10 eluted with water, and lyophilized to give **20a**. The material was treated with 0.1 N aqueous NaOH for 30 minutes, neutralized with H^+ resin, and lyophilized to give **20b**. Yield: 38.7 mg, 0.095 mmole, 86%.

$[\alpha]_D$= -3.8° (c 0.4 H_2O); mp 245°C (dec); Rf 0.52 E, 0.48 D, 0.43 C; IR (KBr) ν_{max} 3430, 1600, 1410, 1080 cm^{-1}; FAB MS for $C_{14}H_{23}O_{10}N$ 365.34 : (M+1) 366, (M-1) 364; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Allyl 5-bromoacetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **21b**

Compound **19** (67 mg, 0.22 mmol) was dissolved in methanol (2 mL) and triethylamine (0.5 mL) was added. The solution was colled to -70°C and one drop of bromoacetyl chloride was added. The reaction was monitored by t.l.c., and was judged complete by the disappearance of starting material. The solution was vacuum evaporated to dryness, dissolved in water and desalted on a 3 cm X 60 cm column of Sephadex G10 eluted with water. The product **21b** was obtained as a white powder after lyophilization. Yield: 75.2 mg, 0.18 mmol, 80%.

$[\alpha]_D$= -8.0° (c 0.1 H_2O); Rf 0.53 D, 0.51 D; FAB MS for $C_{14}H_{22}O_9NBr$ 428.24: (M-1) 428, 426; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Methyl (allyl 5-bromoacetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate **21a**

Compound **21b** (35. mg, 0.082 mmol) was dissolved in methanol, and treated with a freshly prepared solution of zomethane in ether. Compound **21a** was isolated by silica gel chromatography with 8:2 choloroform methanol. Yield: 19.1 mg, 0.043 mmol, 52%. This material was sent for elemental analysis.

For ^{1}H and ^{13}C n.m.r, see tables 3-1 and 3-2; Analysis calculated for $C_{15}H_{24}O_9NBr$: C 40.73, H 5.47, N 3.17, Br 18.07 Found: C 35.52, H 5.48, N 2.49, Br 18.39.

Allyl 5-(carboxymethyl)amino-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid, **22**

Compound **21a** (24.9 mg) was dissolved in water (2 mL) and aqueous sodium hydroxide (0.1 N) was added until the pH was 10.5. The reaction was allowed to proceed for 3 hours. The solution was neutralized with acetic acid, then desalted on Sephadex G10 eluted with water. Compounds **21b** (15%), **20b** (28%) and **22** (58%) were isolated by preparative t.l.c. using solvent system C.

Rf 0.28 C, 0.20 D; FAB MS for $C_{14}H_{23}O_{10}N$ 365.34: (M-1) 364; For ^{1}H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Allyl 5-acetamido-9-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid, **23**

Compound **7b** (45 mg, 0.129 mmol) was dissolved in DMSO (0.5 mL, dried over 4A molecular sieves). Trimethylorthoacetate (60 mg) was added with toluenesulfonic acid (30 mg). An additional 120 mg trimethylorthoacetate was needed to drive the reaction to completion, as followed by tlc. The solution was loaded on a 0.5 cm . X 5 cm. column of Dowex IRA 400 anion exchange resin (formate form) and washed with water (100 mL). The product **23** was eluted with formic acid (1 M, 100 mL) and lyophilized immetely. Yield: 27.9 mg, 0.071 mmol, 55%.

$[\alpha]_D$= +2.7° (c 0.55 H_2O); Rf. 0.57 D; IR (KBr) ν_{max} 3300, 1730, 1610, 1580, 1410, 1120, 1040 cm^{-1}; FAB MS for $C_{16}H_{25}C_{10}N$ 391.38: (M-1) 390; For ^{1}H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Methyl (allyl 5-acetamido-3,5-dideoxy-β-L-*arabino*-2-heptulopyranosid)onate, **24a**

Sodium borohydride (5 g) was dissolved in water (20 mL) and mixed with Amberlite IRA 400 anion exchange resin (Cl- form)(10 g). H_2 gas was released. After one hour, the resin was decanted and fresh borohydride in water was added. After ten minutes the beads were filtered, rinsed with water and allowed to dry at room temperatue.

Similarly, a fresh portion of Amberlite IRA 400 anion exchange resin (5 g, Cl^- form) was treated with 3 x 30 mL portions of 0.5 N sodium metaperiodate over several hours. The beads were finally rinsed with water and allowed to dry at room temperature.

Compound **7a** (20 mg, 0.055 mmol) was dissolved in dry methanol (2 mL). Then each of the above resins (0.5 g IO_4^- form, 0.5 g BH_4^- form) were rinsed with methanol and added to the solution and stirred at room temperature. The reaction was monitored by tlc and showed a number of intermediates. After 3 hours the reaction was deemed complete and the solution was filtered and vacuum evaporated to give **24a** as an oil. Yield: 9.8 mg, 0.032 mmole, 59%.

$[\alpha]_D$= +2.0° (c 0.5 H_2O); mp 67.5-72.4°C; Rf. 0.72 B; IR (KBr) ν_{max} 3390, 1650, 1725, 1560, 1440, 1040 cm^{-1}; FAB MS for $C_{13}H_{21}O_7N$ 303.31: (M+1) 304; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Allyl 5-acetamido-3,5-dideoxy-β-L-*arabino*-2-heptulopyranosidonic acid, **24b**

Compound **24a** (10 mg, 0.034 mmol) was dissolved in aqueous sodium hydroxide (0.1 N, 5 mL) and allowed to react for 30 minutes. The solution was neutralized with Rexyn 101 cation exchange resin (H^+ form), then adjusted to pH 6 with aqueous sodium hydroxide. Yield: 8.0 mg, 0.028 mmol, 81%.

$[\alpha]_D$= -21.3° (c 0.5 H_2O); Rf 0.31 D; FAB MS for $C_{12}H_{19}O_7N$ 289.29: (M-1) 288; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Methyl (4-nitrophenyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate, **25a**

To a solution of **5** (200 mg, 0.40 mmole), *p*-nitrophenol (2 eq., 131 mg, 0.94 mmole), $Ph_3EtP^+Br^-$ (1 eq., 175 mg, 0.47 mmole) in CH_2Cl_2 (1 mL) was added aqueous NaOH (1.0 M, 1.0 mL). The reaction mixture was stirred vigorously at room temperature. The starting material had disappeared after 10 minutes.

The organic phase was diluted with chloroform and extracted with saturated bicarbonate, and then water. After drying over sodium sulfate the organic phase was vacuum evaporated to give a yellow foam. The product **25a** was crystallized from ethanol. Yield: 63.8 mg, 0.104 mmol, 26%. Optimized yield 40%.

Rf.=0.3 EtOAc; mp 98.4-115°C (Lit. 104-108°C Eschenfelder 1987).

Methyl (4-nitrophenyl-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate, **25b**

Compound **25a** (259 mg, 0.42 mmole) was dissolved in methanol (10 mL), and several drops of sodium methoxide were added to make the pH≈10. After 2 hours the solution was neutralized with Rexyn 101 cation exchange resin (H^+ form) and vacuum evaporated. Compound **25b** was crystallized from methanol-ether. Yield: 156 mg, 0.35 mmole, 84%.

$[\alpha]_D$= +44.9° (c 1.0 MeOH)(Lit.+48.5° Eschenfelder 1987); mp 159.6-161.9°C (Lit. 162-164°C); Rf.= 0.52 H

4-Nitrophenyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-*D-galacto*- 2-nonulopyranosidonic acid, **25c**

Compound **25b** (56 mg, 0.13 mmol) was dissolved in aqueous sodium hydroxide (0.1 M, 10 mL) and stirred for 30 minutes at room temperature. The solution was neutralized with Dowex 50W-X8 (H^+ form) to pH 4, filtered and lyophilized to yield **25c** as a slightly yellow powder. Yield: 40 mg, 0.09 mmol, 71%.

$[\alpha]_D$= +63.3° (c 1.0 H_2O)(Lit. +69.° Eschenfelder 1987); mp 121.7-124.3°C (Lit. 113-115°C); FAB MS for $C_{17}H_{22}O_{11}N_2$ 430.36: (M-1) 429 (weak); For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-2,6-anhydro-3,5-dideoxy-D-*glycero* -D-*galacto*-non-2-enonate, **26**

Compound **5** (29 mg, 0.054 mmol) was dissolved in toluene (20 mL) and refluxed for 1 hour. Compound **26** was purified by chromatography on silica gel with EtOAc. Yield 20 mg, 0.042 mmol, 78%.

$[\alpha]_D$= +67.2° (c 0.7 $CHCl_3$ lit. +79.0 Okamoto, 1987); Rf.= 0.32 EtOAc; ^1H n.m.r.(200MHz) ($CDCl_3$) δ: 6.02 (d, 1H, $H_{2,3}$=3.2 Hz, H3), 5.70 (d, 1H, $J_{NH,5}$=10Hz, NH), 5.50-5.54 (m, 2H, H7 and H4), 5.37 (m, 1H, H8), 4.57 (dd, 1H, $J_{8,9'}$=3.2, $J_{9,9'}$=12.3, H9'), 4.38-4.44 (m, 2H, H5 and H6), 4.17 (dd, 1H, $J_{8,9}$ = 7.0, $J_{9,9'}$=12.3, H9) 3.81 (s, 3H, COOMe), 2.14, 2.07, 2.06, 2.05, 1.94 (5xs, 5x3H, NAc,OAc) ^{13}C n.m.r. ($CDCl_3$) δ: 170.9, 170.8, 170.3, 170.2 (N, O-Ac), 161.8 (CO_2Me), 145.1 (C-2), 107.9 (C-3), 76.6 (C-6), 70.5 (C-8), 67.7 (C-4), 67.0 (C-7), 61.9 (C-9), 52.5 ($CO_2\underline{Me}$) 46.5 (C-5), 23.0 (N-Ac), 29.6 20.6, 20.5,(O-Ac).

Methyl 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*erythro*-L-*manno*-nononate **27a**

Compound **1b** (0.16 g, 0.50 mmol) was treated as described to give compound **5**. Pd on charcoal (100 mg, 10% w/w, Aldrich) was added to toluene (5 mL) with pyridine (1 mL) and H_2 was bubbled through the suspension. Compound **5** was added and stirred overnight with H_2 atmosphere. The suspension was filtered through celite and rinsed with CH_2Cl_2 and the combined solution and washings were vacuum evaporated to dryness. The residue was dissolved in CH_2Cl_2 , extracted twice with 0.1 N HCl, once with saturated sodium bicarbonate, once with water, dried over sodium sulfate and vacuum evaporated to a light yellow oil. Crude yield: 0.117 g, 0.25 mmole, 50%, $\alpha:\beta$ = 6.5:1 based on ^1H n.m.r. Half of this material was treated with sodium methoxide in methanol to give a mixture of the de-O-acetylated material. After neutralization, the pure α isomer **27a** was obtained from a silica gel column eluted with an 8:2 mixture of choroform methanol. Yield: 17 mg, 0.054 mmole, 55%.

$[\alpha]_D$= -24° (c 0.6 H_2O); Rf. 0.49 B; IR (KBr) ν_{max} 3400, 1730, 1640, 1560, 1440, 1040 cm^{-1}; FAB MS for $C_{12}H_{21}O_8N$ 307.11: (M-1) 306; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

5-Acetamido-2,6-anhydro-3,5-dideoxy-D-*erythro*-L-*manno*-nononic acid **27b**

Compound **27a** (6.2 mg, 0.020 mmole) was dissolved in aqueous NaOH (0.1 N, 10 mL) for 30 minutes, then the solution was neutralized with H^+ resin and lyophilized. Yield: 5.5 mg, 0.019 mmole, 94%

Rf. 0.35 E; FAB MS for $C_{11}H_{19}O_8N$ 293.27: (M+1) 294; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Methyl (propyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate, **28a**

Compound **7a** (26 mg, 0.072 mmol) was dissolved in methanol (10 mL) and 5% Pd on charcoal (25 mg) was added. Hydrogen gas was bubbled through the solution for one hour. The reaction was centrifuged to remove the catalyst, then vacuum evaporated. Crystals formed upon standing. Yield: 25 mg, 0.068 mmol, 95%.

$[\alpha]_D$= -8.9° (c 0.7 H_2O); mp 145.5°C; Rf. 0.52 D; IR (KBr) ν_{max} 3390, 1650, 1725, 1560, 1440, 1040 cm^{-1}; Analysis Calculated for $C_{15}H_{27}O_9N.H_2O$: C 46.99, H 7.10, N 3.65. Found: C 46.56, H 6.93, N 4.78; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

Propyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-*D*-*galacto*-2-nonulopyranosidonic acid **28b**

Compound **28a** (10.0mg, 0.028 mmole) was dissolved in aqueous sodium hydroxide (0.1 N, 3 mL) for 30 minutes, then neutralized with H^+ resin, filtered, neutralized to pH 6 with aqueous sodium hydroxide and lyophilized to a white powder. Yield: 9.1 mg, 0.026 mmole, 92%

$[\alpha]_D$= -4.1 (c 0.37 H_2O); FAB MS for $C_{14}H_{25}O_9N$ 351.35: (M+1) 352; For ^1H and ^{13}C n.m.r, see tables 3-1 and 3-2.

N-Acryloyl-1,6-diaminohexane **29**

1,6-Hexanediamine (2.17g, 0.02 mol) was dissolved in water (50 mL) and cooled to 4°C with an ice bath. Acryloyl chloride (1.6 mL, 0.02 mol) was added all at once and the solution was stirred for 24 hours. The solution was lyophilized yielding 4.09g of white powder.

The crude mixture (100 mg) was dissolved in water and loaded on a silica gel column (20g) and eluted with acetone:water:acetic acid (9:1:1). The fractions containing **29** (hydrochloride) (Rf 0.36) as detected by ninhydrin reagent and permanganate spray were vacuum evaporated to dryness at 25°C, then dissolved in water and lyophilized. A solution was treated with anion exchange resin (hydroxide form), then acidified with *p*-toluenesulfonic acid. It was lyophilized, then crystalized from methanol. Yield: 49.2 mg, 49%.

mp 149-155°C (lit. 142-143°C Kojima 1966); Rf. 0.36 J; MS for $C_9H_{18}ON_2$ 170.25: (M+1) 171.; ^1H n.m.r. (D_2O) δ: 6.263 (dd, 1H, $J_{c,b}$=17.1, $J_{c,a}$=9.7, H_c), 6.160 (dd, 1H, $J_{b,a}$=1.8, $J_{b,c}$=17.2, H_b), 5.745 (dd, 1H, $J_{a,b}$=1.9, $J_{a,c}$=9.7, H_a), 3.27 (t, 2H, $J_{\phi,\epsilon}$=6.8, H_ϕ), 2.98 (t, 2H, $J_{\alpha,\beta}$=7.8, H_α) 1.657-1.358 (m, 6H, H_β to H_δ).

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-3)β-D-galactopyranosyl(1-4)1-[(6-acrylamido-1-hexyl)amino]-1-deoxy-D-glucitol, **30**

Compound **2a** (45 mg, 0.077 mmol) was dissolved with **29** (48.9 mg, 0.42 mmole) in borate buffer (0.4 M, pH 8.0, 5 mL). Then sodium cyanoborohydride (10 mg, 0.17 mmol) was added and the reaction was stirred for 2 days at room temperature. The pH was adjusted to 4 with acetic acid and the solution was vacuum evaporated at 30°C to dryness. The residue was co-evaporated with methanol five times to remove borate esters. The residue was then dissolved in water, and acetone was added to precipitate the product **30** as an oil. The material was purified on a silica gel column eluted with solvent system G. Yield: 26.6 mg, 0.046 mmol, 60%.

$[\alpha]_D$= -3.8° (c 0.4 H_2O); Rf 0.4 I; FAB MS for $C_{32}H_{58}O_{19}N_3$ 788.83: (M-1) 788; ^{13}C n.m.r data appear in table 3-3, ^1H n.m.r. (D_2O) δ: 6.263 (dd, 2H, $J_{c,b}$=17.1, $J_{c,a}$=9.7, H_c) 6.160

(dd, 2H, $J_{b,a}$=1.8, $J_{b,c}$=17.2, H_b), 3.745 (dd, 2H, $J_{a,b}$=1.9, $J_{a,c}$=9.7, H_a), 4.599 (d, 1H, $J_{1,2}$=8.0, Gal-H1), 4.115 (dd, 1H, $J_{3,2}$=9.6, $J_{3,4}$=3.2, Gal-H3), 3.269 (t, 2H, $J_{\phi,\epsilon}$=6.8, H_ϕ), 3.349 (t, 1H, $J_{2,3}$=13.1 $J_{2,1}$=2.3, Glc-H2(?)), 3.080 (t, 2H, $J_{\alpha,\beta}$=7.7, H_α), 2.761 (dd, 1H, $J_{3a,3e}$=11.9, $J_{3e,4}$=4.3, NeuAc-H3eq), 2.030 (s, 3H, NAc), 1.802 (t, 1H, $J_{3a,3e}$=$J_{3a,4}$=12.6, NeuAc-H3ax), 1.657-1.358 (m, 6H, H_β to H_δ).

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-3)β-D-galactopyranosyl(1-4)1-allylamino-1-deoxy-D-glucitol, **31**

Compound **2a** (64.6 mg, 0.102 mmole) was dissolved in methanol (20 mL, and allylamine (0.08 g) was added. The pH was adjusted from 9.5 to 8.0 with acetic acid. Then sodium cyanoborohydride (0.056 g, 1.1 mmole) was added and the solution was refluxed for one hour, then vacuum evaporated to dryness, and the yellow residue was dissolved in water, and lyophilized. The resulting oil was dissolved in methanol, and **31** was precipitated as an oil using acetone. Yield: 50 mg, 0.0741 mmole, 73%.

$[\alpha]_D$= -1.5° (c 0.4 H_2O); Rf. 0.25 I; FAB MS for $C_{26}H_{46}O_{18}N_2$ 674.65: (M-1) 673; ^{13}C n.m.r data appear in table 3-3. ^1H n.m.r. (D_2O) δ: 5.927 (m, 1H, H_c), 5.541 (dd, 1H, $J_{a,c}$9.7, $J_{a,b}$1.3, H_a), 5.493 (dd, 1H, $J_{b,a}$=1.3, $J_{b,c}$10.0, H_b), 4.597 (d, 1H, $J_{1,2}$=7.7, Gal-H1), 4.112 (dd, 1H, $J_{3,2}$=9.7, $J_{3,4}$=3.2, Gal-H3), 3.339 (t, 1H, $J_{1,2}$=13.3, Glc-H2(?)) 2.763 (dd, 1H, $J_{3a,3e}$=12.5, $J_{3e,4}$=4.5, NeuAc-H3eq), 2.033 (s, 3H, NAc), 1.801 (t, 1H, $J_{3a,3e}$=$J_{3a,4}$=12.3, NeuAc-H3ax).

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-3)β-D-galactopyranosyl(1-4)N-acryloyl-β-D-glucopyranosylamine, **32**

Compound **2b** (34.1 mg, 0.054 mmole) was dissolved in water (5 mL) saturated with ammonium bicarbonate and stirred at 37°C for 7 days. (Solid ammonium bicarbonate was added to keep the solution saturated.) The solution was then lyophilized three times to remove excess ammonium bicarbonate. The white powder was dissolved in water (1 mL) with sodium carbonate (0.2 g), and then methanol (1 mL) was added and the resulting slurry was cooled to 0°C and acryloyl chloride in dioxane (14% v/v, 0.5 mL) was added over a five minute period. The solution was stirred for one hour, and an additional 70 μL acryloyl chloride was added. After five hours, a grain of BHT was added, and the solution was vacuum evaporated to dryness, dissolved in water (1 mL) and desalted on a 3 cm X 80 cm column of Sephadex G10 eluted with water. The first fraction to elute was lyophilized to give the white powder **32**. Yield: 19.4 mg, 0.028 mmole, 52%.

$[\alpha]_D$= -3.1 (c 1.0 H_2O); Rf. 0.31 E; HPLC Rt.= 5.5 min. (0.5 cm . X 25 cm RP C18

column, 1.5 mL/min. 10 mM triethylamine/acetic acid pH 4.8); IR (KBr) ν_{max} 3420, 1630, 1560, 1380, 1030 cm^{-1}; FAB MS for $C_{26}H_{42}O_{19}N_2$ 686.62: (M-1) 685; ^{13}C n.m.r data appear in table 3-3, ^1H n.m.r. (D_2O) δ: 6.326 (ABX, 1H, H_c), 6.307 (ABX, 1H, H_a), 5.872 (ABX, 1H, H_b) 5.096 (d, 1H, $J_{1,2}$=9.2 Hz, βGlc-H1), 4.545 (d, 1H, $J_{1,2}$=7.9, Gal-H1), 4.116 (dd, 1H, $J_{3,2}$=9.8, $J_{3,4}$=3.1, Gal-H3), 3.489 (t, 1H, $J_{2,3}$=$J_{2,1}$=9.0, βGlc-H2), 2.761 (dd, 1H, $J_{3a,3e}$=12.4, $J_{3e,4}$=4.6, NeuAc-H3eq), 2.030 (s, 3H, NAc), 1.800 (t, 1H, $J_{3a,3e}$=$J_{3a,4}$=12.0, NeuAc-H3ax).

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-8)5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-3)β-D-galactopyranosyl(1-4)N-Acryloyl-β-D-glucopyranosylamine 33

Compound 4 (69 mg) was dissolved in water (5 mL) saturated with ammonium bicarbonate and stirred at 37°C for 3 days. (Solid ammonium bicarbonate was added to keep the solution saturated.) The solution was then lyophilized three times to remove excess ammonium bicarbonate. (Yield 50.1 mg) The white powder was dissolved in water (1 mL) with sodium carbonate (0.2 g), and then methanol (1 mL) was added and the resulting slurry was cooled to 0°C and acryloyl chloride in dioxane (14% v/v, 0.5 mL) was added over a five minute period. The solution was stirred for one hour, and an additional 70 μL acryloyl chloride was added. After five hours, a grain of BHT was added, and the solution was vacuum evaporated to dryness, dissolved in water (1 mL) and desalted on a 3 cm X 80 cm column of Sephadex G10 eluted with water. The first fraction to elute was lyophilized to give a white powder. Crude yield: 32.0 mg. Integration of the h.p.l.c. chromatogram of this material indicated that 66% was the desired product 33.

Rf. 0.42 F; HPLC Rt.= 7.2 min. (0.5 cm X 25 cm RP C18 column, 1.5 mL/min. 10 mM triethylamine/acetic acid pH 4.8); FAB MS for $C_{37}H_{59}O_{27}N_3$ 977.88: (M-1) 977; ^{13}C n.m.r data appears in table 3-3, ^1H n.m.r. (D_2O) δ: 6.329 (ABX, 1H, H_c), 6.310 (ABX, 1H, H_a), 5.873 (ABX, 1H, H_b) 5.092 (d, 1H, $J_{1,2}$=9.3 Hz, βGlc-H1), 4.549 (d, 1H, $J_{1,2}$=8.0, Gal-H1), 4.105 (dd, 1H, $J_{3,2}$=9.5, $J_{3,4}$=3.1, Gal-H3), 3.491 (t, 1H, $J_{2,3}$=$J_{2,1}$=8.9, βGlc-H2), 2.790 (dd, 1H, $J_{3a,3e}$=12.4, $J_{3e,4}$=4.6, NeuAc"-H3eq), 2.678 (dd, 1H, $J_{3a,3e}$=12.4, $J_{3e,4}$=4.6, NeuAc'-H3eq), 2.030 (s, 3H, NeuAc"-NAc), 2.067 (s, 3H,NeuAc'-NAc), 1.763 (t, 1H, $J_{3a,3e}$=$J_{3a,4}$=12.0, NeuAc"-H3ax), 1.744 (t, 1H, $J_{3a,3e}$=$J_{3a,4}$=12.0, NeuAc'-H3ax).

Table 3-1. ^{1}H NMR chemical shifts (ppm) and coupling constants (Hz) of the NeuAc derivatives in D_2O at 25°C

Comp. No.	$H3_{ax}$ (J_{a-e},J_{3-4})	$H3_{eq}$ (J_{e-a},J_{3-4})	H5 (J_{4-5},J_{5-6})	H6 (J_{5-6},J_{6-7})	H7 (J_{6-7},J_{7-8})	H9 (J_{8-9},$J_{9-9'}$)	H9' (J_{8-9},$J_{9-9'}$)	Methyl ester (s)	NAc (s)	Aglycon: Allyl, Others				
										H_a	H_b	H_c	H_d	H_e
1a	1.819 (12.8,11.2)	2.214 (12.8,4.8)	3.907 (10.0,9.5)	3.984 (9.0,1.0)	3.505 (1.0, 9.0)	3.793 (2.5,11.7)	3.605 (6.2,11.7)	-	2.049	-	-	-	-	-
1b	1.913 (12.0,11.6)	2.315 (12.0,4.6)	3.916 (9.8,10.2)	4.067 (10.2,1.0)	3.552 (1.0, 8.9)	3.834 (2.4,9.1.9)	3.619 (5.1,11.2)	3.838	2.049	-	-	-	-	-
7a	1.826 (12.1,12,2)	2.724 (12.2,4.6)	a	a	3.559 (- , 8.9)	a	3.654 (6.0,11.9)	3.866	2.034	5.253	5.324	5.913	4.313	4.071
7b	1.663 (12.1,11.7)	2.753 (12.4,4.5)	3.820 (9.8,10.2)	3.708 (10.2,1.9)	3.586 (1.7, 8.9)	3.869 (- , 9.6)	3.640 (6.3,12.2)	-	2.033	5.225	5.324	5.939	4.243	4.007
8a	1.800 (12.3,11.7)	2.681 (12.3,4.5)	a	a	3.557 (1.6, 8.9)	a	3.650 (6.3,12.2)	3.879	2.033	-	-	-	-	3.384
8b	1.626 (12.3,11.7)	2.718 (12.3,4.6)	3.803 (10.1,10.1)	3.689 (10.1,1.9)	3.586 (1.8, 9.0)	3.869 (2.6, 9.7)	3.641 (6.8,12.5)	-	2.033	-	-	-	-	3.341
9a	1.789 (12.2,11.9)	2.392 (12.3,4.5)	3.924 (9.7,10.3)	a	3.582 (0.8, 8.7)	a	3.664 (5.7, -)	3.869	2.050	-	-	-	-	3.277
9b	1.646 (13.1,11.3)	2.339 (13.1,4.7)	3.888 (10.0,9.9)	3.788 (10.3,1.0)	3.531 (0.8,9.3)	3.853 (2.7,9.9)	3.661 (6.0,12.1)	-	2.047	-	-	-	-	3.201
10a	1.831 (12.6,11.9)	2.730 (12.8,4.5)	a	a	3.553 (- ,8.9)	a	3.648 (6.7,12.5)	3.876	2.033	-	-	5.119[b] (4.8)	3.760 (10.1,4.7)	3.468 (10.2,4.9)
10b	1.704 (12.6,11.9)	2.736 (12.8,4.5)	3.832 (9.8,10.3)	3.697 (9.5,1.7)	3.578 (1.6, 8.8)	3.872 (- ,9.8)	3.634 (6.6,12.5)	-	2.033	-	-	5.118[b] (4.9)	3.681 (11.4, -)	3.425 (10.2,4.8
11	1.730 (12.3,11.9)	2.719 (12.3,4.6)	a	3.733 (10.5,1.9)	3.571 (1.6,8.9)	3.829 (1.8,9.7)	3.634 (6.6,12.5)	-	2.030	-	-	a	a	a
12	1.726 (12.2,11.9)	2.723 (12.3,4.5)	3.830 (9.7,10.3)	3.697 (9.7,2.0)	3.585 (1.8,8.7)	3.861 (2.7,9.8)	a	-	2.030	-	-	3.185 (4.8,5.4)	a	3.967 (10.6,4.9)

Table 3-1. (Cont'd)

Comp. No.	$H3_{ax}$ (J_{a-e},J_{3-4})	$H3_{eq}$ (J_{e-a},J_{3-4})	H5 (J_{4-5},J_{5-6})	H6 (J_{5-6},J_{6-7})	H7 (J_{6-7},J_{7-8})	H9 (J_{8-9},$J_{9-9'}$)	H9' (J_{8-9},$J_{9-9'}$)	Methyl ester (s)	NAc (s)	Aglycon: Allyl, Others H_a	H_b	H_c	H_d	H_e
14	a	2.715 (12.3,4.6)	a	3.701 (10.2,1.7)	3.404 (1.5, 8.8)	a	3.651 (5.6,12.1)	-	2.030			c		
15a	1.811 (12.0,11.6)	2.705 (12.0,4.6)	a	a	3.566 (2.1,10.4)	a	3.646 (5.1,11.2)	3.875	2.030			c		
15b	1.651 (12.1,12,2)	2.748 (12.2,4.9)	3.811 (10.3,9.8)	3.685 (9.8,1.6)	3.591 (2.9,9.3)	3.869 (2.1,11.6)	a	-	2.030			c		
16a	1.816 (12.1,11.7)	a	a	a	3.557 (1.7, 8.9)	a	a	3.870	2.030			c		
16b	1.645 (12.0,12.0)	2.748 (12.0,4.9)	3.806 (9.7, 9.6)	3.675 (9.5,1.6)	3.584 (1.6, 9.5)	3.862 (2.0,12.0)	a	-	2.030			c		
17	1.763 (11.2,12.8)	2.748 (12.9,3.8)	a	a	3.636 (- , 9.7)	a	a	-	2.023	5.232	5.326	5.937	4.262	4.087
18	1.743 (11.0,11.2)	2.299 (13.0,4.7)	3.846 (9.9,10.5)	3.681 (9.9,10.5)	3.518 (1.2,9.37)	3.906	3.613 (6.0,11.5)		2.033	5.209	5.339	5.956	a	4.232
19	1.699 (12.4,12.5)	2.797 (12.5,4.6)	3.241 (9.7,10.3)	4.045 (10.3,2.1)	3.773 (2.2,11.0)	3.900 (2.5,12.0)	3.721 (5.7,12.1)	-	-	5.227	5.325	5.931	4.242	a
20a	1.679	2.752	a	a	3.556	a	3.640	-	4.647 (2.187)	5.225	5.326	5.941	4.252	4.013
20b	1.681 (11.8,13.1)	2.767 (12.4,4.6)	a	a	3.583 (1.7, 8.9)	3.87 (1.1,9.8)	3.639 (6.5,12.3)	-	4.121	5.224	5.328	5.941	4.251	4.014
21a	1.833 (12.1,11.9)	2.729 (12.6,4.0)	a	a	3.579 (- ,8.6)	a	3.645 (6.9,12.6)	3.869	3.930	a	a	5.915	4.345	4.074
21b	1.677 (12.2,12.5)	2.755 (12.5,4.5)	a	a	3.611 (- ,9.0)	3.660 (2.3,7.0)	3.702 (6.0,11.9)	-	3.927	5.228	5.329	5.940	4.327	4.016

Table 3-1. (Cont'd)

Comp. No.	$H3_{ax}$ (J_{a-e},J_{3-4})	$H3_{eq}$ (J_{e-a},J_{3-4})	H5 (J_{4-5},J_{5-6})	H6 (J_{5-6},J_{6-7})	H7 (J_{6-7},J_{7-8})	H9 (J_{8-9},$J_{9-9'}$)	H9' (J_{8-9},$J_{9-9'}$)	Methyl ester (s)	NAc (s)	Aglycon: Allyl, Others H_a	H_b	H_c	H_d	H_e
23	1.645 (12.3,11.6)	2.745 (12.5,4.6)	3.825 (10.1,9.8)	3.724 (9.5,1.9)	3.632 (1.9, 9.1)	4.405 (2.4,11.7)	a	-	2.033 (2.125 OAc)	5.214	5.316	5.931	4.227	4.075
24a	1.842 (11.4,12.5)	2.711 (13.5,3.5)	a	a	a	-	-	3.866	2.028	a	a	5.917	4.333	4.075
24b	1.626 (11.7,12.4)	2.693 (12.4,4.0)	3.688 (9.3,9.6)	a	3.616 (5.0,12.9)	3.760 (1.9,12.9)	-	-	2.025	5.229	5.336	5.940	4.276	3.969
25c	2.022 (13.1,10.6)	2.825 (12.8,4.5)	3.940 (10.4,9.8)	3.615 (- ,1.5)	3.570 (1.5, 10.2)	4.140 (1.6,10.4)	a	-	2.030			8.200[d] (9.4)	7.265[d] (9.4)	
27a	a	2.530 (12.0,4.9)	a	a	3.530 (1.6, 9.5)	a	a	3.800	2.030			4.750[e] (6.5,1.4)		
27b	1.859 (12.8,11.2,6.6)	2.550 (12.9,4.3,1.5)	3.791 (9.8, 9.8)	3.637 (10.3,1.8)	3.541 (1.6,9.0))	3.860 (2.5,11.0)	3.682 (4.0,11.4)	-	2.023			4.423[e] (6.5,1.4)		
28a	1.799 (11.0,11.2)	2.705 (13.0,4.7)	3.759 (9.9,10.5)	a (9.9,10.5)	3.553 (1.2,9.37)	a	3.644 (6.0,11.5)	3.864	2.030	0.869 (7.4)		1.553 (7.0)	3.434 (6.9,9.3)	3.403 (6.8,9.3)
28b	1.639 (11.7,12.3)	2.739 (12.3,4.5)	3.816 (9.7,9.1)	a	3.684	a	a	-	2.030	0.877 (7.4)		1.555 (7.0)	3.371 (6.9,9.3)	a

a) H4, H8 and others not assigned due to complexity of the spectrum.
b) Aldehyde protons as a hydrate. c) See experimental section. d) Aromatic protons
e) H2.

Table 3-2 ^{13}C NMR Chemical Shift (ppm) of Sialic Acid Derivatives (D_2O, 25°C)

No.	C-1	NHAc	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	CH_3	Me	Aglycon
1a	175.7	174.0	96.1	39.7	67.5	52.9	71.3	69.0	70.9	64.0	22.9		
1b	173.0	176.4	96.7	39.9	68.0	53.0	71.7	69.5	71.5	64.5	23.3	54.8	
7a	171.2	176.1	100.0	40.6	68.3	53.0	74.1	69.5	71.8	64.3	23.3	54.6	67.0, 119.7, 134.3
7b	174.6	176.3	101.8	41.7	69.4	53.1	73.9	69.4	72.9	63.8	23.2		67.1, 119.4, 135.0
8a	171.1	176.1	100.3	40.1	68.2	52.9	74.0	69.0	71.7	64.3	23.3	54.5	52.9
8b	174.6	176.3	101.9	41.3	69.5	53.2	73.8	69.5	72.9	63.9	23.3		52.8
9a	171.7	176.1	100.3	40.2	67.5	52.0	71.6	69.1	70.9	64.4	23.0	54.2	52.7
9b	176.0	175.4	101.2	40.7	67.9	53.0	71.0	69.3	70.9	64.4	23.1		53.0
10a	171.2	176.1	100.5	40.8	69.9	53.4	74.5	68.8	72.2	64.8	23.8	55.1	68.4, 89.7
10b	174.6	176.3	101.7	41.3	69.4	53.1	73.8	69.4	72.9	63.9	23.3		68.2, 89.6

Table 3-2 Cont'd

No.	C-1	NHAc	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	CH_3	Me	Aglycon
11	173.2	175.7	100.6	40.6	69.1	52.7	73.5	68.7	72.1	63.7	23.0	-	66.4, 61.4
12	174.1	175.9	101.3	40.7	69.0	52.8	73.6	69.0	72.5	63.6	23.0	-	40.3, 61.1
13	a	a	97.5	a	69.6	53.4	74.2	69.0	71.5	64.7	23.3	-	56.1, a
14	174.1	175.8	101.3	40.5	69.0	52.8	73.6	69.0	72.5	63.5	23.0	-	b
15a	171.0	175.8	100.0	40.2	69.2	52.7	73.9	68.0	74.7	64.0	23.1	54.3	b
15b	174.6	176.2	101.7	41.4	69.3	53.0	73.7	69.3	72.9	63.7	23.1	-	b
16a	170.8	175.6	99.9	40.1	69.1	52.6	73.7	68.0	71.5	63.9	23.0	54.2	b
16b	173.9	175.9	101.3	41.3	69.1	52.9	73.7	69.3	72.7	63.8	23.2	-	b
17	172.3	176.3	101.1	40.1	68.8	52.8	74.6	68.3	72.2	63.8	23.3	-	66.6, 119.7, 134.7
18	61.3	176.4	101.7	37.4	69.0	53.4	71.7	69.5	72.8	64.1	23.2	-	64.2, 119.0, 135.7

Table 3-2 Cont'd

No.	C-1	NHAc	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	CH_3	Me	Aglycon
19	173.5	-	101.3	41.3	67.4	53.9	72.3	68.6	71.9	63.0	-	-	66.9, 119.0, 134.3
20a	a	a	a	40.9	68.7	52.2	72.9	68.4	72.3	63.4	63.2 (20.4)[c]	-	66.5, 119.0, 134.3
20b	a	a	101.7	41.5	69.2	52.6	73.4	69.0	72.7	63.6	61.9	-	67.1, 119.4, 135.0
21a	171.3	172.1	100.0	40.4	68.0	53.1	73.7	68.0	71.7	64.2	29.0	54.4	66.9, 119.8, 134.3
21b	170.8	177.2	100.7	40.7	69.5	53.4	73.7	68.6	72.4	63.9	29.0	-	67.0, 119.8, 134.6
22	a	(179.7)[d]	102.1	38.4	69.1	51.9	78.0	73.1	73.4	63.6	(78.0)[d]	-	67.3, 119.0, 135.0
23	174.9	176.3 (175.8)[c]	101.8	41.6	69.3	53.0	73.6	69.4	70.4	66.8	23.1 (21.3)[c]	-	67.2, 119.5, 135.0
24a	171.3	176.3	100.1	40.1	68.4	54.4	76.6	62.2	-	-	23.1	53.0	66.8, 120.0, 134.3
24b	174.6	176.0	101.9	41.4	68.6	53.0	75.6	62.0	-	-	22.8	-	67.7, 119.4, 134.6
25c	a	176.9	103.9	42.2	69.1	53.1	75.2	69.7	72.8	64.2	23.3	-	127.0, 121.5

Table 3-2 Cont'd

No.	C-1	NHAc	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	CH_3	Me	Aglycon
27a	175.0	176.3	74.7	34.6	68.6	54.0	73.0	69.3	72.1	64.3	23.1	53.5	-
27b	a	a	74.8	35.2	69.3	53.4	74.4	69.1	72.5	63.7	22.9	-	-
28a	a	176.4	100.3	40.4	68.4	54.4	74.1	69.4	71.8	64.2	23.1	52.9	67.9, 23.3, 10.6
28b	175.0	176.3	101.7	41.4	69.3	52.8	73.5	69.1	72.7	63.5	23.3	-	67.7, 22.9, 10.6

a) Not observed. b) See experimental section. c) Acetate d) Methylene and carboxyl

Table 3-3 ^{13}C NMR Data for Sialyllactose Derivatives

Atom	2a	30	31	32	33	
NeuAc						
C1	174.9	174.9	174.9	175.0	174.6	174.4
C2	101.0	101.0	101.0	101.1	101.3	101.5
C3	40.9	41.0	41.0	41.6	41.0	41.9
C4	69.6	69.6	69.6	69.7	69.8	75.3
C5	53.0	53.0	53.0	53.1	53.1	53.6
C6	74.2	74.2	74.2	74.2	74.0	69.2
C7	69.4	69.4	69.4	69.5	69.5	70.5
C8	73.0	73.1	73.1	72.9	72.9	79.4
C9	63.9	63.9	63.9	64.0	63.9	62.8
C=O	176.3	176.1	176.1	176.2	176.1	176.1
Ac	23.3	23.4	23.4	23.5	23.4	23.7
Gal						
C1	103.9	103.7	103.7	103.9		104.0
C2	70.7	70.6	70.6	70.7		70.6
C3	76.4	76.4	76.4	76.5		76.8
C4	68.8	68.9	68.9	68.8		68.8
C5	76.8	76.7	76.7	76.8		76.8
C6	62.3	62.5	62.5	62.4		62.5
αGlc						
C1	93.1	50.7[a]	51.0[a]			
C2	72.5	71.9	71.9			
C3	72.7	72.1	72.1			
C4	79.6	79.5	79.5			
C5	71.4	69.0	69.0			
C6	61.4	63.2	63.2			
βGlc						
C1	97.0			80.6		80.6
C2	75.1			73.1		73.1
C3	75.6			76.3		76.3
C4	79.5			79.4		78.9
C5	76.1			76.6		76.6
C6	61.3			61.2		61.2
Aglycon or other		49.0	50.2	170.6		170.6
		29.3	128.5	130.5		130.6
		26.6	125.1			
		26.8				
		40.5				
		176.3				
		131.2				
		128.1				

a) Aminodeoxyglucitol resonances

Chapter 4

Polyvalent Sialic Acid Macromolecules

4.1 Introduction

The preparation of polyvalent macromolecular forms of NeuAc was of central importance in this thesis, first as immunogens to elicit immune responses and second as antigens to screen for anti-sialic acid antibodies. They may also be useful for the investigation of avidity, or even the inhibition of viral hemagglutinin (Weis 1988).

Although NeuAc is present in nature as a macromolecule, the carbohydrate quantity and linkage type are heterogeneous. The advantage of synthetic NeuAc macromolecules is that these two factors can be controlled. As discussed in section 1-4, it is desirable for the immunogen to have a molecular weight greater than 5000 Da and have at least 10 NeuAc per macromolecule. Two types of macromolecules have been made which satisfy these criteria: Protein conjugates (neoglycoproteins) and polyacrylamide copolymers. First, the synthesis of protein conjugates with NeuAc will be described. Three new conjugates have been introduced, and a new method of conjugation using a Michael-type addition has been studied. This is followed by a description of the synthesis of polyacrylamide copolymers with pendant NeuAc. A new highly reactive monomer has been introduced, and its properties in radical polymerization are presented.

4.2 Neoglycoproteins

4.2.1 Synthesis of NeuAc Protein Conjugates by Reductive Amination with 2-Oxoethyl Glycosides

Direct covalent attachment of the aldehyde **10** onto bovine serum albumin (BSA) and tetanus toxiod (TT) could be achieved using sodium cyanoborohydride because this reagent is selective for the imine functionality while being inert toward carbonyl functions. This procedure was pioneered by Gray (1974) on reducing sugars.

As well as the terminal amino group in the protein, the ε-amines of lysine residues are the only possible sites for the reaction. BSA contains 59 lysine residues and has a molecular

weight of 67,000 Da (Benjamin 1984) and TT has 100 lysine residues and a molecular weight of 150,000 Da (Veronesi 1981). The TT obtained from Institute Armand Frappier had been denatured with formaldehyde, which reduces the number of lysine residues available to ≈60 (Bizzini 1970). The reaction scheme is presented in figure 4-1 to give conjugate 34.

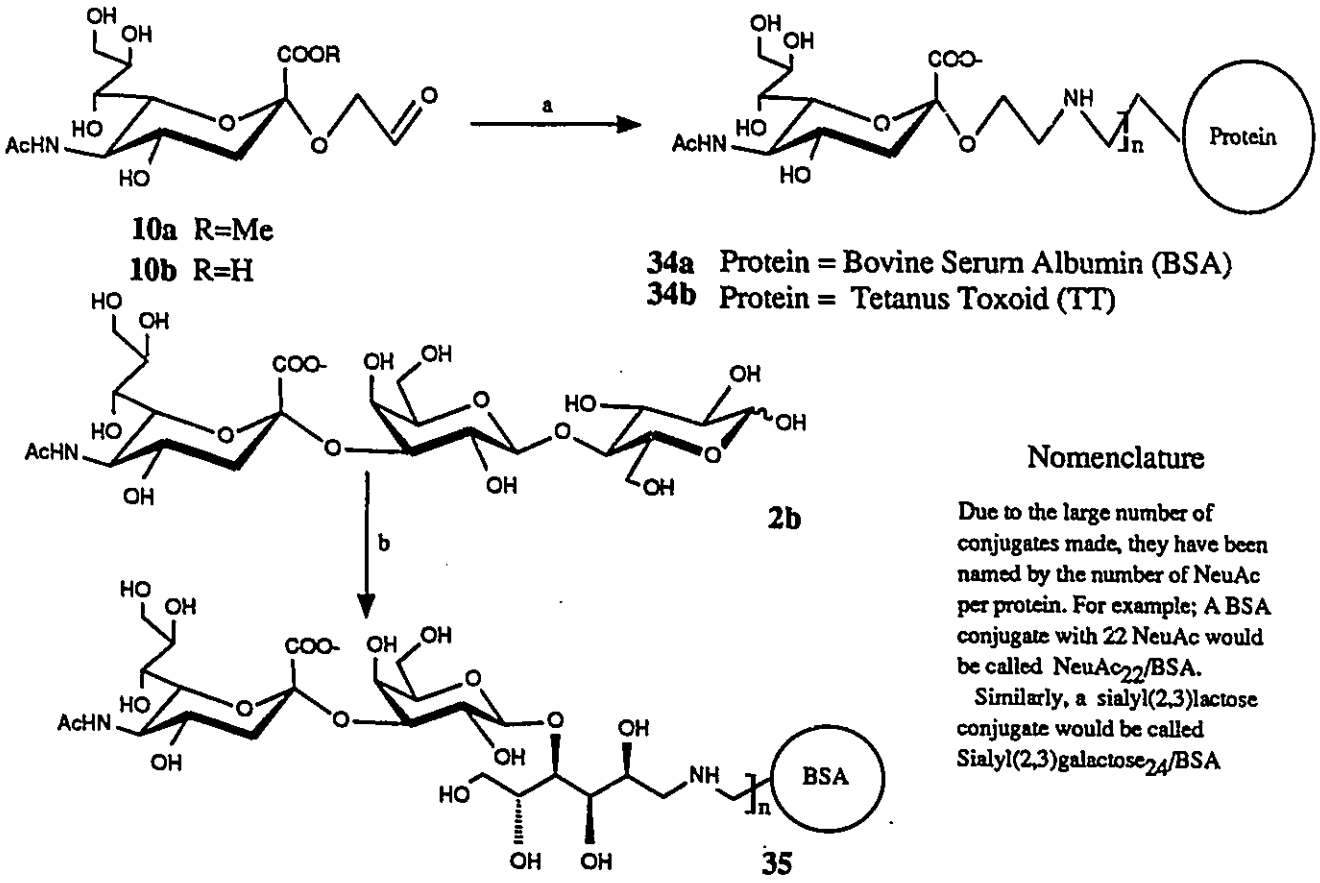


Figure 4-1 Conjugation of NeuAc and Sialyllactose to Protein

a) 0.2M Phosphate buffer pH 7.0, $NaCNBH_3$,37°C b) 0.4M Borate buffer pH 8.2, $NaCNBH_3$,37°C

4.2.1.1 Kinetics and Incorporation

The reaction was found to be quite slow, and this is because both the imine formation and cyanoborohydride reduction are possible as rate limiting steps. Several days were usually required to functionalize more than 15 lysines per protein molecule. Though it was reported that pre-incubation before the addition of cyanoborohydride did not improve yields (Lee 1980), we still felt this technique was desirable. Using borate buffer (0.2 M, pH 9.0) as suggested by Roy et al. (1984) for reducing sugars and BSA as a model protein, the level of incorporation onto the protein was marginal even after 6 days. It is apparent that the free aldehyde in **10** did

not behave like a reducing sugar. Therefore, the phosphate buffer suggested by Gray (1974) was re-investigated. As observed from table 4-1, there was no improvement in coupling efficiency when only the molar ratio of the aldehyde to the amine was increased at 50°C (entry 3 and 4). There was a slight improvement when both the aldehyde and hydride ratios were increased relative to the amine (entry 1 and 2). There was also no temperature effect observed between 37°C and 50 °C in the same buffer (entry 2,3 and 6,7). A detrimental effect could be seen when the level of hydride reagent was too high (entry 4 and 7) at 50°C. A slight improvement was observed with the addition of a cosolvent as seen in figure 4-2. Thus the maximum coupling of NeuAc was to 23 out of 59 lysines on BSA, and to only 16 out of ≈60 lysine residues on TT.

Table 4-1 Factors influencing the Reductive Amination of **10** onto BSA

Entry	Molar ratio[a] $CHO/HN_2/H^-$	Temp. (°C)	Conditions[b]	NeuAc/Protein[c] 3 days (6d)
10b				
1	1:1:10	37	P	15
2	5:1:25	37	P	22
3	5:1:25	50	P	23
4	10:1:25	50	P	21
5	5:1:25	37	P+20% DMSO	20
6	10:1:100	37	P	18(18)
7	10:1:100	50	P	17(22)
8	10:1:100	37	P+20% DMSO	19(21)
9	5:1:25	37	B	6
10a				
10	7:1:33	37	P	32(35)
11	7:1:33	37	P+ 5% MeOH	36(42)
12	7:1:33	37	P+25% DMSO	36(41)
13	8:1:33	37	P pH6	33
14	8:1:33	37	P pH7	36(45)
15	8:1:33	37	P pH8	38(42)
16	8:1:33	37	P pH9	36(38)

[a]Reaction mixtures. H^- refers to $NaCNBH_3$. [b]P= 0.2 M phosphate buffer, pH 7 unless stated otherwise. B= 0.2 M borate buffer, pH 7.
[c] As determined by colorimetric methods, see appendix 1.

The level of NeuAc incorporation was measured by a specific colorimetric test using resorcinol (Svennerholm 1957), and the amount of protein was determined by the Lowry method (see appendix 1).

We postulated that the restricted protein incorporation of **10b** in comparison to neutral sugars studied by others (Lee 1980, Roy 1984) could be due to electrostatic repulsion from the anionic nature of the acid function in **10b**. Therefore, conjugations with the aldehyde ester **10a** were initiated in the hope to minimize these repulsive interactions. As shown in table 4-1, there

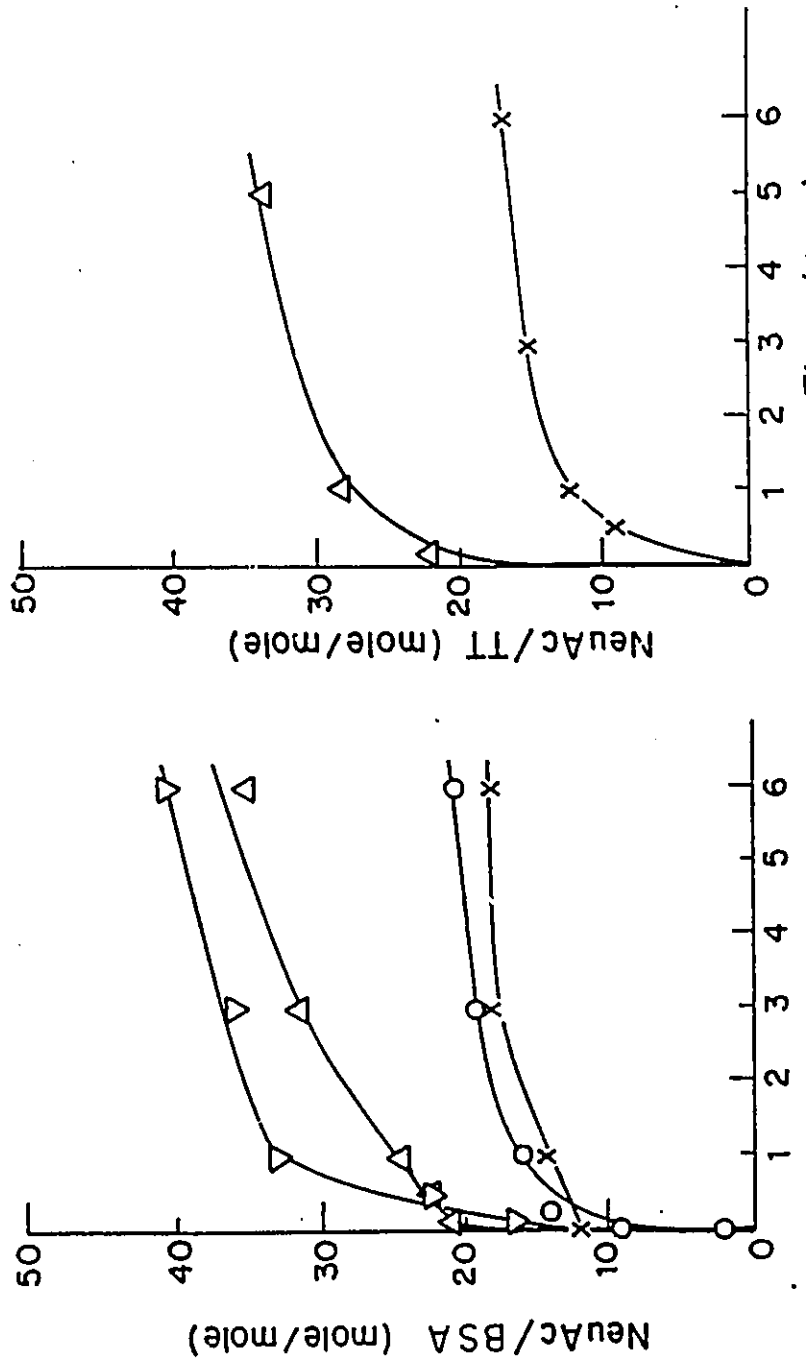


Figure 4-2 Rate of Incorporation of **10** to Bovine Serum Albumin (BSA) or Tetanus Toxoid by Reductive Amination

x ≡ Entry 6 (Table 4-1) ▽ ≡ Entry 10

o ≡ Entry 8 Δ ≡ Entry 12

was a noticeable improvement in the level of reductive amination. This was further increased when methanol or dimethylsulfoxide were used as cosolvents. There was almost no pH efffect in phosphate buffer. For example, at pH 8.0 the initial rate of reaction was greater than at pH 6.0, but after 3 days all the incorporations were essentially the same. This result is consistent with previous findings (Lundblad 1984).

Under the optimum conditions, a conjugate having 53 NeuAc residues per BSA was achieved. The actual rates were studied with time and the results are illustrated in figure 4-2.

4.2.1.2 Ester Hydrolysis During Reductive Amination

When the aldehyde methyl ester **10a** was used for reductive amination, it was assumed that after conjugation the methyl ester would still be present in the NeuAc on the protein. It should have been necessary to remove the methyl ester by base hydrolysis; however, ^{1}H n.m.r., immunoelectrophoresis and a colorimetric test specific for esters (see appendix 1 for the Hestrin test) failed to reveal the presence of methyl ester in the protein conjugates. Further verification was obtained using neuraminidase from *Vibrio cholerae* which was able to remove 96% of the sialic acid on the protein. This enzyme is unreactive with the methyl ester of sialic acid (Brossmer 1971). Furthermore, analysis of reductive amination of **10a** with N^{α}-acetyl-(L)-lysine showed a single product **14** (see section 3.4.2), which was isolated and

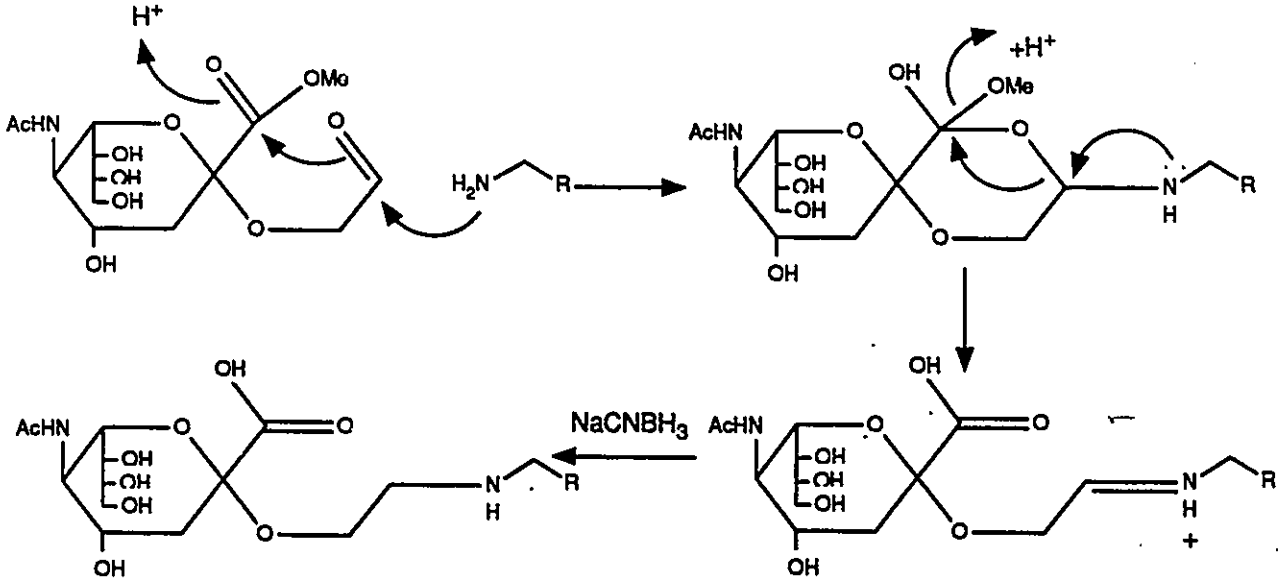


Figure 4-3 Mechanism for Hydrolysis of Methyl Ester During Reductive Amination

identified as the free acid. Hydrolysis of the ester of **7a** was not accomplished by either cyanide or protein acting alone, however it was found that the aldehyde ester **10a** was converted into **10b** in phosphate buffer. Hydrolysis could have been catalyzed by

intramolecular assistance of the neighboring heteratom as shown in figure 4-3.

4.2.2 Synthesis of Sialylgalactose Protein Conjugates using Reductive Amination with the Reducing Sugar

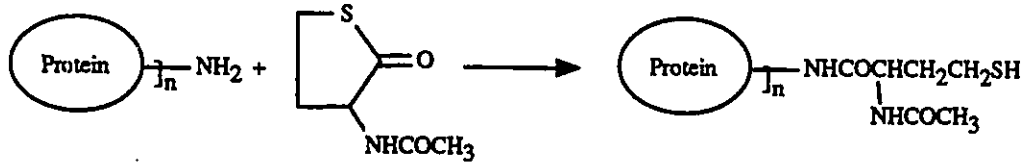
Previous studies (Smith 1980) of the coupling of NeuAcα2-3Galβ1-4Glc **2a** to protein with a spacer was reviewed in section 1.4.1. We felt this two step method was not necessary, and that direct conjugation (Kamicker 1977) could be accomplished using improved procedures (Roy 1984). This improved procedure is based on the use of borate buffer, which increases the equilibrium concentration of the aldehyde form of the carbohydrate, and thus allows a greater chance of imine formation (Roy 1984b).

The method worked very well, giving rise to a conjugate **35**. The structure of the resulting sialylgalactosedeoxyaminosorbitol is shown in figure 4-1. As with the conjugate **34**, there was a maximum obtained at ≈24 oligosaccharides per protein molecule.

4.2.3 Synthesis of Sialyllactosylamine Protein Conjugates by Michael Addition

Alternative methods of conjugation of saccharides to proteins are often useful. For example, it may be possible to obtain antibodies with diminished specificity for the spacer by an initial innoculation with one spacer and booster innoculation with a different spacer (produced by one of the alternate methods). We have developed a new method of conjugation using a Michael addition.

Haptens with leaving groups have been covalently linked to proteins thiolated with homocysteinethiolactone, as illustrated below (Reiner 1977). Only 10 of the thiol groups on



each protein were available to react, and there was a problem of crosslinking due to oxidation. The lesson we drew from this study was that the ϵ-amino group of lysine was nucleophilic enough to attack the carbonyl of homocysteinethiolactone and cause ring opening. We supposed these ϵ-amino groups would therefore be nucleophilic enough to react with activated double bonds in a Michael-type addition.

A Michael-type addition is a nucleophilic 1,4 addition to an activated double bond. Nucleophiles include thiols, carboxylic acids, primary or secondary amines and alkoxides. The double bond may be activated by a carbonyl, nitrile, nitro or sulfonyl group (March 1979).

Compounds in this thesis which have a conjugated double bond system are **16** and **32** which would result in NeuAcSpacer/BSA **36** and Sialyllactosylamine/BSA **37** as shown in figure 4-4.

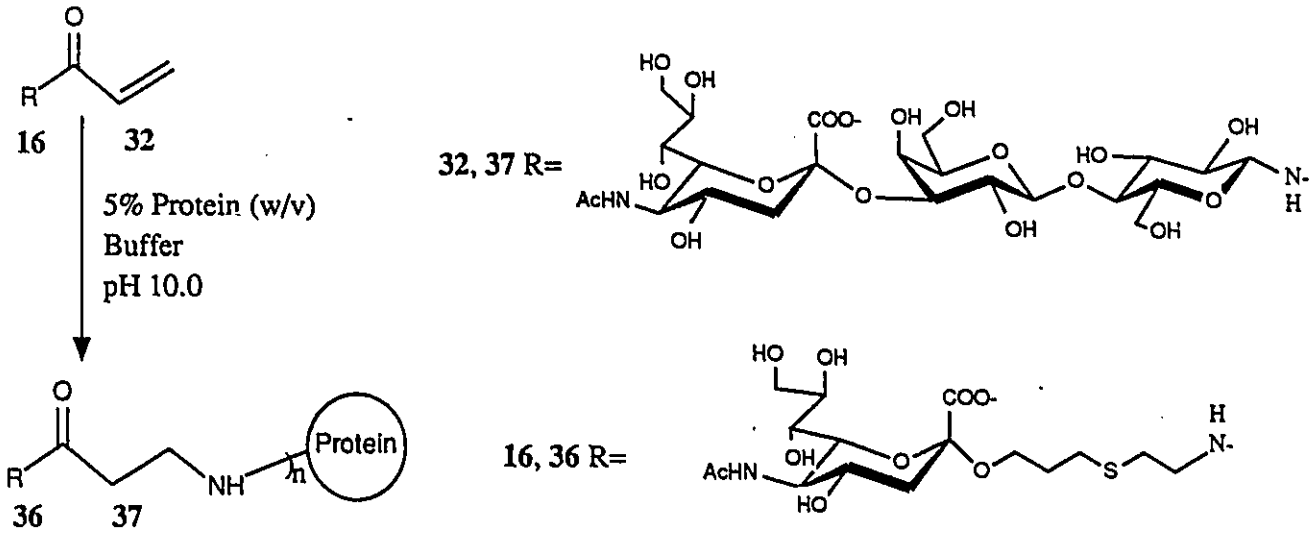


Figure 4-4 Michael-type Addition of the ϵ-amino of Lysine to the N-Acryloyl group

Indeed, we found that compounds with N-acryloyl groups could be covalently linked to protein by simply mixing at high pH. As seen from table 4-2, the highest incorporation of carbohydrate was made when the concentration of protein was high (compare entry 2 and 3). We found we could dissolve BSA as high as 10% (w/v), but the final concentration was 5% after addition of the sugar. The reaction occurred as low as pH 8.8, and even at pH 10.5 it did not cause significant hydrolysis of the protein, as determined by SDS PAGE electrophoresis. Borate buffer gave a slightly improved incorporation.

Table 4-2 Optimization of the Michael Addition to Protein.

Entry	Concentration[a] **16b** (mg/mL)	Protein (mg/mL)	Temp. (°C)	Buffer[b]	Incorporation[c] NeuAc/BSA
1	1	4	21	NaOH pH 9.5	0.
2	9	10	21	0.1 M Carb pH10.5	9.
3	25	50	37	0.1 M Carb pH10.5	14.
4	25	50	37	0.1 M Carb pH8.8	13.
5	25	50	37	0.4 M Bor pH10.	16.

[a]Total volume was 200 µL. [b] Carb indicates carbonate buffer, Bor indicates borate buffer. [c]Measured by resorcinol colorimetric test.

Figure 4-5 shows the rate of incorporation of two different acryloyl monomers. The acryloylated glycosylamine **32** reacts faster, in spite of the fact that the concentration of **16** was

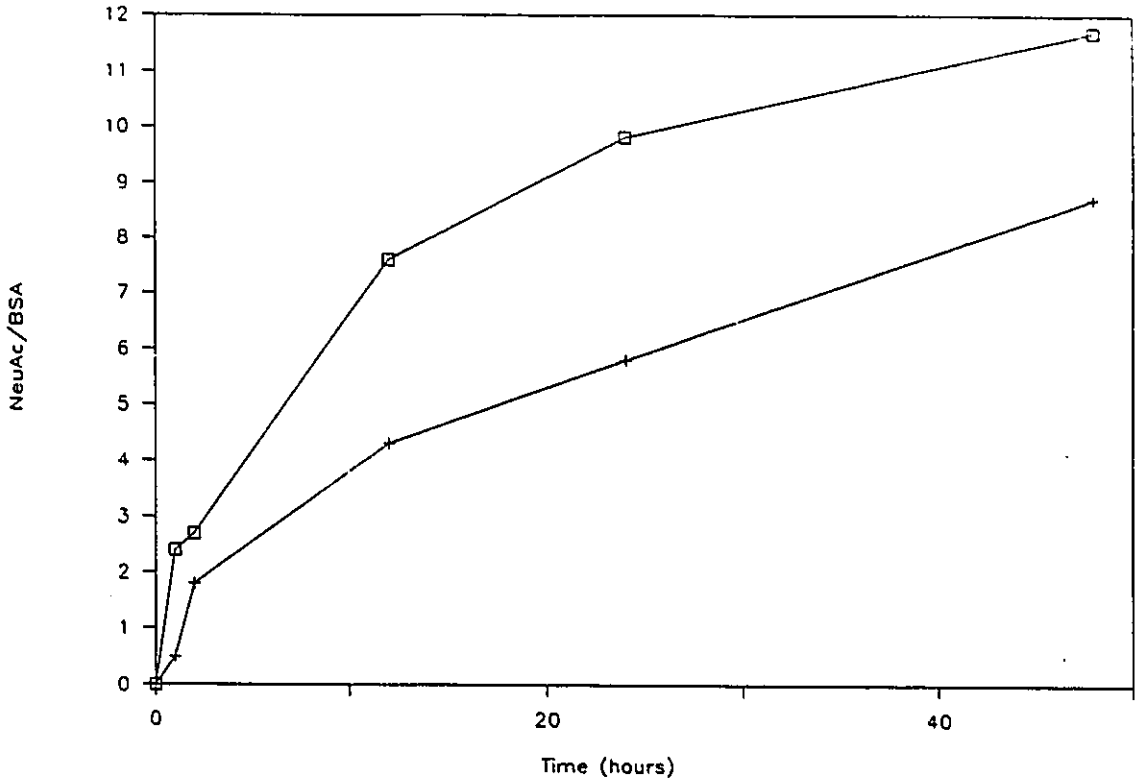


Figure 4-5. Rate of Michael Addition with two Different N-Acryloyl NeuAc Derivatives

Conditions: 7.5% (w/v) BSA in 0.1 M Carbonate Buffer, pH 10.
+ ≡ 16b 25 mg/mL
□ ≡ 32 10 mg/mL

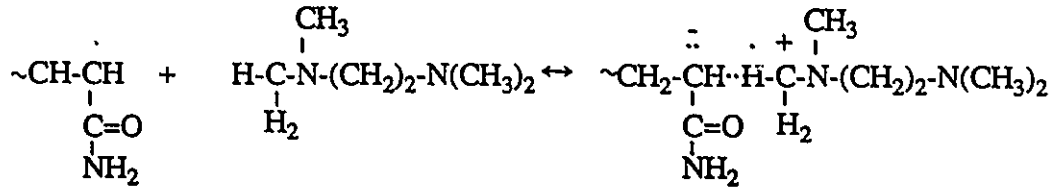
thrice as large. This may be due to the electron withdrawing nature of the anomeric nitrogen.

Conjugate 37 was sent for amino acid analysis (courtesy of BioMega Ltd). The results (not shown) indicate that only the lysine residues had been modified in 37 compared to the starting protein BSA. This gives direct proof that there is a covalent link between the carbohydrate and the lysine residues. This confirms the Michael addition occuring at the ϵ-amino of the lysine residues. In addition, the integration of the lysine peak in the h.p.l.c. of the amino acid analysis (PICOTAG) when normalized indicated that 15 out of 59 lysine residues had been modified. This result matched closely with the resorcinol colorimetric test which indicated there were 19 NeuAc per BSA.

4.3 Polyacrylamide Copolymers

4.3.1 Radical Polymerization: Mechanism and Theoretical Considerations

Radical polymerization occurs by the step by step addition of monomers onto a growing polymer chain. Radical polymerization requires stringently clean conditions to prevent radical scavenging (BioRad 1987). The radical is initated (in this work) from the persulfate ion which produces both a sulfate ion radical and a hydroxyl radical when heated (Sandler 1974). The reaction may also be catalyzed by the addition of tetramethylethylenediamine (TEMED) which stabilizes the transition state for the transfer reactions by contributions from polar structures such as



in which there is a partial charge transfer between an electron donor and an electron acceptor (p 237, Odian 1981)

In simple cases, the rate of polymerization at steady state may be used to determine the molecular weight of a polymer since the chain length will be the ratio of the polymerization rate to initiation rate. This result leads to the relation:

$$\text{Mol. wt.} \propto \text{chain length} = k_p[M]/2(R_i[I])^{1/2} \qquad 4\text{-}1$$

where:

k_p is the polymerization rate constant.
M is monomer.
R_i is the initiation rate.
I is the initiator

This equation indicates that the molecular weight of a radical initiated polymer will vary directly with the concentration of monomer and inversely with the root of the initiator concentration.

4.3.2 Co-polymers

When two different monomers are used, the following reactions may be considered:

$$\sim M_1\cdot + M_1 \xrightarrow{k_{11}} \sim M_1\text{-}M_1\cdot$$

$$\sim M_1\cdot + M_2 \xrightarrow{k_{12}} \sim M_1\text{-}M_2\cdot$$

$$\sim M_2\cdot + M_2 \xrightarrow{k_{22}} \sim M_2\text{-}M_2\cdot$$

$$\sim M_2\cdot + M_1 \xrightarrow{k_{21}} \sim M_2\text{-}M_1\cdot$$

This will result in the following types of polymers:

i) If $k_{12}=k_{11}$ and $k_{21}=k_{22}$, then the incorporation of M_1 or M_2 into the polymer will be determined solely on the ratio of monomers, and an "ideal" polymer will result which has a statistically random mixture of M_1 and M_2 units in the chain.

ii) If k_{12} and k_{21} are larger than k_{11}or k_{22}, then an alternating polymer will result.

iii) In all other cases, copolymers with structures intermediate between 1 and 2 result.

By steady state analysis, the composition of the copolymer (m_1/m_2) may be related to the ratio of the monomers in the reaction mixture.

$$m_1/m_2 = [M_1]/[M_2]\,x\,(r_1[M_1]+[M_2])/(r_2[M_2]+[M_1]) \qquad 4\text{-}2$$

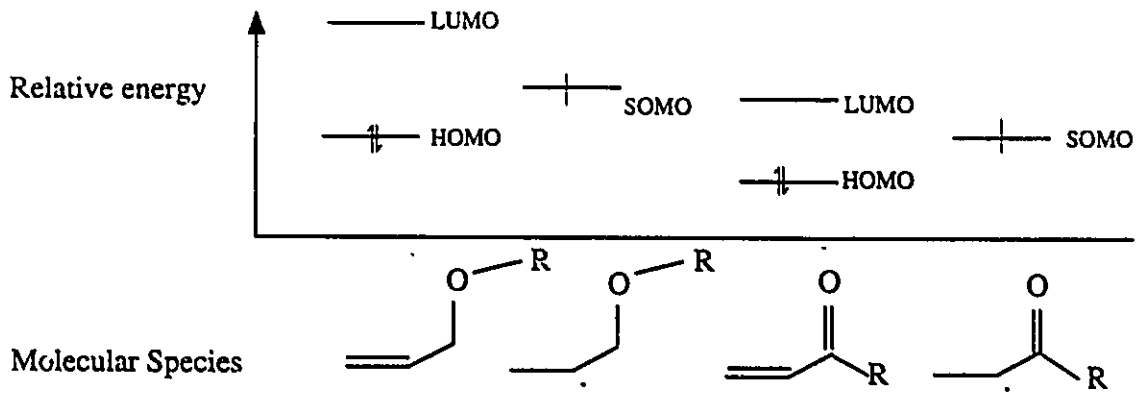
where r_1 and r_2 are the monomer reactivity ratios,

$r_1=k_{11}/k_{12}$

$r_2=k_{22}/k_{21}$

For ideal copolymers $(r_1r_2)=1$, while for an alternating copolymer $(r_1r_2)\approx 0$. When r_1 is larger than r_2, there will be a tendancy to form long homopolymer sequences interspersed with brief alternating sequences.

Different factors determine the value of r_1 and r_2, such as resonance stabilization, steric effects, and polarity (Cowie 1984). The reactivity ratio may also be rationalized by Frontier Molecular Orbital analysis. Consider the case of two types of radical, one resonance stabilized and the other not. The ability of the singly occupied molecular orbital (SOMO) of the radical to interact with the highest occupied molecular orbital (HOMO) or lowest unoccupied molecular orbital (LUMO) of the monomer depends on their relative energies (Struble 1968). In general, orbitals in the resonance stabilized species will be lower in energy (Beckwith



1986). As shown in the diagram above, the smallest energy gap occurs between the SOMO of the non-resonance stabilized radical and the LUMO of the resonance stabilized monomer. In this case, the π system is electrophilic-like and the radical is nucleophilic-like.

This diagram is biased to show how the resonance stabilized radical will preferentially interact with a resonance stabilized monomer; however, any number of possible reactivity ratios could be predicted based on the relative energies of the SOMO with the LUMO.

There are many possible combinations of monomers and it would be impossible to study them all. Attempts have been made to place radical-monomer reactions on a quantitative basis. To this end, the Q-e scheme was developed which provides an empirical calculation for predicting reactivity ratios. Briefly,

$$r_1 = Q_1/Q_2 \exp[-e_1(e_1-e_2)] \qquad 4\text{-}3$$

$$r_2 = Q_2/Q_1 \exp[-e_2(e_2-e_1)]$$

where
Q_n is the resonance stabilization of the radical.
e_n is the polar property.

Each monomer has unique Q and e values, and its reactivity with any other monomer can be calculated using equation 4-3. Tables of Q-e values are available (p465 Odian 1981) which may help in the prediction of polymer type.

4.3.3 Synthesis of NeuAc Copolymers

The first NeuAc polyacrylamide copolymers were made from allyl NeuAc **7b** to give **38b** as illustrated in figure 4-6. After exhaustive dialysis to remove unreacted monomers and small molecular weight materials, the incorporation of NeuAc in the polymers was determined by the resorcinol colorimetric test (Svennerholm 1957), and the values were roughly in accord with estimates based on the integration in ^1H n.m.r spectra of the polymers. We were disappointed to find that even a large excess of the NeuAc monomer in the reaction mixture did not increase the incorporation of NeuAc in the polymer beyond 17% (w/w) as in table 4-3.

Based upon the reactivity ratios calculated from the Q-e values of a model compound (allyl acetate), we knew that the allyl function did not cross react well with acrylamide. We then designed (see section 3.3) the N-acryloylaminoethylthiopropyl spacer in **16b** which shared the acryloyl function with acrylamide, and hence should have a reactivity ratio of ≈1. We were again disappointed to find that the incorporation was limited to 15% (w/w) NeuAc in the copolymers **39b**, as shown in table 4-3.

Table 4-3. Incorporation of NeuAc into Copolymers compared to Reaction Mixture Concentration.

Concentration NeuAc Monomer (mg/mL)	Concentration Acryl-amide (mg/mL)	Ratio Reactants (w/w)	Incorporation NeuAc (% w/w)[a]	Incorporation NeuAc (% m/m)[b]
7b				
21	66	0.3	6.	1.4
52	59	0.9	11.	2.8
100	74	1.4	16.	4.3
50	25	2.0	17.	4.6
16b				
29	31	0.9	14.	3.8
50.	14	3.6	15.	4.3

[a] Weight NeuAc per total weight. [b] Moles NeuAc per moles acrylamide in polymer.

Concurrent work with protein conjugation also showed a similar ceiling for the incorporation of NeuAc. It was postulated that the negative charge of the carboxylate functional group in NeuAc caused electrostatic repulsion between the monomer and the growing polymer chain, thus preventing the approach of NeuAc monomers. This hypothesis was confirmed when the methyl ester of these monomers (**7a** and **16a**) were polymerized and resulted in higher incorporation and improved yields. These data are illustrated in figure 4-7, and appear in table 4-4 below. A maximum of 33% NeuAc (w/w) could be obtained. The

methyl esters were then hydrolyzed by dissolution in aqueous sodium hydroxide to produce essentially the same polymer as if the acid monomers had been used.

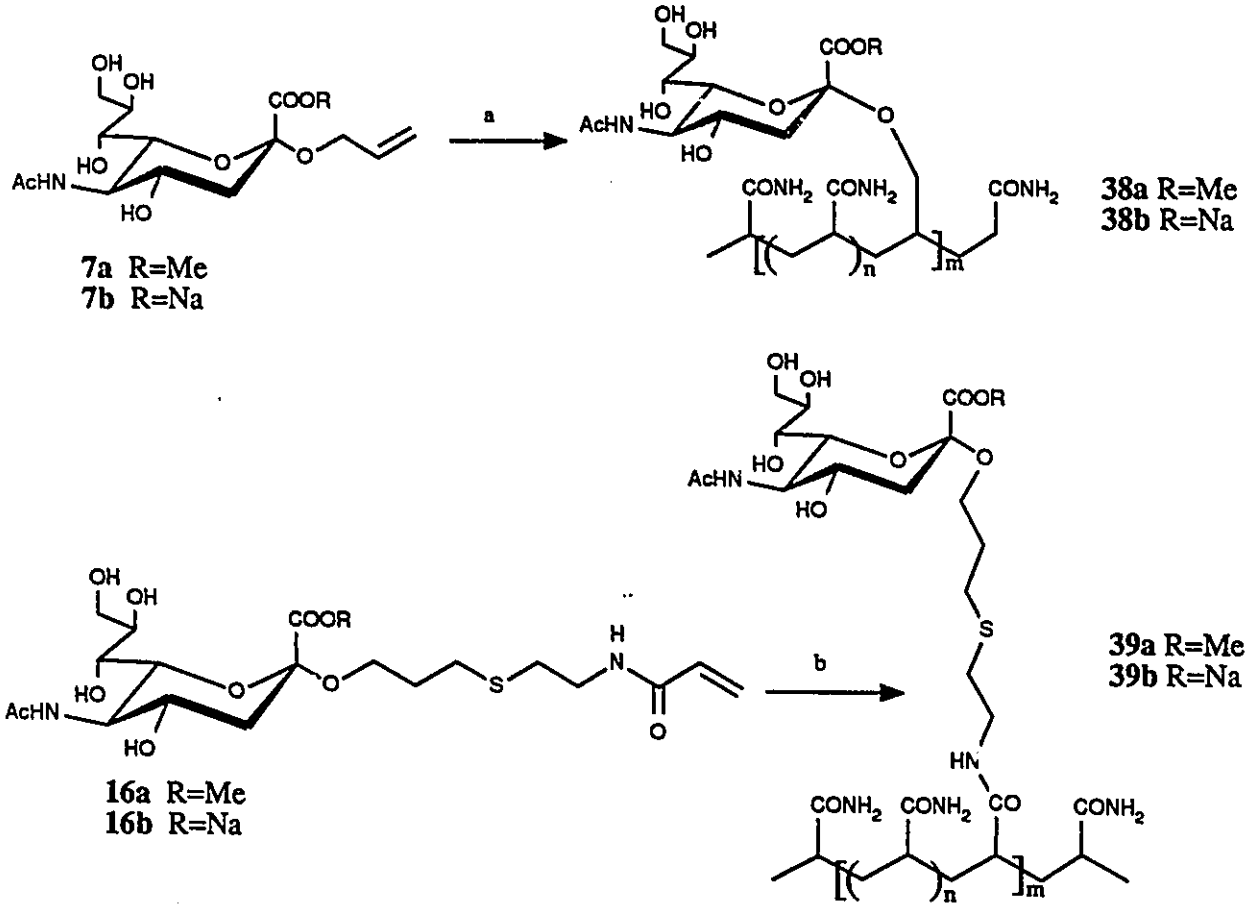


Figure 4-6 Polymerization of Monomers with Acrylamide

a) acrylamide, 1 mg/ml $(NH_4)_2S_2O_8$,100°C,5. min. b) acrylamide, 1 mg/ml $(NH_4)_2S_2O_8$,10 µl/ml TEMED, r.t.,o.n.

4.3.4 Comparison of Allyl vs Acryloyl Reactivity

A comparison of the two NeuAc monomers is useful to demonstrate the advantage of the N-acryloylaminoethylthiopropyl spacer in terms of polymer synthesis. The immunochemical advantages of this spacer will be considered in chapter 5.

Table 4-4 below gives the results of the the synthesis of two types of copolymers made from monomers **7a** and **16a** with acrylamide. The yield of NeuAc in the polymer **39** is an order of magnitude higher than the yield in the allyl NeuAc copolymer **38**. This reflects the higher reactivity of **16a** compared to **7a**.

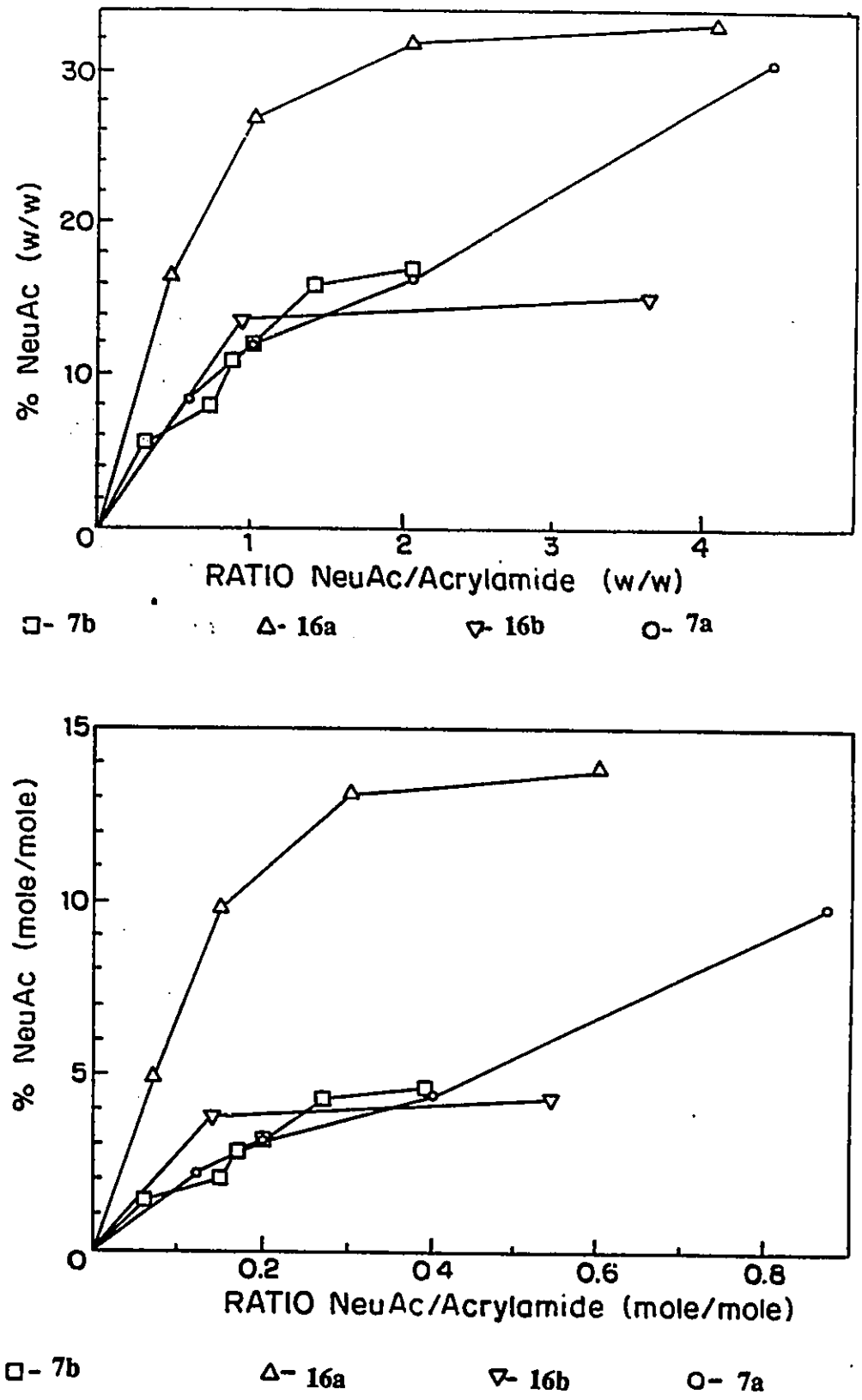


Figure 4-7 Incorporation of NeuAc in Copolymers Compared to Reaction Mixture Ratios

(Data from tables 4-3 and 4-4)

Table 4-4 Properties of NeuAc Acrylamide Copolymers

Ratio reactants (w/w)	Yield NeuAc (%)	Incorporation NeuAc resorcinol[a] (%w/w)[b]	(%m/m)[c]	NMR[d] (%m/m)[c]	$[\alpha]_D$
16a/acrylamide					
0.5:1	64	16.6	4.9	7.8	-2.7
1:1	70	26.7	9.8	15.8	-4.9
2:1	61	32.0	13.1		
4:1	47	33.0	13.8	36.	-8.3
7a/acrylamide					
0.5:1	6.3	8.4	2.1	1.9	-0.8
1:1	7.2	12.0	3.1	3.6	-1.6
2:1	7.4	16.3	4.4	7.1	-1.7
4:1	7.4	30.4	9.8	12.9	-2.2

[a] See appendix 1. [b] Weight NeuAc per total weight. [c] Moles NeuAc per moles acrylamide in polymer. [d] Based on integration of NAc singlet(2.03 ppm) vs. the polymer methylene (1.6 to 1.8 ppm).

The estimation of NeuAc content by ^1H n.m.r. matches quite well with the resorcinol results in the simple allyl NeuAc copolymers; however, there is a large difference found with the ethylthiopropyl NeuAc copolymers. This could be the result of interference of the spacer protons which appear in the same part of the n.m.r. spectrum as the acrylamide protons. Calculations were done to correct for this, and these are the results reported above. It is unlikely that the resorcinol test was influenced by the ethylthiopropyl function, as the monomer responded as expected in quantitative tests reported in appendix 1. Despite this discrepancy, the polymer **39** has a higher incorporation of NeuAc, especially when considering a molar comparison.

The reactivity of the allyl function can be compared to the conjugated double bond system through the model compound (allyl acetate) for which the constants Q and e are known. The data in table 4-4 were fit to equations 4-2 and 4-3 to obtain Q-e values. Only the value of Q was varied since this constant encorporates resonance stabilization and steric effects. The data for compound **7a** fit the equation very well, but those for compound **16a** did not fit, especially as the incorporation levelled off at 30%. The values obtained are reported in table 4-5.

Values are calculated for r_1 and r_2 from these data. For example, monomer **7a** with acrylamide would have values 0.001 and 7.2 respectively. These values indicate the polymer **38** has large stretches of acrylamide with an occasional allyl NeuAc.

For the monomer **16a**, its reactivity ratios with acrylamide are 0.5 and 2.0 respectively,

which indicates the polymer 39 is "ideal", essentially having randomly positioned acrylamide or pendant NeuAc in the chain.

Table 4-5 Q-e Values Calculated for NeuAc Monomers

Compound	Q	e	Reference
Acrylamide	1.12	1.19	Cowie 1985
Allyl acetate	0.028	-1.13	Odian 1981
Allyl NeuAc **7a**	0.010	-1.13	
Spacer NeuAc **16a**	0.55	1.19	

4.3.5 Effect of the Initiation Method

Two methods of radical initiation were employed with ammonium persulfate: heat and chain transfer with TEMED. At first they were used interchangeably (Roy 1988c), but when later experiments with the methyl esters of the monomers gave higher incorporations, a substantial difference could be observed depending on the initiation method. A comparison of the two methods is shown in table 4-6 which gives the results in terms of yield and incorporation into the polymer.

Table 4-6 Comparison of Initiation Methods in Radical Copolymerization

Acrylamide (mg/mL)	Allyl NeuAc **7a** (mg/mL)	Method of Polymerization TEMED[a] Incorp[c]. (%)	TEMED[a] Yield[d] (%)	Heat[b] Incorp[c]. (%)	Heat[b] Yield[d] (%)
20	10	3.0	31.6	8.4	36.
20	20	5.4	20.7	12.0	26.
20	40	9.0	20.7	12.0	26.
20	80	13.8	6.4	30.4	17.

[a]Polymerized in 1 mL H_2O, 1 mg/mL $(NH_4)_2S_2O_8$, 10µl/mL TEMED, r.t., o.n. [b]Polymerized in 1 mL H_2O, 1 mg/mL $(NH_4)_2S_2O_8$, heated 100°C, 5 min.
[c]Incorporation of NeuAc in the polymer (w/w) as determined by resorcinol colorimetric test. [d]Yield of polymer based on total reactants (w/w).

It is apparent that for this system the chain transfer with TEMED results in lower yield and incorporation. The electron rich allyl radical is not stabilized by the electron rich transfer agent, and it is therefore more likely to result in termination. Furthermore, heating may supply the energy to overcome some of the steric hindrance associated with the large NeuAc monomer.

4.3.6 Polyacrylamide with Hexanediamine Spacer Arms

Commercial polyacrylic acid of known molecular weights was readily available (inexpensive), and we decided to use this material to prepare NeuAc macromolecules via carbodiimide coupling. This method was considered advantageous because the molecular weight of the polymer could be easily controlled.

Treatment of the polyacrylic acid in aqueous solution with EDC resulted in a precipitate which did not redissolve even after addition of amine nucleophiles, however, if the nucleophile (hexanediamine in large excess with ammonium) at pH 7 was added before EDC, there was no precipitation and coupling resulted as determined by the ninhydrin test for primary amines after exhaustive dialysis. ^{1}H n.m.r. of the polymer indicated an unexpected resonance at 2.71 ppm which may be the methyl groups from residual O-acylisourea from the EDC (Inoue 1982, Tenforde 1972). Treatment of this polymer at pH 10 with ammonium hydroxide at 50°C for 48 hours resulted in the loss of most of the ^{1}H n.m.r. signal at 2.71 ppm. The method was then optimized using the 90 kDa and 50 kDa polyacrylic acid polymers to produce polyacrylamide and polyacrylamide with pendant aminohexane arms **40**, as shown in figure 4-8. Quantitation of the residual carboxylic acid groups on the polymer was attempted by titration, but no conclusive results were obtained. The number of amino groups was determined by a quantitative ninhydrin test (see appendix 1).

The polymer **40** was then used (as with protein) for reductive amination. For example, reductive amination with NeuAcα2-3Galβ1-4Glc **2a** gave polymer **41**. A similar polymer was also prepared by radical polymerization of **30** with acrylamide which resulted in higher incorporation, though it had an unknown molecular weight. Note that the reductively aminated **41** would still contain some free pendant amino groups. In fact, this latter polymer was found to have a poor shelf life and became insoluble after only a few months at 4°C, possibly due to oxidation of the residual primary amines or cross linking.

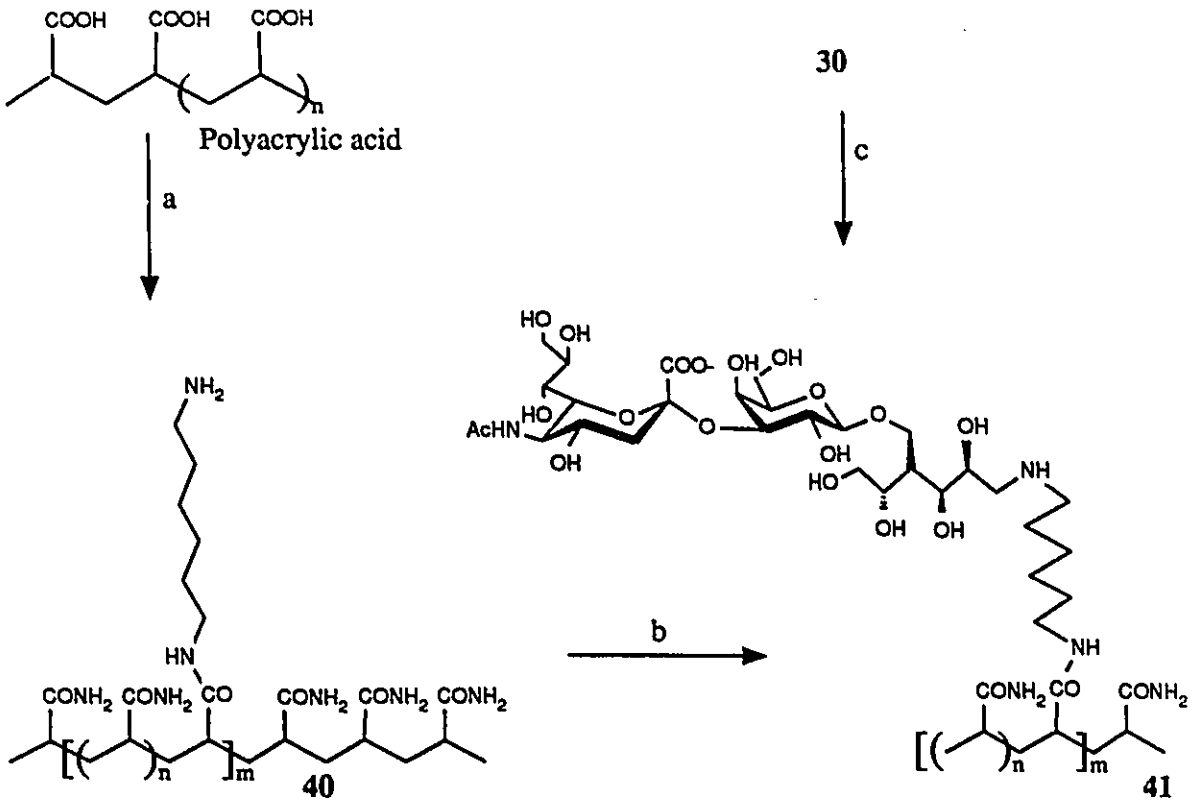


Figure 4-8 Synthesis of Sialyllactose Polyacrylamide Copolymer
a) Hexanediamine, ammonia, pH 7 , EDC b) 2a,Borate buffer, $NaCNBH_3$
c) Acrylamide, $(NH_4)_2S_2O_8$, 100°C, 5 min.

4.3.7 Molecular Weight Determination

Several methods were employed to estimate the molecular weight of the copolymers, including calculations based on initiator concentration, membrane extrusion, osmosis, double immunodiffusion and gel permeation chromatography. From the method of polymerization, a distribution of molecular weights was expected, and this was confirmed by membrane extrusion and gel permeation. No meaningful results could be obtained using osmometry.

4.3.7.1 Initiator Concentration

As equation 4-1 indicates, the molecular weight of a polymer may be calculated on the basis of the amount of initiator used. Results from previous work (Kosma 1987) showed that polymers made using 1 mg/mL of ammonium persulfate had molecular weights in the vicinity of 100 kD. These are the same conditions used here, and hence the same value is expected.

4.3.7.2 Membrane Extrusion

Concentrators (Amicon, Amicon Corp. Danvers M.A. 01923) equipped with membranes with a specified molecular weight cut-off were filled with solutions of polymers. After concentration, water was added and the solutions were again allowed to concentrate. Finally water was added and the solutions were then recuperated and tested by resorcinol for polymer concentration. Two cut-off ranges were used, and the results are shown in table 4-7. This method suffers from two problems. First, the molecular weight cut-off is based upon protein; and second, the distribution of NeuAc in the polymer may not be constant with molecular weight.

Table 4-7 Estimation of Molecular Weight Distribution of Allyl NeuAc Copolymer by Membrane Extrusion

	Material recuperated >25,000 g/mole	Material recuperated >125,000 g/mole
16% (w/w) Allyl NeuAc copolymer **38b**	100%	20%

4.3.7.3 Double Immunodiffusion

The rate of radial diffusion of a polymer in a gel is dependant upon the molecular weight. The polymer is detected by immunochemical precipitation, and the results of these experiments are presented in full in section 5.3.1. It was found that the molecular weight of copolymer **38** was dependant upon the NeuAc incorporation, whereas copolymer **39** appeared to have the same molecular weight regardless of the NeuAc incorporation. Based on comparisons with the distance migrated using NeuAc/BSA conjugates, the molecular weight was estimated to be between 80 and 120 kD for copolymer **39**. Copolymer **38** would be lower.

4.3.7.4 Gel Permeation Chromatography

Following the results of Kosma (1987), we found that the copolymers were retained by Sepharose CL-4B gel filtration column with a molecular weight cut-off of 5×10^6 dalton, and that the copolymers appeared in the void volume of other extrusion gels with lower molecular weight cut-offs (Sephadex G-100 and Sephacryl S-300). The chromatograms appear in figure 4-9, which also shows calibration of the column using a commercially purchased (Polyscience Inc.) polyacrylic acid polymers of known molecular weights modified to give polyacrylamide according to the procedure outlined in section 4.3.6. Evidently there are some portions of the polymers which still appear in the void volume, and this region of the chromatogram is subject to interpretation (Ezrin 1973).

On the basis of this, and the other results, the molecular weight of the polymer is approximately 100 kD, with a M_W/M_M of 5 to 10.

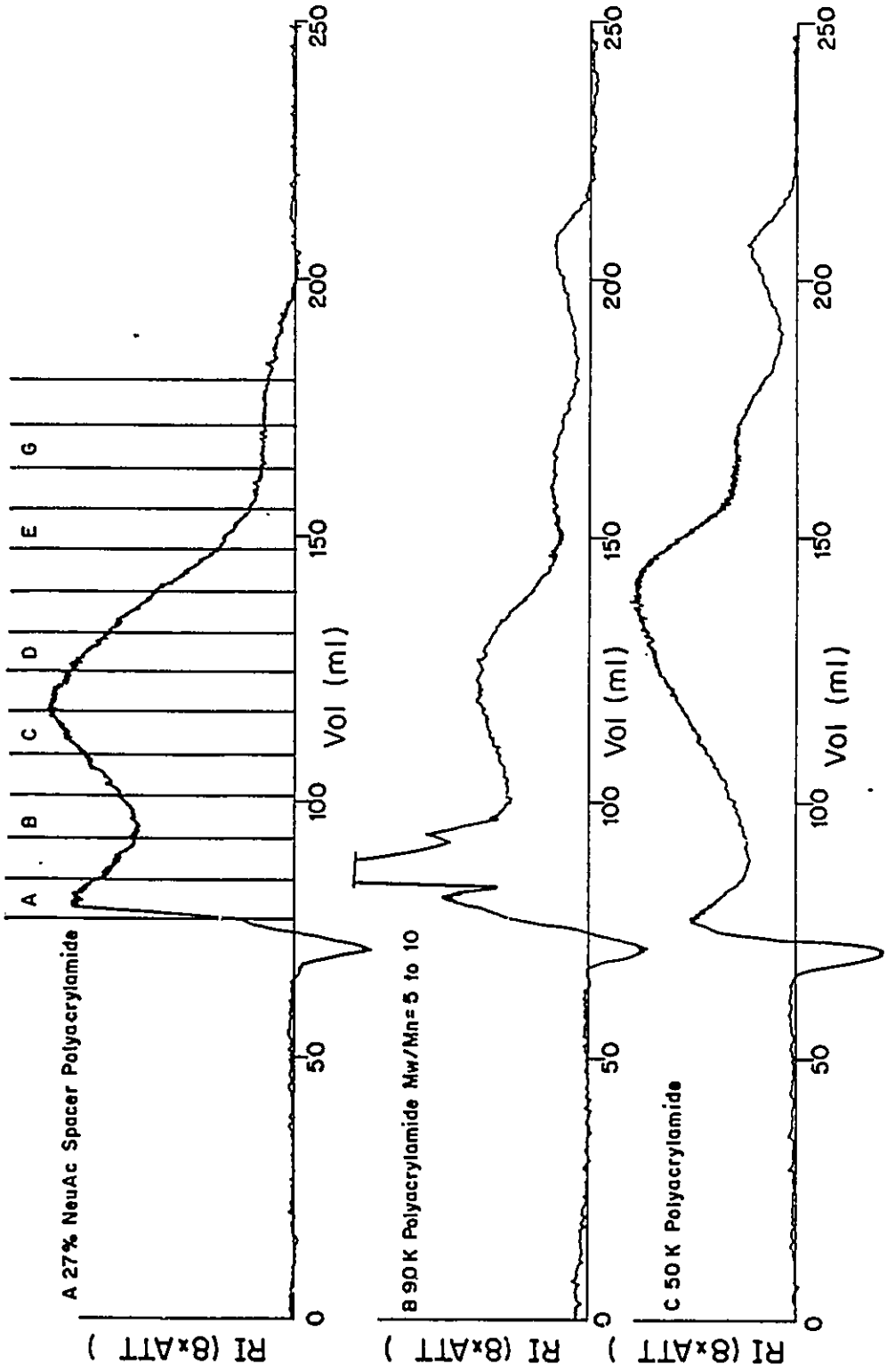


Figure 4-9 Molecular Weight Determination of NeuAc Copolymer (39a) by Gel Extrusion Chromatography

(Sepharose CL 4B 1.5 x 80 cm 0.03 M NH_4HCO_3)

4.4 Experimental Methods

General Method for NeuAc Protein Conjugate 34 by Reductive Amination

Compound **10** (20 mg) was dissolved in phosphate buffer (0.2M, pH 7.0, 2.0 mL). Then protein (10.0 mg) was added and the mixture was stirred for 1 hour at 37°C (to allow formation of Schiff base). Then $NaCNBH_3$ (20 mg) was added. Aliquots were taken at appropriate time intervals and dialyzed against running water for 24 hrs, then distilled water for 48 hrs. The sample was then diluted to a known volume, the sialic acid content measured by resorcinol (Svennerholm 1957) and the protein content measured by Lowry (see appendix 1). The sample was lyophilized and weighed and characterized with wheat germ agglutinin by double immuno-diffusion, immunoelectrophoresis, quantitative precipitation and ELISA, and by SDS-PAGE (see chapter 5).

$NeuAc_{53}$/BSA **34a**

Bovine Serum Albumin (Pentex, Miles Laboratories) (10.7 mg, 0.16 μmole) was dissolved in phosphate buffer (0.2 M,pH 7.0, 2.0 mL). Compound **10a** (22 mg, 60 μmole) was dissolved in 0.3 mL of the same buffer and added to the protein solution with methanol (1 mL) and the solution was stirred for 20 minutes at 37°C. Then $NaCNBH_3$ (20.4 mg, 0.29 mmole) was added and the reaction was stirred for 4 days at 37°. The reaction mixture was then placed in a dialysis bag and dialyzed for 3 days against water. The solution was diluted to 10.0 mL and three 0.25mL aliquots were tested with resorcinol for sialic acid content. The solution was lyophilized giving a white powder. Yield: 14.0 mg, 53 NeuAc/BSA (Note that the increase in weight of the protein is consistent with an increase in molecular weight of 15,000 g/mole.)

Sialyl(2,3)$galactose_{24}$/BSA **35**

Compound **2a** (10.4 mg, 16.5 μmole) and BSA (10.7 mg, 0.16 μmole) were dissolved in borate buffer (0.2 M, pH 8.7, 1.5 mL). The solution was stirred at 55°C for 1 hour, then $NaCNBH_3$ (26 mg, 470 μmole) was added and reacted for 24 hours. The solution was dialyzed and lyophilized giving a white powder. Yield: 13.2 mg, 24 NeuAc/BSA, 28% based on NeuAc. (Note that the increase in weight of the protein is consistent with an increase in molecular weight of 15,000 g/mole.)

Sialyl(2,3)lactosylamine$_{19}$/BSA **36**

BSA (100 mg) was dissolved in carbonate buffer (0.1 M, pH 10.5, 1.0 mL) and dialyzed against the same buffer for 2 days to give a 10% BSA solution.

Then compound **32** (2.0 mg, 2.9 μmole) was dissolved in water (50 μL), and the 10% BSA solution (100 μL) was added. The test-tube was sealed and set at 37°C for 48 hours.

After reaction, the solution was dialyzed against water for 3 days, then diluted to 5 mL and lyophilized to give a white powder.

Yield: 5.0 mg, 19 NeuAc/BSA, 50% based on NeuAc. 15 NeuAc/BSA based on amino acid analysis. SDS PAGE indicated an increase of 7000 in molecular weight.

General method for allyl-NeuAc copolymers **38a**

Compound **7a** (10, 20, 30 or 40 mg) was dissolved in water (1.0 mL) with acrylamide (20 mg). Then ammonium persulfate (1.0 mg) was added and the solution deaerated under an aspirator for 10 minutes and then heated at 100°C for 5 minutes.

The solution was dialyzed against water for three days, then lyophilized to give a white foam. The methyl esters were hydrolyzed when the polymer was dissolved in 0.1N aqueous sodium hydroxide for two hours, then dialyzed against water for three days followed by lyophilization. NMR analysis showed the loss of signal at 3.58 ppm, indicating complete hydrolysis.

General Method for ethylthiopropyl-NeuAc copolymers **39a**

Compound **16a** (10, 20, 30 or 40 mg) was dissolved in 1 mL distilled water with acrylamide (20 mg, J.T.Baker Chem. Co.). Then distilled TEMED (10 μL) was added with ammonium persulfate (1.0 mg), and the solution was deaerated for 10 minutes by aspirator, then sealed under N_2.

The solution was stirred for 6 hours, and additional ammonium persulfate (1 mg) was added, and the solution was stirred overnight. The solution was dialyzed against water for three days, then lyophilized to yield a white foam.

The methyl esters were hydrolyzed when the polymers were dissolved in 0.1N aqueous sodium hydroxide for two hours. After dialysis against water for three days the samples were lyophilized. NMR analysis showed the loss of the signal at 3.58 ppm, indicating complete hydrolysis.

Hexanediamine Copolymer **40**

90 kD polyacrylic acid (0.40 g, 1.4 meq., 25% aqueous Polyscience Inc.) was diluted with water (5 mL) and hexanediamine (0.30 g, 2.8 meq.) and ammonium hydroxide (1 mL, 14

meq.) were added. The pH was adjusted to 7.5 with concentrated HCl. Then EDC (0.36 g, 1.9 meq.) was added and the solution was stirred at room temperature. After 24 hours, ammonium hydroxide (2 mL) was added and the solution was kept at 50°C for 5 days. It was the dialyzed for three days and lyophilized to a white powder.

Yield: 115 mg. Incorporation: 40% mol/mol as determined by ^{1}H n.m.r.

NeuAcα2-3gal-hexanediamine copolymer **41** method 1

Copolymer **40** (10.0 mg) was dissolved in borate buffer (0.3 M, pH 8.7, 2.5 mL) and **2a** (19.5 mg, 0.03 mmol) was added. Then sodium cyanoborohydride was added (1.0 mg) and the reaction was allowed to proceed for 5 days. The solution was dialyzed and lyophilized to a white powder.

Yield: 9.0 mg. Content: 9% NeuAc (w/w)

NeuAcα2-3gal-hexanediamine copolymer **41** method 2

Compound **30** (8.1 mg, 0.010 mmole) was dissolved in 0.5 mL water with acrylamide (3.5 mg, 0.049 mmole). Then ammonium persulfate (0.3 mg) was added and the solution was heated to 100°C for 10 minutes.

The solution was dialyzed against water for three days, then lyophilized to give a white powder.

Yield: 2.8 mg, Content: 18% NeuAc w/w.

Chapter 5

Wheat Germ Agglutinin Interaction with Sialic Acid Neoglycoproteins and Copolymers

5.1 Introduction

Hemagglutinin from *Triticum vulgaris* (WGA) has been used in a wide variety of techniques to determine the presence of NeuAc (Liener 1986). It binds to the trans equatorial hydroxyl and acetamido groups as found in NeuAc and GlcNAc (see section 1.2.4.4). We decided to use this lectin to determine the antigenicity of the polymers and neoglycoproteins prepared as described in Chapter 4. The Svennerholm resorcinol method of sialic acid determination demonstrated the presence of sialic acid in the macromolecules, but we did not know if it was accessible for binding.

As we used the lectin more, we found that not only did it give information about the conjugates, but the conjugates gave information about the lectin which had not appeared in the literature. These results are valuable in that a more complete understanding of the lectin's binding site was obtained, and the characteristics of our polymers were related to a commercially available product.

5.2 Wheat Germ Agglutinin and NeuAc/Protein Conjugates

Several immunochemical methods used in the study of antibodies (as discussed in section 1.2.4.2) were applied to the study of the binding interaction of NeuAc/protein conjugates with WGA. The experimental methods are given in section 5.4, and the results are presented below followed by a discussion.

5.2.1 Double Immunodiffusion

Double immunodiffusion studies were performed mostly with BSA conjugates **34a** since sharp precipitin bands were formed, as compared to the diffuse bands obtained with the Tetanus Toxoid conjugates **34b** shown in figure 5-1. The ability to form a precipitin band with

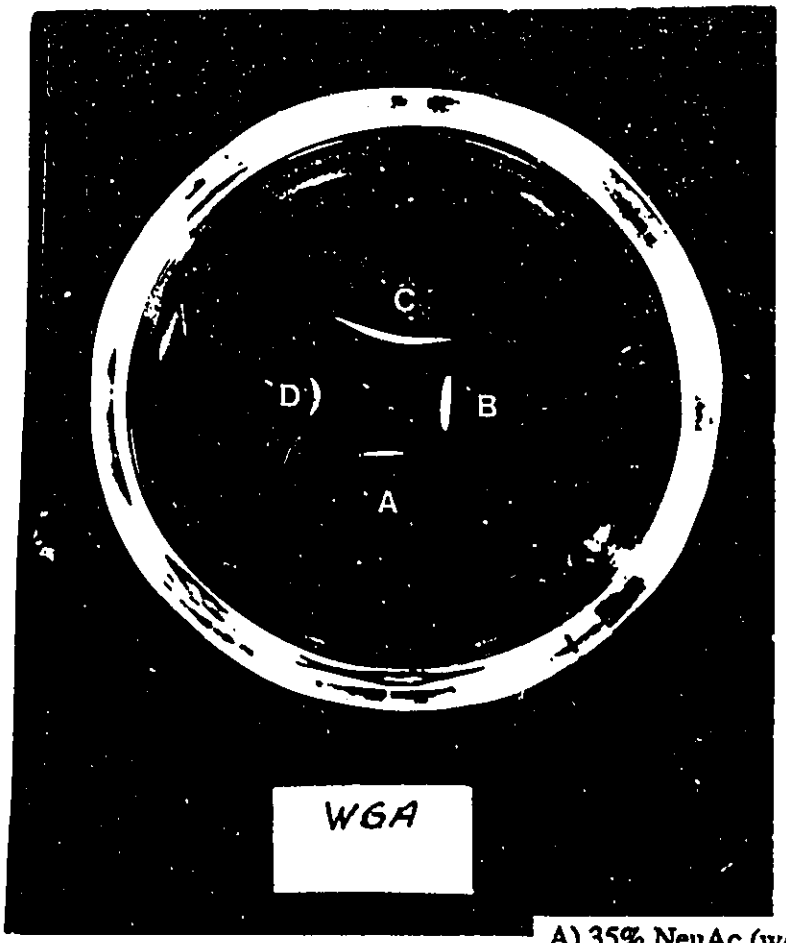


Figure 5-1 Double Immunodiffusion of WGA with NeuAc Conjugates

A) 35% NeuAc (w/w) 39b
B) $NeuAc_{53}$/BSA 34a
C) $NeuAc_{23}$/hexanediamineBSA (Pon 19
D) $NeuAc_{10}$/TT 34b

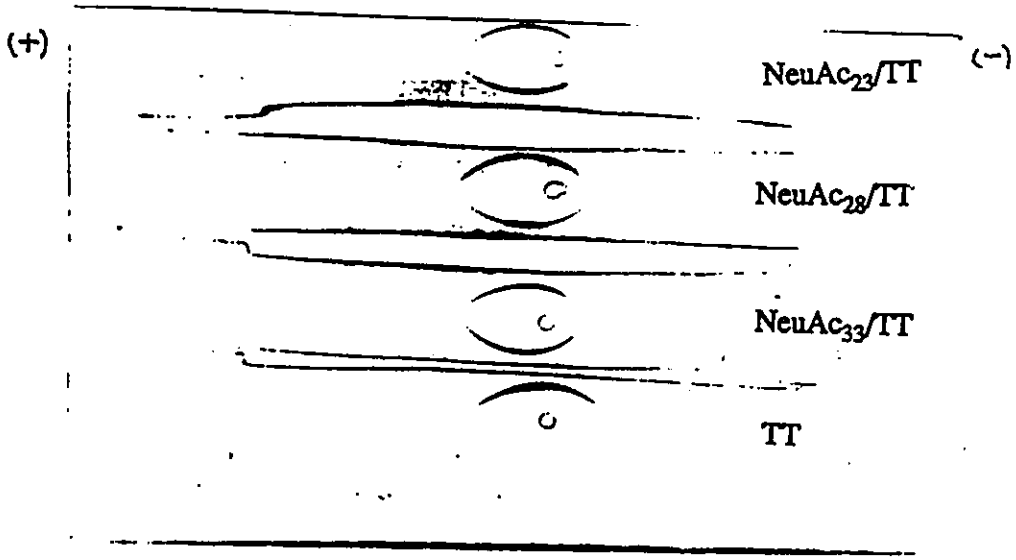


Figure 5-2 Immunoelectrophoresis of NeuAc/TT Conjugates (34b) with Different NeuAc Content

WGA was dependent upon the incorporation of NeuAc in the protein. The results, which are not shown, are described here. For example, protein conjugates **34a** required a minimum of between 14 and 20 NeuAc per BSA molecule before precipitation was observed at a standard concentration of 1 mg/mL. A conjugate with only 14 NeuAc/BSA did not form a precipitin band. This threshold phenomenon also applied to the sialylgalctose/BSA conjugate **35**. A study is underway to prepare many conjugates with different levels of incorporation to determine the exact cut-off point.

5.2.2 Immunoelectrophoresis

Immunoelectrophoretic mobilities of the proteins Bovine Serum Albumin (BSA) and Tetanus Toxiod (TT) were changed upon conjugation to sialic acid, as would be expected due to the negative charge associated with the sugar. Indeed, the conjugates **34a** and **34b** did migrate towards the positive end of the gel, and this technique was used to confirm that hydrolysis of the methyl ester of NeuAc occurred during reductive amination (see section 4.2.1.2).

It was hoped that the distance migrated in electrophoresis would be related to the amount of NeuAc coupled to the protein, but no differences were observed, as can be seen in figure 5-2.

5.2.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE (results not shown) of the protein carbohydrate conjugates showed a slight decrease in their mobility as would be expected with an increased molecular weight. The bands for TT were too diffuse to see any difference, but with BSA it was possible to see enough difference to estimate the increase in molecular weight. The values have been reported in the experimental sections where relevant.

5.2.4 Quantitative Precipitation

Studies were undertaken to determine the effect of the NeuAc content in the conjugate **34a** on the ability to precipitate WGA. Care was taken in the procedure to add the lectin to already diluted solutions of conjugates so that if kinetic effects were important in inhibition of the precipitation, there would be no anomalous results.

When the quantity of protein precipitated is plotted against the weight of conjugate added, skewed curves are obtained as shown in figure 5-3; however, when the data are plotted

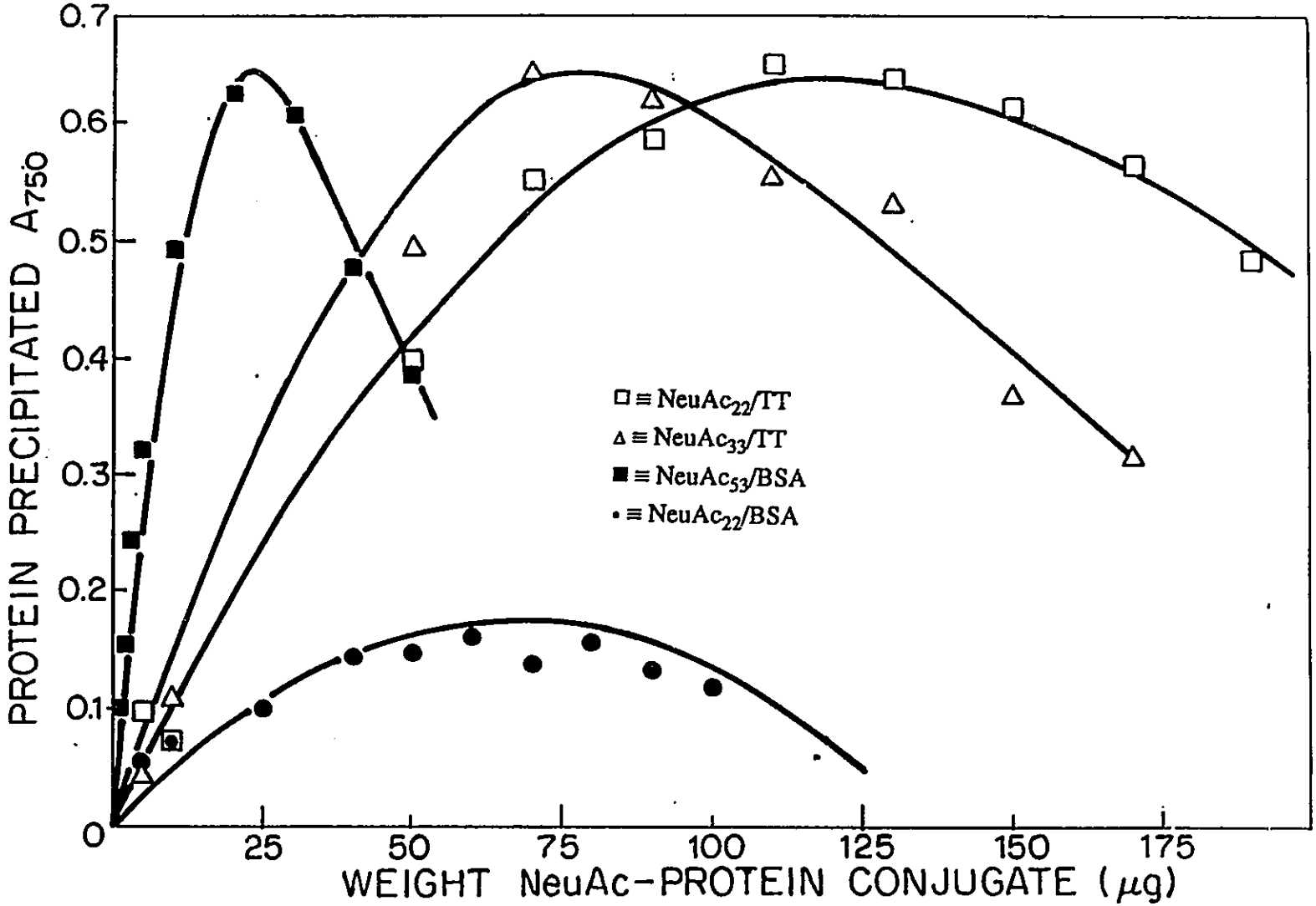


Figure 5-3 Quantitiative Precipitation of WGA with NeuAc/Protein Conjugates (34)

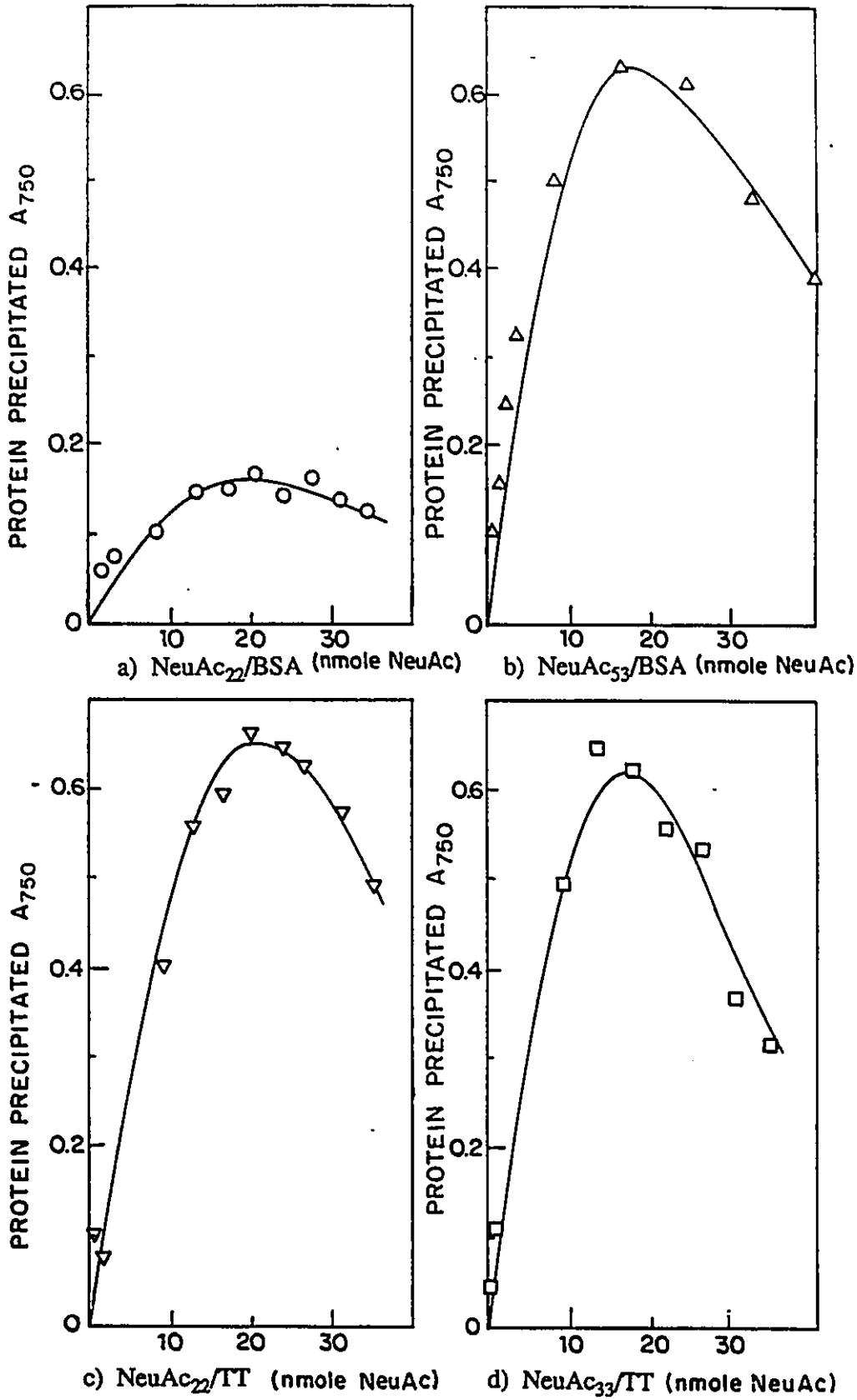


Figure 5-4 Quantitative Precipitation of WGA with NeuAc/Protein Conjugates Plotted as a Function of Total nmoles NeuAc

versus the total moles of NeuAc added, all the curves reach a maximum at 16±2 μmole NeuAc, as shown in figure 5-4.

At this point, assuming the calibration curve for the Lowry protein analysis is applicable to the precipitate, all of the protein was precipitated except for the case of 22 NeuAc/BSA (figure 5-4a) when less than half of the protein was precipitated. For 14 NeuAc/BSA (not shown) no precipitate was formed.

It was apparent that the protein conjugates were able to intertact with WGA, however; it had yet to be determined if the binding was non-specific.

5.2.5 ELLA

Enzyme linked lectin assays (ELLA) were performed using commercially available WGA linked to the enzyme horseradish peroxidase (WGA-HRP). (See section 1.2.4.2 for a review of the principles involved.) The results in figure 5-5 show NeuAc/BSA **34a** and sialylgalactose/BSA **35** conjugates coated on ELISA plates at the same concentration. As expected, decreasing amounts of WGA-HRP resulted in decreasing optical density after washing and addition of the substrate. There was only a slight effect due to the amount of carbohydrate on the protein.

Inhibition of ELLA was done using sugar monomers to verify the specificity of the interaction. An initial inhibition test with both **34a** and **35** as coating antigens on the microplate showed similar results, hence only **34a**, due to its greater availability, was used for the complete study.

When plotted as percentage inhibition versus inhibitor concentration on a logarithmic scale, typical sigmoidal curves were obtained. The best inhibitor was α-allyl-GlcNAc, as shown in figure 5-6, and the worst inhibitor tested was α-allyl-NeuGc **20** which did not inhibit at all. NeuAcα2-3Galβ1-4Glc **2a** and NeuAcα2-6Galβ1-4Glc **3a** were able to inhibit the interaction, but these two compounds gave different slopes as compared to the monosaccharides tested. The concentrations required to give 50% inhibition are reported in table 5-1 below together with the relative changes in free energies ($\Delta\Delta G^{o}$). Relative thermodynamic parameters obtained from sorbent assays have gained wide acceptance (Spohr 1985) and even absolute thermodynamic parameters can be determined (Li 1986, Day 1990).

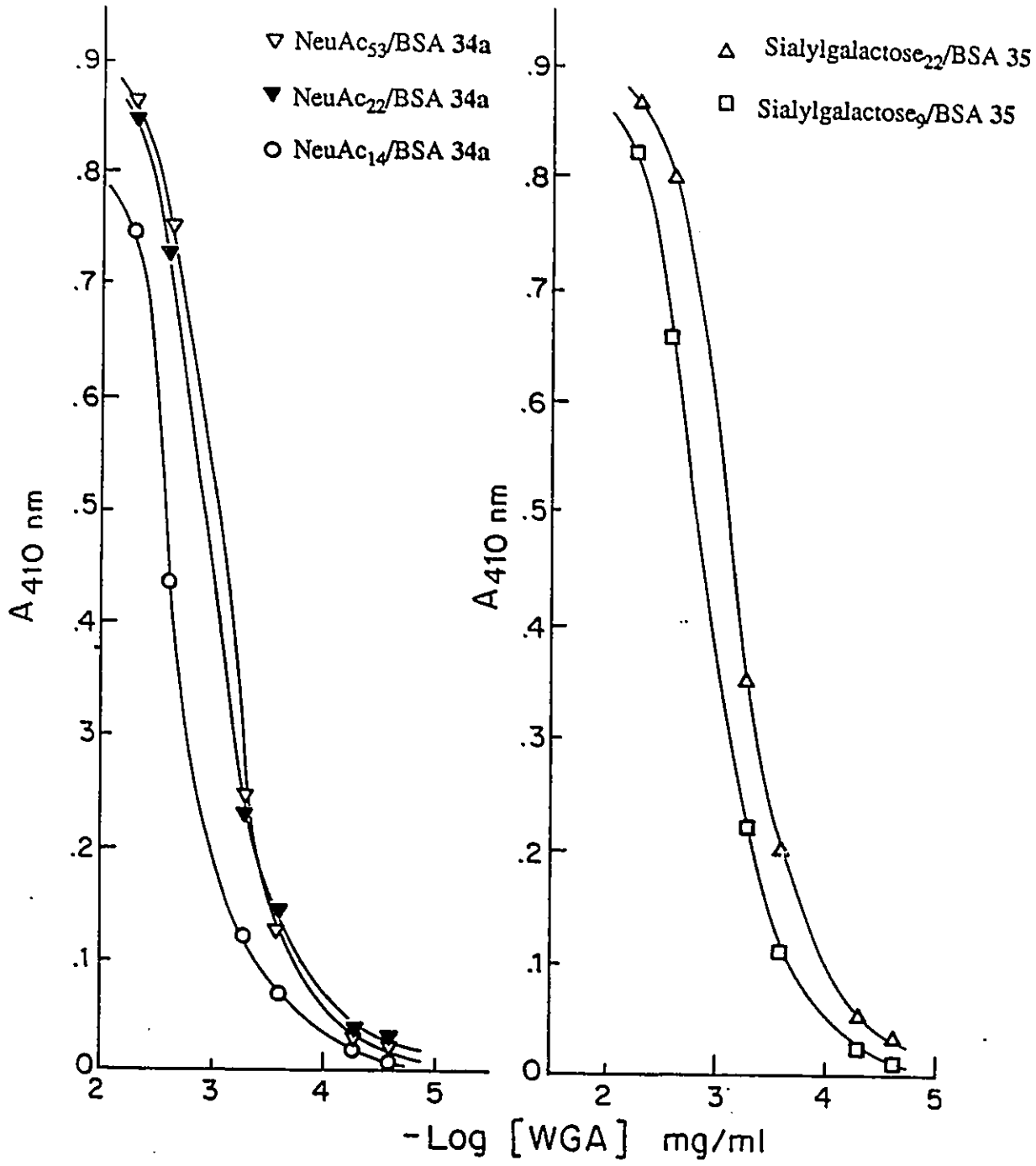


Figure 5-5 ELLA using WGA-HRP on NeuAc/BSA (34a) or Sialylgalactose/BSA (35) as Coating Antigen

Lectin: 1.0 μg/mL HRP-WGA 100μL/well

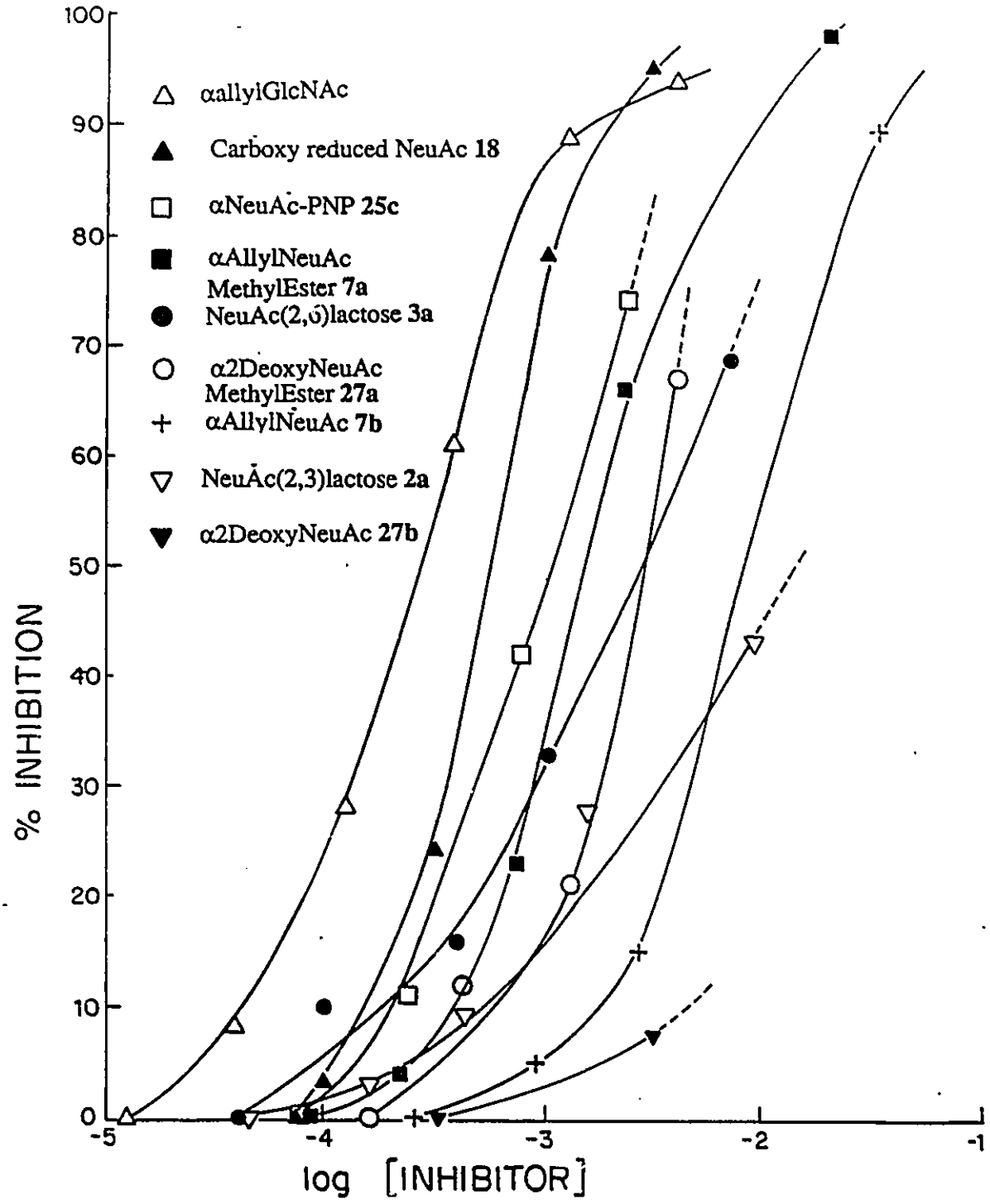


Figure 5-6 Inhibition of WGA-HRP ELLA on NeuAc/BSA (34a) with NeuAc Derivatives

(Coating: 1.0 μg/well with $NeuAc_{22}$/BSA (34a)

Lectin: 1.0 μg/mL HRP-WGA 100μL/well)

Table 5-1 Inhibition of ELLA of WGA-HRP with NeuAc/BSA by Monomer NeuAc Derivatives

Inhibitor	50% In-hibition (μM)	Relative Effectiveness to GlcNAc	to **7b**	ΔΔG° to GlcNAc (kCal/mol)
αallylGlcNAc	320	1.0	25	0.0
Carboxy reduced NeuAc **18**	560	0.57	14	0.29
αNeuAc-PNP **25c**	1100	0.29	7.2	0.73
αAllylNeuAc MethylEster **7a**	1600	0.20	4.9	0.95
NeuAc(2,6)lactose **3a**	3200	0.10	2.5	1.36
α2DeoxyNeuAcMethylEster **27a**	3200	0.10	2.5	1.36
αAllylNeuAc **7b**	7900	0.04	1.0	1.90
NeuAc(2,3)lactose **2a**	16000	0.02	0.49	2.31
α2DeoxyNeuAc **27b**	a	-	-	-
αAllylNeuGc **20**	a	-	-	-

[a] No inhibition at 3 mM.

5.2.6 Discussion

The ability to form precipitates with WGA either in gel or in solution is related to the quantity of sialic acid present on the protein. The results obtained reveal the following pattern: A minimum of 17±3 NeuAc/protein is needed to form a precipitin band in double immunodiffusion.

Other groups have described a logarithmic increase in the binding affinity with sugar density using other lectins (Lee 1987), and threshold type phenomenon have been invoked to explain antibody activity against cancer cells (Hakomori 1986). It is an interesting phenomenon which must be explained.

It may be relevant that the maximum incorporation attainable of NeuAc (free acid) during conjugation is ~22 NeuAc/BSA. This is likely due to electrostatic repulsion (see section 4.2.1.1), and indicates the spatial orientation is close. In such a case, when considering a dynamic model of lectin binding, the close proximity of another binding site reduces the ability of the lectin to diffuse away. This would result in an effective increase in binding strength (Li 1985).

The quantitative precipitation results presented indicate that maximum precipitation is related to the numer of haptens (binding sites) rather than number of protein molecules. If the amount of WGA can be estimated based on the weight of the protein, there are 14±2 NeuAc

per WGA at the point of maximum precipitation. An analogy made with antigen antibody precipitates indicates that at the region of maximum precipitation (the equivalence point) all the lectin is precipitated with all the carbohydrate (Kabat 1961). In the antibody antigen case, it has been found that the ratio of antibody to antigen in the precipitate increases with molecular weight of the antigen. In this case, it is the number of haptens that is more relevant. Unfortunately, quantitative precipitation of lectins has not been reported in the literature in the manner we have employed and it is difficult to extend these results to other antigens with WGA.

If the dynamic model of lectin binding proposed above is applied to quantitative precipitation, and if the ratio of 14:1 found for maximum precipitation is true, then WGA (with 4 binding sites per dimer) requires approximately three NeuAc to be within diffusion range to ensure "irreversible" binding. This would explain why conjugates with low incorporation are unable to cause precipitation (Kronis 1982). Furthermore, this explains why a pentasaccharide of GlcNAc is able to inhibit 5900 times more than the correspond monomer (Liener 1986). Mathematical analysis of diffusion rates compared to binding affinity may be used to obtain data on the distances between the haptens in the conjugates.

The enzyme linked lectin asssay (ELLA) results show a marked difference between surface and solution effects. While a conjugate with only 14 NeuAc/BSA is unable to cause precipitation in solution, there is a response in ELLA. Furthermore, the ELLA technique could not differentiate between 22 and 50 NeuAc/BSA. It is likely that the surface of the microplate well is saturated with sialic acid under the conditions employed. This effect has been studied in more detail using limiting quantities of NeuAc polyacrylamide copolymers, and will be discussed later (section 5.3) The fact that NeuAc/BSA **34a** and sialylgalactose/BSA **35** conjugates gave similar results indicates the relative equal accessibility of the carbohydrates on the plate surface to the lectin.

To prove the specificity of the interaction, addition of sugar monomers were able to cause inhibition, indicating the sugar portion of the NeuAc/BSA conjugates was the locus of binding.

The inhibition studies also gave results in agreement with the binding site proposed by Wright (1980) (see section 1.2.4.4). It is apparent that the negative charge of the carboxyl group reduces the binding affinity of α-allyl-NeuAc **7a** because both the ester **7b** and carboxy reduced derivative **18** inhibited 4.9 and 14 times more, respectively. This is consistent with the negative charge of glutamate or aspartate present in the primary and secondary binding sites of WGA (Wright 1980) which would cause charge repulsion.

It is interesting to note that the 2-deoxy derivative **27b** binds with less affinity than the allyl glycoside, indicating that some binding energy is obtained from the aglycon. This is

consistent with a possible van der Waals interaction with tyrosine 61 in the binding site. This is confirmed with the *p*-nitrophenyl aglycon **25c** which had a very high inhibitory effect. This aromatic moiety would also be in the vicinity of tyrosine 61 of the binding site, and could even undergo dipole stacking.

The glycolyl derivative **20** of sialic acid did not inhibit binding, as has been previously noted (Liener 1986). This confirms that the N-acetyl group provides much of the energy for binding.

The unusual result found here is that NeuAcα2-6Galβ1-4Glc **3a** is a better inhibitor than the 2,3 derivative **2b**, contrary to what would be expected on the basis of equilibrium studies done by Kronis and Carver (1982) in which the 2,3 isomer was found to have a lower dissociation constant. Several explanations may be offered to explain this difference. First, in this study commercial WGA-HRP conjugate was used which is a mixture of isolectins and modified by the conjugation process. Second, this study was done using solid phase as compared to the n.m.r. study of Kronis and Carver which was done in solution. The surface effect in the solid phase would limit the degrees of freedom, and hence the increased degree of freedom of the 2,6 isomer over the 2,3 isomer could account for the better affinity.

The different slope of inhibition with the trisaccharides as compared to monosaccharides is likely a mass effect, and is consistent with an activation energy for binding as described by Kronis and Carver (1985).

In conclusion, this model study indicated that the NeuAc moieties present on the neoglycoproteins were accessible to binding as indicated by the ability to interact with WGA in a variety of techniques. The specificity of the interaction was localized to NeuAc by inhibition studies, which also gave some insight to the energy differences of different molecules in the binding pocket.

5.3 Wheat Germ Agglutinin and NeuAc Polyacrylamide Copolymers

In a similar fashion to what has already been described with the NeuAc/protein conjugates, NeuAc polyacrylamide copolymers were also tested for antigenicity using wheat germ agglutinin.

In order not to repeat the detailed analysis that had already been performed using the protein conjugates, only a few select experiments were done including double radial diffusion and ELLA.

5.3.1 Double Immunodiffusion

It was established early on that NeuAc polyacrylamide copolymers **38** and **39** were able to cause precipitin bands in gel double immunodiffusion studies. The polymers with the methyl ester intact produced much sharper precipitin bands than the corresponding acids, and hence the ester was frequently used in these studies.

The double immunodiffusion of the polymers reported in table 4-4 with WGA are shown in figures 5-7 and 5-8. Notice that the intensity of the precipitin line is related to the NeuAc content in the polymer. There appeared to be a certain cut-off, corresponding to approximately 17% NeuAc w/w, below which bands did not appear. (Note: The ester was used in figure 5-7 and the threshold appears somewhat lower (≈10%).) This is similar to the result found for the protein conjugates described in section 5.2.1.

Also of importance is the relative distance of migration of the polymers before the precipitin band appears. This distance is related to the molecular weight (Johnstone 1982), and hence can be used to estimate molecular weight (see section 4.3.3). Fractions obtained from the gel permeation chromatography (figure 4-9) were also placed in double radial diffusion gels, and the results are shown in figure 5-9. It is apparent that the higher molecular weight fraction A,B and C diffuse more slowly than the lower weight fractions D,E, and G. This confirms the usefulness of the method for estimating relative molecular weight.

A re-examination of figures 5-7 and 5-8 show that all the ethylthiopropyl NeuAc copolymers (**39**) diffuse the same distance, and hence have approximately the same molecular weight. The allyl NeuAc copolymers (**38**), on the other hand, appear to form bands at different distances and the low NeuAc incorporation is associated with high molecular weight. This can be rationalized in terms of the different reactivities of the allyl function compared to the acrylamide. Evidently the allyl group causes chain termination and hence the higher allyl NeuAc concentration results in lower molecular weight.

5.3.2 ELLA

WGA-HRP was used in the ELLA tests similar to those described for the NeuAc/protein conjugates. In this case, since the copolymers were never established as useful coating agents in adsorbent assays, it was deemed necessary to make a complete study of the ELLA response with respect to coating concentration of polymers, as well as the effect of the NeuAc content in the polymer. The results are summarized in figure 5-10.

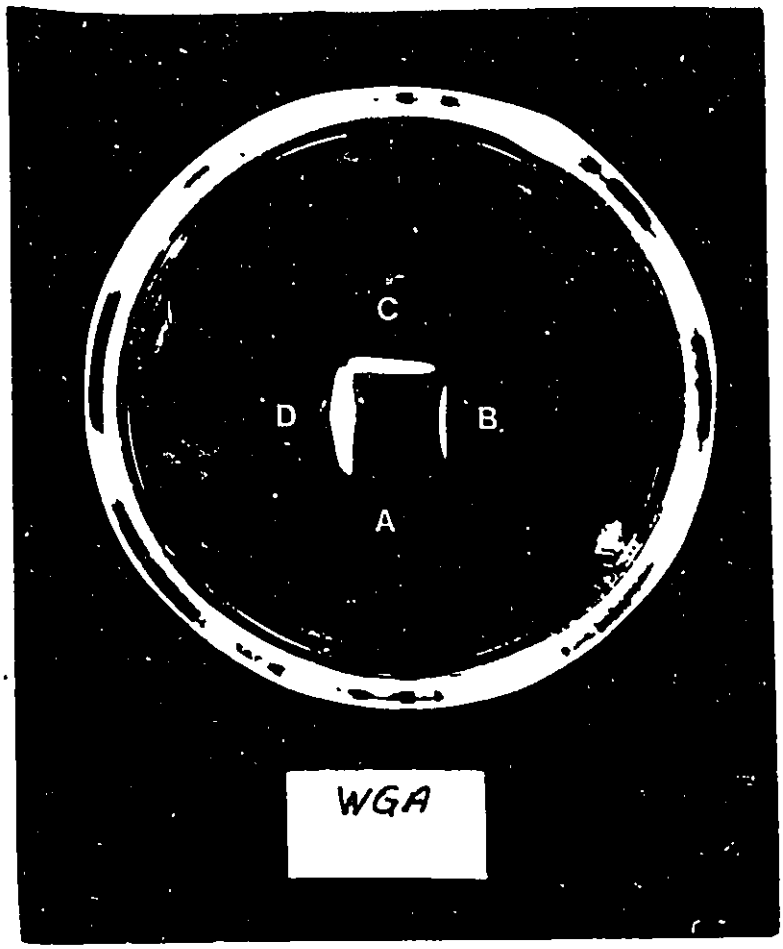


Figure 5-7

Double Immunodiffusion of Copolymer 38a with WGA

A) 8% NeuAc (w/w)
B) 12% NeuAc (w/w)
C) 16% NeuAc (w/w)
D) 30% NeuAc (w/w)

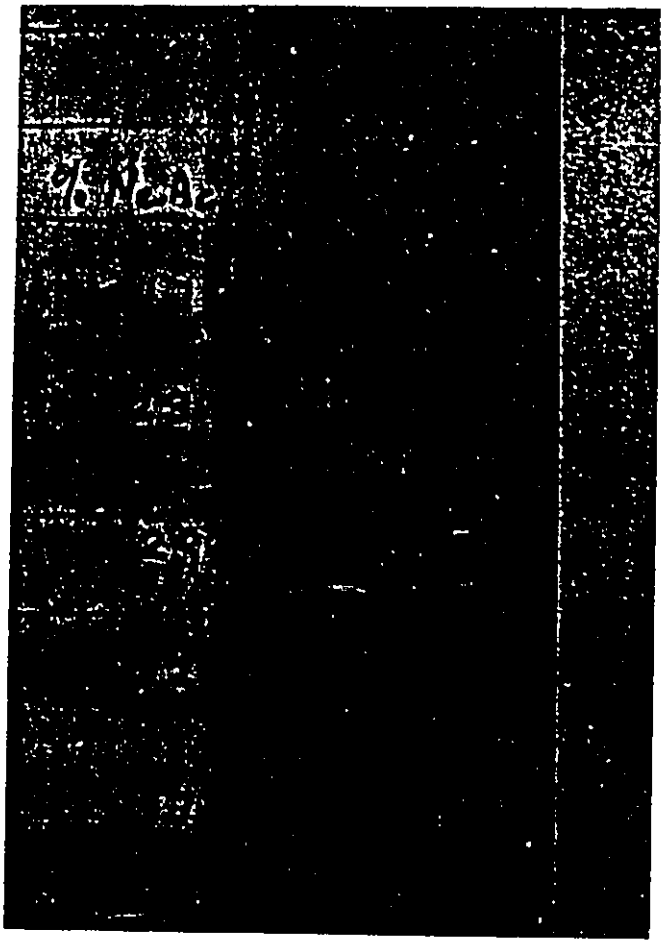


Figure 5-8 Double Immunodiffusion of Copolymer 39b with WGA
NeuAc content (w/w) as indicated

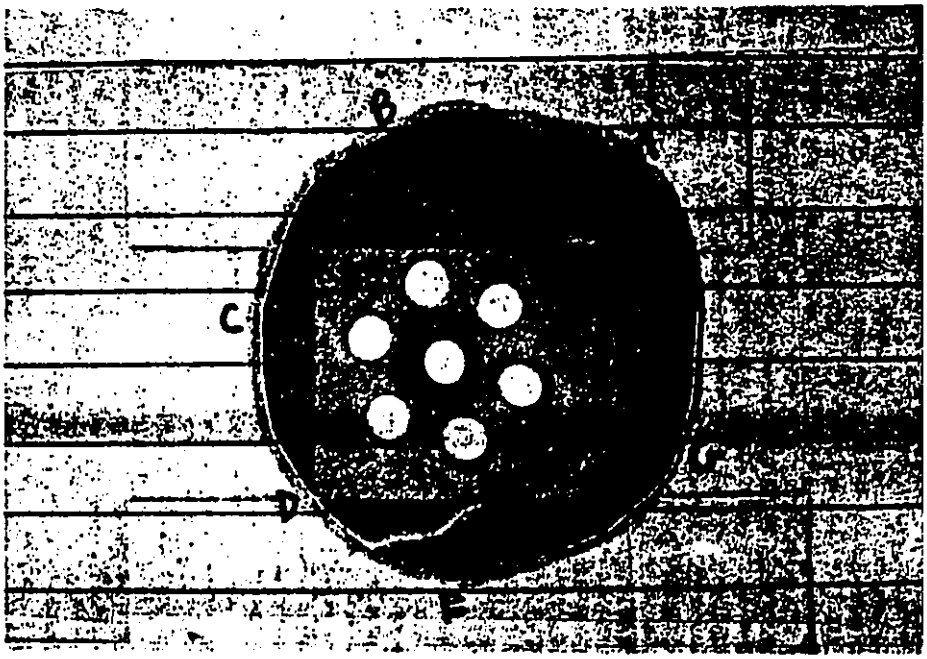


Figure 5-9 Double Immunodiffusion of Copolymer 39a with WGA
Letters correspond to fractions in figure 4-9a

These results show that ethylthiopropyl NeuAc copolymers (39) are much more effective as coating antigens than allyl NeuAc copolymers (38). As incorporation of NeuAc increases, the ELLA response of ethylthiopropyl copolymers (39) increases while that of the allyl copolymers (38) decreases slightly, possibly due to the lower molecular weight found for this polymer.

5.3.3 Discussion

The interaction of the NeuAc polyacrylamide copolymers with WGA was demonstrated by double immunodiffusion and enzyme linked lectin assay.

The gel diffusion studies were useful in providing an indication of relative molecular weights. It is apparent from figure 4-9 that a broad distribution of molecular weights is obtained by the method of polymerization. The lower molecular weight material might have had an effect in causing inhibition of precipitation, but this does not seem to be the case. In fact, the precipitation bands appear darkest with the highest NeuAc content, associated with low molecular weight.

The ELLA studies were useful as an indication that the polymers were indeed sticking to the wells of the plates, and the polymer with the ethylthiopropyl spacer were almost ten times more effective than the corresponding polymer without spacer. This may be due to the "hydrophobic" nature of the spacer, which therefore assists in binding to the polystyrene or PVC plastic surface. Properties of the spacer which may have improved the response are increased degrees of conformational freedom and decreased steric hindrance from the polymer backbone.

The general conclusion from these results is that polymers with spacer arms are more effective and useful in adsorbent type assays.

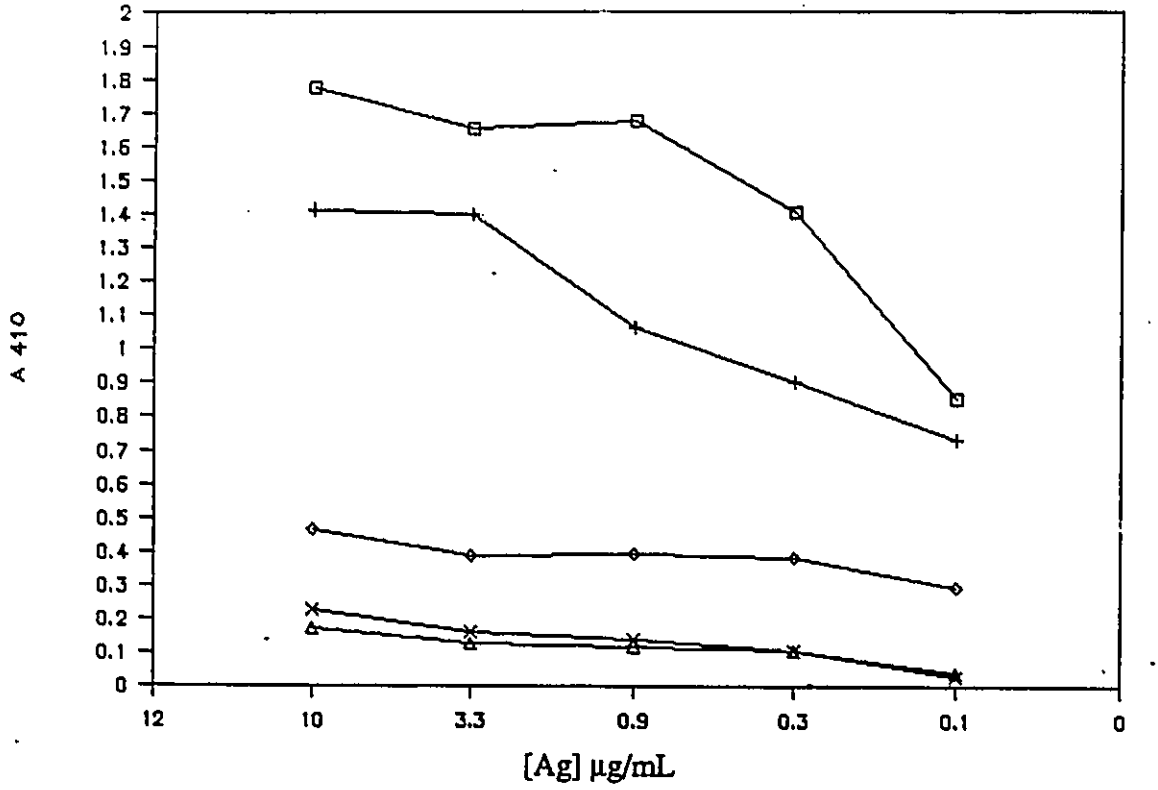


Figure 5-10 Effect of Concentration and NeuAc Content of Copolymers **38b** and **39b** in ELLA using WGA-HRP

□ ≡ 30% NeuAc (w/w) **39b**
\+ ≡ 27% NeuAc **39b**
◇ ≡ 17% NeuAc **39b**
Δ ≡ 30% NeuAc **38b**
x ≡ 17% NeuAc **38b**

Lectin: 1.0 µg/mL HRP-WGA 100µL/well

5.4 Experimental Methods

Double Immunodiffusion (Ouchterlony 1978)

Preparation of plates:

Agarose (200 mg, BDH) was added to phosphate buffered saline (PBS) (16 mL) and heated at 100°C for 20 minutes until the agarose dissolved, then 4 mL of 10% PEG 8000 was added. The hot solution (2.7 mL per dish) was put in 5 cm diameter petri dishes and allowed to cool and gel. Holes were punched in the gel 1.0 cm apart and removed by suction.

Diffusion:

Wheat germ agglutinin (Sigma L-9640) was dissolved in PBS (2.0 mg/mL). NeuAc protein conjugates or copolymers were dissolved in PBS (1.0 mg/mL). Approximately 20 μL of the solutions were placed in the wells and allowed to diffuse for 3 hrs. at room temperature, then overnight at 4°C. Precipitin bands had usually formed by the next day. The bands were stained with coomassie blue.

Immunoelectrophoresis.

Gel Preparation:

Agar (BDH, Coarse powder, 1.5 g) was added to 100 mL 0.05 M barbital buffer pH 8.3 with 0.01% azide and heated at 100°C for 1.5 hours until "dissolved". Then 25 mL of the hot solution was placed on a 10 X 10 cm glass plate and allowed to cool and gel.

Sample Preparation:

NeuAc-protein conjugates were dissolved at 1 mg/mL in PBS and placed in 1 mm dia wells punched in the gel. Whatman #1 Paper was wet with barbital buffer and used to make electrical contact with the gel. The electrophoresis was run at 140 V, 20 mA for 2 hrs. Troughs were then cut between the wells and filled with rabbit anti-NeuAc-TT serum (#308) or WGA (Sigma, 2 mg/mL). The plate was incubated at 4°C for 24 hrs. or longer. Precipitin bands were stained with coomassie blue.

Lectin Quantitiative Precititation (Kabat 1961)

Wheat germ agglutinin (Sigma N° L-9640) was diluted to 0.4 mg/mL in PBS and 100 μL was placed in 1.5 mL MCC tubes (Hughes and Hughes Ltd., Elms Industrial Estate, Church Road, Harold Wood, Romford, RM3 OHR, Essex). Aliquots of 5.0 to 100.0 μL of 1.0 mg/mL NeuAc-protein conjugates in PBS were added to give final volumes of 300 μL. The solutions were agitated and allowed to incubate for 3 days at 4°C. Then the tubes were centrifuged for 5 minutes at 14,000 g in a Fisher MicroCentrifuge Model 235B, and allowed to incubate for 2 more days at 4°C. After centrifuging again, the pellets were washed twice with 300 μL cold

PBS with centrifuging between washes. The protein content of the pellets was determines using Lowry protein determination standardized with both BSA and WGA.

ELLA (Enzyme Linked Lectin Assay)
with Protein Adsorbents

NUNC (Denmark) microanalysis titer plates were rinsed with 95% ethanol, dried, and coated (100 μL/ well) with 10 μg/ml NeuAc-protein conjugates **34a** or **35** in PBS at room temperature overnight. The plate wells were then blocked with 100 μL/well 0.2% BSA in PBS for 20 minutes. After washing, the wells were then filled with 100 μL/well of serial dilutions of WGA-HRP (Sigma L-3892 Lot 375-4050) from 10^{-2} to 10^{-5} mg/mL in PBS and incubated at room temperature for 3 hrs. The plate was then washed 5 times, and 50 μL/well of ABTS (1 mg/mL) in citrate-phosphate buffer (0.2 M, pH 4.0 with 0.01% H_2O_2) was added. After 15 minutes the optical density was measured at 410 nm relative to 570 nm on a Dyntech MR 600. Blank wells did not contain conjugates. For inhibition studies, 50 μL of 2.5 μg/mL Sigma WGA-HRP was added to 50 μL serial dilutions of inhibitors in PBS.

ELLA
with Polymer Adsorbents

The method above was repeated except that copolymers **38** or **39** were used to coat the microplate wells at 10, 3.3, 1.0, 0.3, and 0.1 μg/mL for 3 hours at room temperature.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE was performed with a BIORAD Mini-Protean II Dual Slab Cell. The procedure was followed from the instruction manual. Briefly, a 7.5% acrylamide/0.66% bisacrylamide polymer gel with 0.1% SDS in 0.375 M Tris buffer pH 8.8 was prepared. Protein samples (2 mg/ml PBS) were dissolved in a sample buffer containing Tris buffer, glycerol, SDS, mercaptoethanol, and bromophenol blue. Then 10 μL of the samples were loaded on the gel and run for 30 minutes at 160V, ≈10 mA.

Chapter 6

Anti-Sialic Acid Antibodies

6.1 Introduction

Sialic acid is known to be involved in the binding site of antibodies in auto-immune diseases, and in cancer diagnostic antibodies against GD_3 and GM_3 as discussed in sections 1.2.5.4 and 1.2.5.5. The main objective of this thesis is to determine whether sialic acid can act as an immunogen. Chapter 5 demonstrated the antigenic properties of NeuAc polymers and neoglycoproteins with WGA, but it remains to be seen whether antibodies would be formed against NeuAc.

It is very desirable to produce antibodies with specificity for sialic acid since they could be used as a tool in a wide variety of circumstances where sialic acids are on cell surfaces. It would also be interesting to determine if mammals are able to produce antibodies against NeuAc. Since the carbohydrate is present on all cell surfaces, it may result in autoimmune disease. An animal must be chosen which expresses mostly N-glycolylneuraminic acid .

Alternately, if NeuAc is not immunogenic, it could be useful as a "shield" to increase the lifespan of conjugated drugs in the bloodstream. Or as a "trap" to adhere to viruses and bacteria which use sialic acid as a receptor.

6.2 Immunization of Rabbits

Sialic acid-protein conjugates were used as immunogens in rabbits to elicit antibodies. The protocol for this procedure was established in the early 1900's by Landsteiner who attached small molecules (haptens) to proteins, and was able to obtain antibodies specifically against the hapten moiety (Erlanger 1980).

The synthesis of the NeuAc-protein conjugates (**34**) used was discussed in Chapter 4. The actual immunogens used were prepared starting from compound **10b**, and hence there was no possibility of NeuAc methyl ester being present in the conjugate.

Californian white rabbits (2 to 3 kg) were immunized with 22 NeuAc/BSA (**34a**) and 10 NeuAc/TT (**34b**) conjugates in Freund's complete adjuvant (CFA). The adjuvant, which is a mixture of emulsifier, mineral oil and attenuated mycobacteria, acts to increase the immune

response. The immunization was 50 μg NeuAc conjugate in 250 μL CFA at both intramuscular and subcutaneous sites. On day 14 a booster injection was given, and the serum samples were collected on day 24. The sera collected from four rabbits were labelled as follows: Samples #305, #306 were obtained from rabbits inoculated with NeuAc/BSA (**34a**), and samples #307 and #308 were obtained from rabbits inoculated with NeuAc/TT (**34b**).

6.3 **Immunochemical Properties of Antibodies**

Some preliminary work was done by co-workers on the sera by quantitative precipitation using the NeuAc/protein conjugates, and these results showed there had been an immune response. Cross reactivity of the anti-NeuAc/BSA anti-serum with NeuAc/TT and vice versa gave an indication that there were antibodies against the common epitope, sialic acid. This work will be published elsewhere. Definitive proof of anti-sialic acid antibodies came from the ability of NeuAc polyacrylamide copolymers **39b**, which have only NeuAc in common with the proteins, to interact with antibodies in the sera, and it is these results which shall be discussed in detail hereafter. The general methods are described in section 1.2.4.2, and the details are given in section 6.5.

6.3.1 Double Immunodiffusion

Agarose gel double immunodiffusion following the method of Ouchterlony (Johnstone 1982) was done using the rabbit sera and both allyl-NeuAc polyacrylamide copolymers **38b** and ethylthiopropyl-NeuAc polyacrylamide copolymers. As can be seen from figure 6-1, only the ethylthiopropyl copolymers **39b** were able to cause precipitation in the gel.

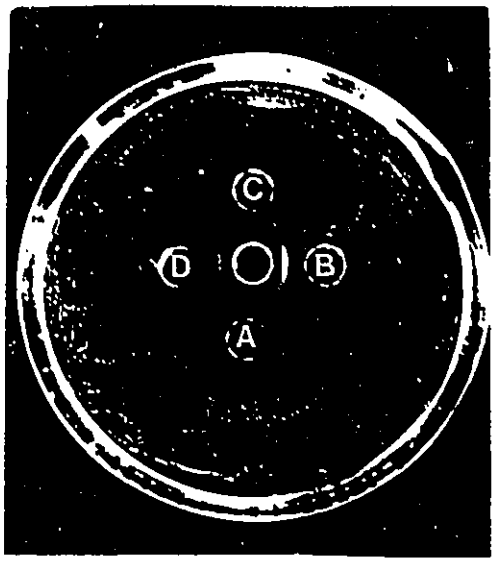
6.3.2 Quantitative Precipitation

The ethylthiopropyl-NeuAc copolymer **39b** was used as an antigen to cause precipitation of antibody protein from undiluted rabbit sera. The samples were refrigerated, then centrifuged , washed, and the protein content of the resulting pellets was measured using Lowry protein test. The advantage of using these polyacrylamide polymers is that they do not react with the Lowry reagents, and hence the method gives a direct measure of the the antibodies precipitated.

The results are shown in figure 6-2 and show maxima as expected with quantitative precipitation (Kabat 1961). The maximum value is approximately 0.5 mg/mL anti-NeuAc

antibodies from the NeuAc/TT immunization, and 0.15 mg/mL from NeuAc/BSA. These results are consistent with the fact that TT is more immunogenic than BSA.

a) Serum # 305



A) 17% NeuAc (w/w) 38b
B) 17% NeuAc 39b
C) 35% NeuAc 38b
D) 15% NeuAc 39b

b) Serum # 308

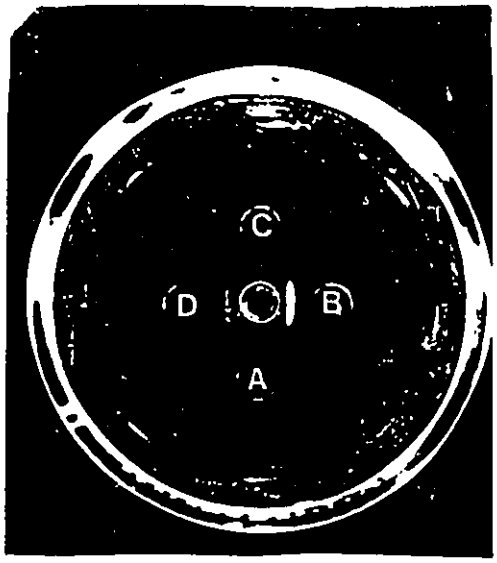


Figure 6-1 Double Immunodiffusion of NeuAc Copolymers with Rabbit Anti-NeuAc/Protein Sera

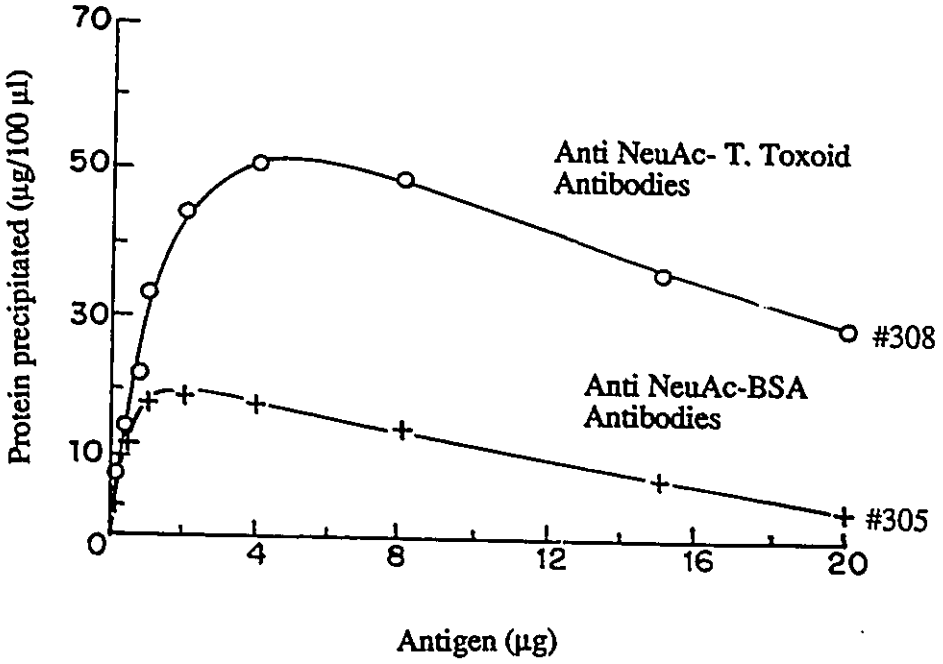


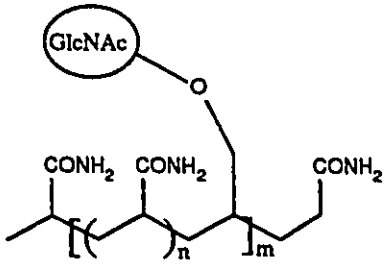
Figure 6-2

Quantitative Precipitation of Rabbit Anti-NeuAc/Protein Sera using NeuAc Copolymer **39b**

Each tube contained 100 µL undiluted sera and increasing amounts of copolymer **39b** as antigen in a total volume of 300 µL.

6.3.4 ELISA

The sera were tested for antibodies against a variety of different polymers using the enzyme linked immunosorbent assay (ELISA) technique which is more sensitive than the methods described so far. The polyacrylamide copolymers tested included NeuAcα2-3gal-hexanediamine copolymer **41**, allyl-NeuAc **38b**, allyl-GlcNAc **42** and ethylthiopropyl-rhammnose **43**, as well as the ethylthiopropyl-NeuAc copolymer **39b**. (**42** and



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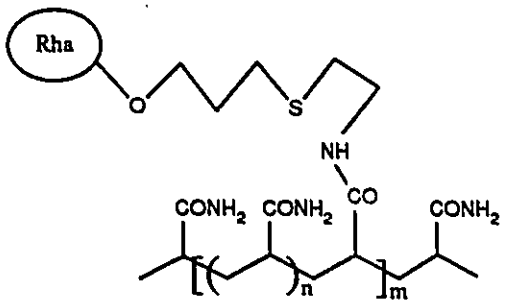


43

43 were obtained from F. Tropper (Roy 1988b, Roy 1988e).) The polymers (dissolved in buffer) were coated overnight in microplate wells, and then after blocking with BSA, the anti-NeuAc/TT serum #308 was added. After incubation, the plates were washed, and Protein A coupled to HRP was added to detect the presence IgG. Only when polymer **39b** was used to coat was there any response above the background. In fact, the GlcNAc polymer **42** was used as a blank in subsequent studies.

A complete study of the ELISA response (Engvall 1980) was then undertaken to determine the titer of the sera and the limiting quantity of polymer required to coat the plate as a function of the NeuAc content in the polymer. The results are presented in figure 6-3 a and b. The 32% (w/w) ethylthiopropyl-NeuAc copolymer **39b** was chosen since it gave the highest response. According to the plateau of the curves, coating at 1 μg per well in PBS overnight should be enough to saturate the well surface and ensure a maximum response.

To optimize the ELISA response, Kirkegaard-Perry-HRP labelled, affinity-purified goat anti-rabbit IgG antibodies were purchased, and found to be four times more sensitive than the equivalent Protein A Sigma product. Using the Kirkegaard-Perry reagent at a dilution of 1/500, a dilution of 1/10,000 anti-NeuAc/TT serum #308, and 1/5,000 anti-NeuAc/BSA serum #305 were found to give 50% full ELISA response, as shown in figure 6-4. This was a definite improvement over the conditions used in figure 6-3 where a dilution of 1/20 was required.

A pre-immune serum was tested to determine the natural presence of anti-sialic acid antibodies. Only background absorbances were obtained.

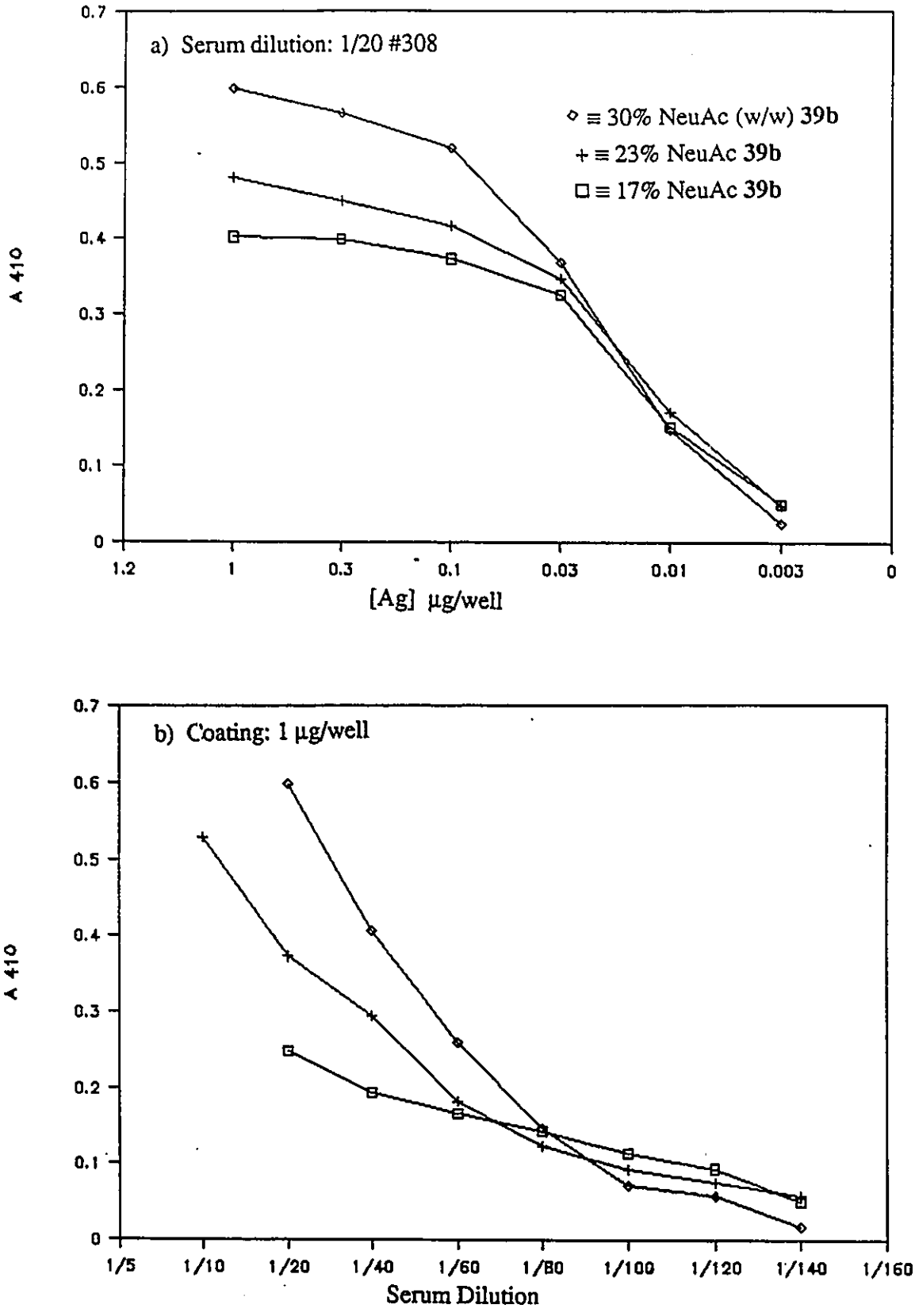


Figure 6-3 Effect of NeuAc content in the Optimization of Antigen Coating Concentration and Serum Dilution in ELISA of Anti-NeuAc/TT Serum

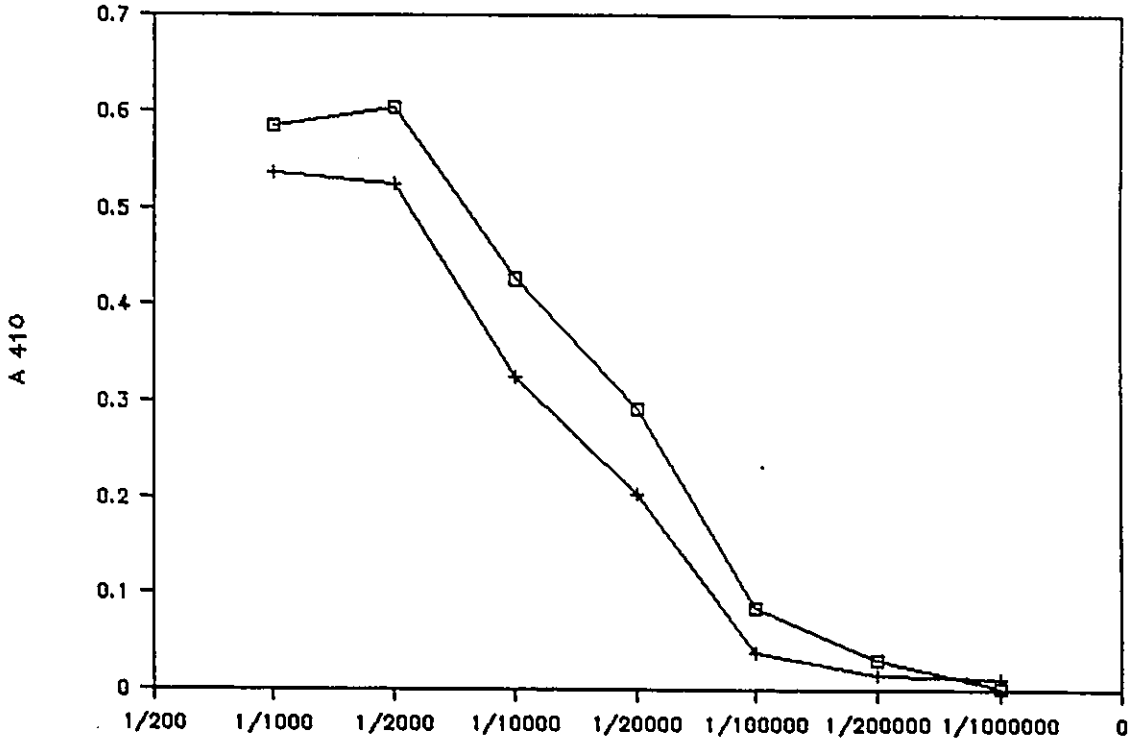


Figure 6-4 Optimization of Sera Dilution in ELISA of Anti-NeuAc/Protein Sera

□ ≡ #308 Anti-NeuAc/TT

\+ ≡ #305 Anti-NeuAc/BSA

Conditions:

Coat: 1 µg/well 32% NeuAc (w/w) 39b.

Incubate: As indicated.

Detect: 1/500 Kirkegaard-Perry anti-Rabbit IgG.

6.3.4 Inhibition of ELISA

To determine the specificity of the antibodies, a variety of derivatives were used to inhibit the interaction of the antibodies with the polymer. If a modification of a functional group of the inhibitor results in an inability to inhibit the ELISA test, this is interpreted as an essential part of the molecule in the binding site.

The ELISA test described above was repeated in the presence of different concentrations of monomers, and the results obtained are shown in figure 6-5a and b.

Chicken serum albumin was used as the blocking reagent, and there was a substantial backround absorbance with anti-NeuAc/BSA antibodies of 0.150 at 410 nm, as compared with a backround of only 0.021 with anti NeuAc/TT antibodies. This is due to the cross-reactivity of chicken serum albumin with anti-bovine serum albumin antibodies. A correction for the blank was made in the calculations to allow for 100% inhibition.

The concentration of monomer required to give 50% inhibition of the ELISA response was found to be relatively independent of the antibody concentration used. (Compare table 6-1 with results in Roy 1988). This indicates that the concentrations of monomer for 50% inhibition may be taken as relative dissociation constants and hence on the basis of the relation between free energy and equilibrium constants ($\Delta G=-RT\ln K$), $\Delta\Delta G$ values may be calculated from these data (Spohr 1985). The data are reported in table 6-1.

The experiments were repeated using gelatin as a blocking agent, and they qualitatively gave the same results. The results are not reported here since they were not done in triplicate, and therefore are not as reliable.

Most inhibitors were able to give a slight inhibition and hence, appear in the graphs in figure 6-5; however, NeuAcα2-3Galβ1-4Glc **2a** and NeuAcα2-6Galβ1-4Glc **3a** were unable to cause inhibition and do not appear in the graphs.

A preliminary analysis of the data presented in table 6-1 clearly shows that modification of both the side chain and the acid group result in a loss of the ability to inhibit, and thus these portions of the molecule appear essential in the binding site.

To a lesser extent, modification of the acetamido group result in only slight decreases in binding affinity. Hence binding to this portion of the NeuAc molecule is less stringent, though it may be required more in the anti-NeuAc/BSA than anti-NeuAc/TT antibodies. A complete loss did occur when there was no carbonyl for the anti-NeuAc/BSA antibodies, indicating the carbonyl function could be required, though a charge effect from the amine in **19** may also reduce the binding. It should be noted here that these are polyclonal antibodies (ie. heterogenous), thus a population of antibodies is being described rather than a single antibody with a well defined binding site. It may be that within this population some antibodies have a

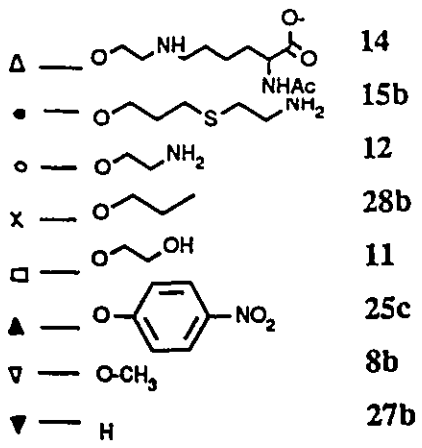
high specificity for the amide moiety.

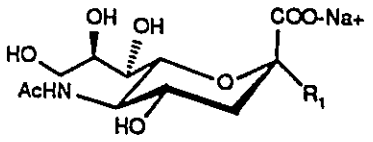
Table 6-1 Inhibition of ELISA Data for Rabbit Anti-NeuAc Antibodies to NeuAc Copolymer.

Epitope	Anti NeuAc-BSA Antibodies			Anti NeuAc-T.Toxoid Antibodies		
	Conc. for 50% Inhibition (μM)	Rel.	ΔΔG (kCal)	Conc. for 50% Inhibition (μM)	Rel.	ΔΔG (kCal)
Acid =						
COO^-Na^+ **7b**	240.	1.	0.0	100.	1.	0.0
$CONH_2$ **17**	a	a	-	a	a	-
COOMe **7a**	a	a	-	a	a	-
CH_2OH **18**	a	a	-	a	a	-
Side Chain =						
7b glycerol	240.	1.	0.0	100.	1.	0.0
23 9'OAc	a	a	-	1000.	0.1	1.36
24b CH_2OH	a	a	-	a	a	-
Amide =						
$COCH_3$ **7b**	240	1.		100.	1.	-
$COCH_2Br$ **21b**	830.	0.29	0.73	170.	0.6	0.30
$COCH_2OH$ **20**	1200.	0.20	0.95	250.	0.4	0.54
H 19	a	a	-	630.	0.16	1.08
Spacer =						
14 lysine	75.	3.2	-0.69	1.8	56.3	-2.38
15bethylthiopropyl	160.	1.5	-0.24	70.	1.41	-0.20
12 ethylamine	240.	1.0	0.0	14.	7.04	-0.15
11 ethanol	480.	0.5	0.41	50.	1.97	-0.40
28b propyl	270.	1.05	-0.03	90.	1.11	-0.06
7b allyl	240.	1.00	0.0	100	1.0	0.0
8b methyl	a	a	-	700.	0.14	1.26
25c nitrophenyl	a	a	-	1100.	0.08	1.59
27b H	a	a	-	a	a	-
2a →3lactose	a	a	-	a	a	-
3a →6lactose	a	a	-	a	a	-

[a] No inhibition at a concentration of 3 mM.

Antibody binding to the spacer portion of the molecule shows that the response varies with the immunogen used. For example, NeuAc methyl glycoside **8b** is able to inhibit the anti-NeuAc/TT antibodies more than the anti-NeuAc/BSA antibodies, indicating a greater requirement for the spacer longer than a methyl in the latter.





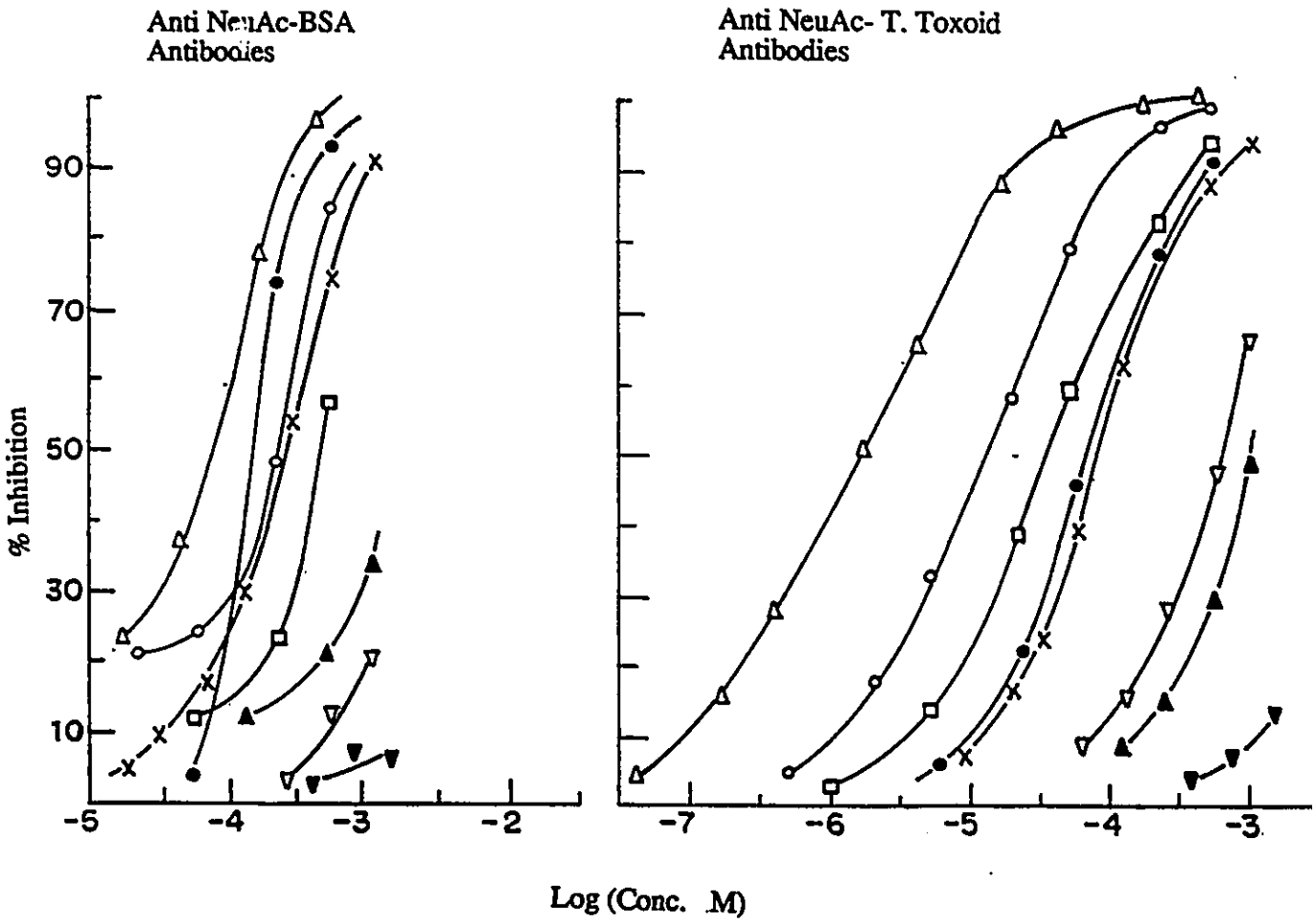


Figure 6-5a Inhibition of ELISA with Spacer Derivatives

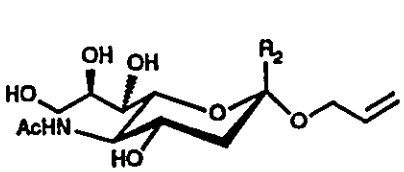
Conditions:

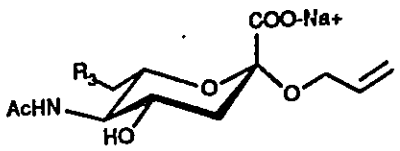
Coat: 1 µg/well 32% NeuAc (w/w) 39b.

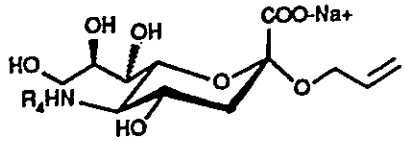
Incubate: 1/5000 #305

or 1/10,000 #308

Detect: 1/2000 Kirkegaard-Perry anti-Rabbit IgG.







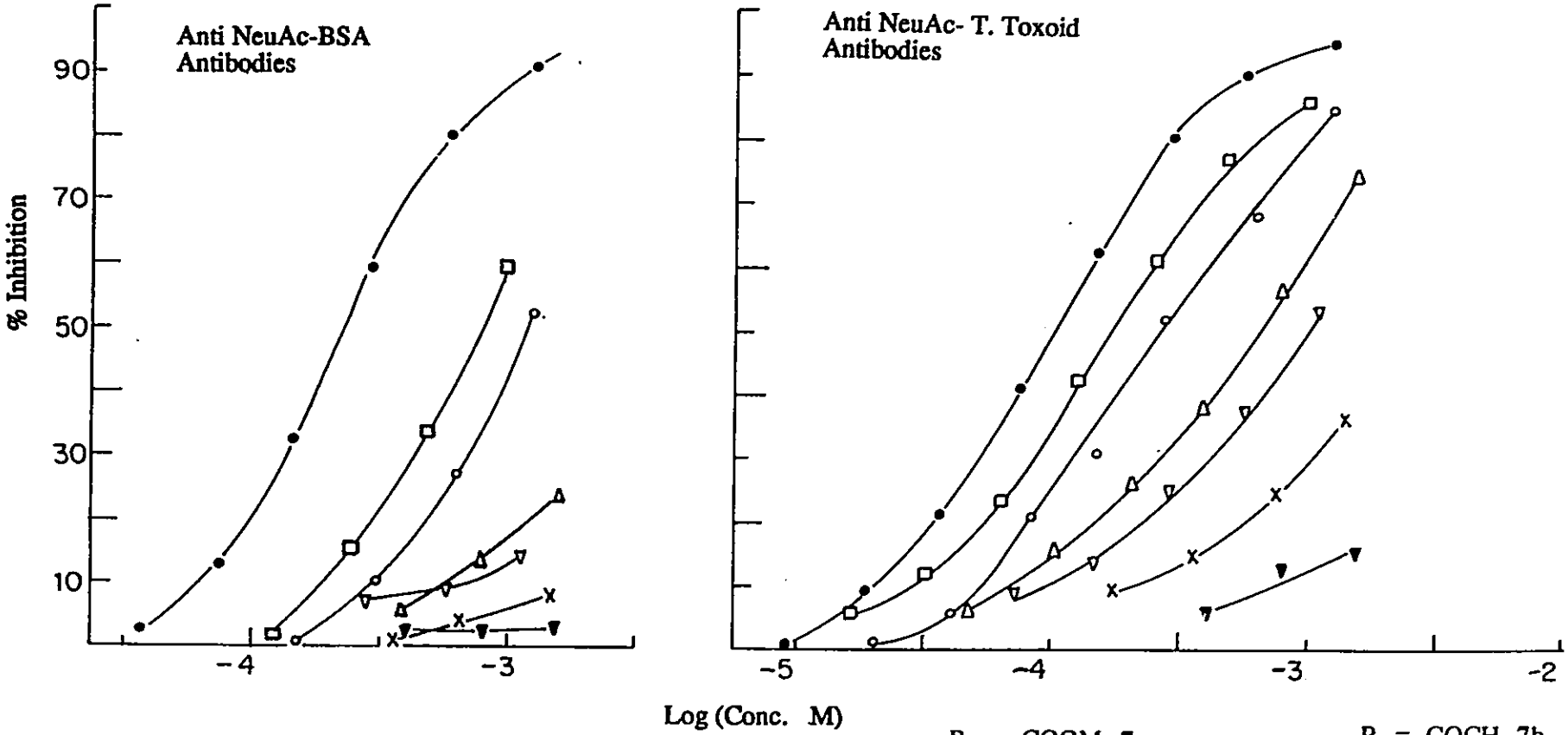


Figure 6-5b

Inhibition of ELISA with NeuAc Functional Group Derivatives

x $R_2 \equiv$ - COOMe 7a

▽ $R_3 \equiv$ -CHOHCHOHCH$_2$OAc 23

▼ $R_3 \equiv$ -OH 24b

• $R_4 \equiv$ -COCH$_3$ 7b

□ $R_4 \equiv$ -COCH$_2$Br 21b

o $R_4 \equiv$ -COCH$_2$OH 20b

△ $R_4 \equiv$ -H 19

There is also some requirement for a heteroatom in the aglycon as the ethyllysine aglycon (**14**) has a higher binding energy than the ethylthiolpropyl derivative (**15b**). For example, the anti-NeuAc/TT antibodies bind the ethyllysine derivative **14** with 2.18 more kCal than with the ethylthiopropyl derivative **15b**. On the other hand, the anti-NeuAc/BSA antibodies do not require the heteroatom as much, there being only a 0.45 kCal difference between those two inhibitors. This indicates that while the anti-NeuAc/BSA antibodies require at least an ethyl aglycon, the next atom in the chain is not significant, and only a slight amount of energy (0.69 kCal) is obtained from anything beyond the length of an allyl group.

In both cases the 2-deoxy derivative **27b** was unable to cause inhibition at a concentration of 5 mM, indicating a possible requirement for the anomeric oxygen in the binding site. Neither the NeuAcα2-3Galβ1-4Glc **2a** or NeuAcα2-6Galβ1-4Glc **3a** were able to inhibit at all, though the *p*-nitrophenyl glycoside **25c** did, indicating that the hydrophobic spacer had less spatial requirements than the hydrophilic ones. Again these results confirm a degree of binding with the spacer.

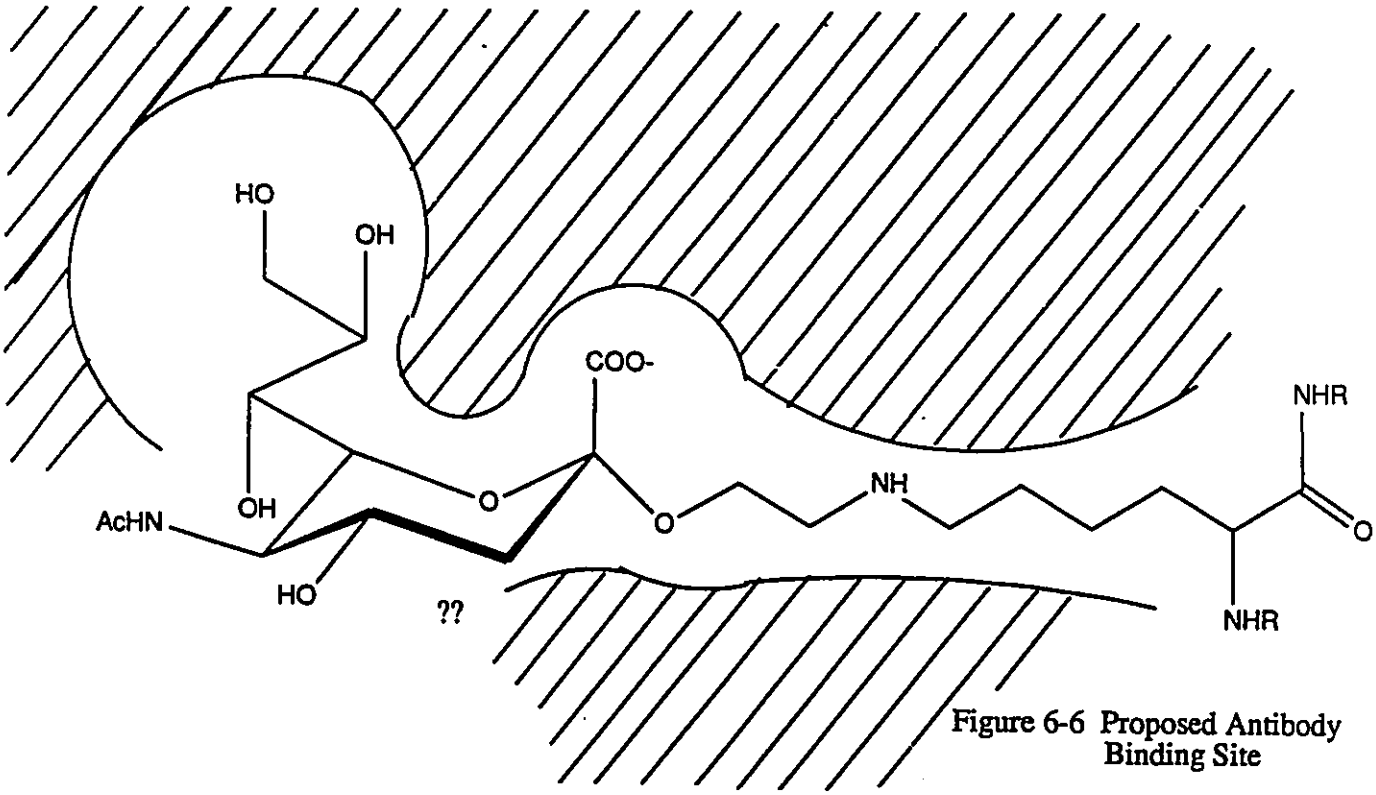


Figure 6-6 Proposed Antibody Binding Site

6.4 Discussion

It is apparent from the results obtained with rabbit antibodies that immunization of rabbits with NeuAc/protein conjugates resulted in the production of antibodies which have specificity to sialic acid. The double immunodiffusion studies indicate that there is some spatial requirement in the aglycon, as no interaction could be observed with polymers where NeuAc was linked closely to the polymer backbone. This result was confirmed with the more sensitive ELISA experiments.

On the basis of inhibition studies, a model of the binding site is presented in figure 6-6. This model shows a relatively loose binding around the acetamido group, because modification of this functionality did not result in a total loss of inhibitory power with the anti-NeuAc/TT antibodies, though the amide carbonyl may be required for the anti-NeuAc/BSA antibodies. The glycerol side chain, acid function and anomeric oxygen are essential in binding, and have been shown in the model. No information was obtained concerning the ring oxygen or the C4 hydroxyl.

It should be stressed that the antibodies discussed here are polyclonal, and hence what is observed is an "average" which is biased to the coating antigen used. In this study, the coating antigen had a long hydrophobic spacer, and naturally antibodies with affinity for this spacer would be selected.

A preliminary screening (results not shown) for antibodies with affinity for NeuAcα2-3Gal showed some promise, but the background levels were quite high, and the results were not reproducible. If antibodies with this specificity are present in the sera, their concentration is quite low. Affinity chromatography would be required to purify them. These results point to the importance of the spacer arm in affecting the immune response towards a particular hapten. In the case of NeuAcα2-3Gal, the galactose is probably too bulky to fit in the binding site of most of the antibody clones selected in our ELISA test. These results must be kept in mind in the future design of immunogens to obtain antibodies with decreased dependence on the spacer.

6.5 Experimental Methods

Antibody Quantitiative Precititation (Kabat 1961)

Undiluted anti-NeuAc/TT (#308) or anti-NeuAc/BSA (#305) anti-sera (100 µL) were placed in 1.5 mL MCC tubes (Hughes and Hughes Ltd., Elms Industrial Estate, Church Road, Harold Wood, Romford, RM3 OHR, Essex). Aliquots (5.0 to 100.0 µL) of NeuAc-spacer-polyacrylamide copolymer **39b** (14% NeuAc (w/w), 1.0 mg/mL in PBS) were added to give final volumes of 300 µL. The solutions were agitated and allowed to incubate for 3 days at 4°C. Then the tubes were centrifuged for 5 minutes at 14,000 g in a Fisher MicroCentrifuge Model 235B, and allowed to incubate for 2 more days at 4°C. After centrifuging again, the pellets were washed twice with 300 µL cold PBS with centrifuging between washes. The protein content of the pellets was determined after addition of 100 µL 0.1N NaOH using Lowry protein test standardized with human gamma globulin.

ELISA (Enzyme-linked Immunosorbent Assay)

Different conditions were employed to optimize the sensitivity of ELISA as noted in the figures. The following is the procedure followed to obtain inhibition results.

NUNC (Denmark) microanalysis plates were rinsed with 95% ethanol, dried, and coated overnight with NeuAc-spacer-polyacrylamide copolymer **39b** (32% NeuAc (w/w), 10 µg/mL in PBS, 100 µL/well).

The wells were blocked with chicken serum albumin (0.1% in PBS, 100 µL/well, Sigma, Fraction V, N° A-3014) for 20 minutes.

Inhibitors were added in serial dilutions from 2 mg/mL at 50 µL/well in PBS. Then diluted rabbit anti-NeuAc/TT antiserum (1/5000 dilution, 50 µL/well, #308) was added. Or alternately, pre-absorbed diluted rabbit anti-NeuAc/BSA antiserum (1/1250 dilution, 50 µL/well, #305 pre-absorbed with BSA) was added. (Pre-absorption was done as follows: 1 mg BSA was added to 2 mL serum, and then incubated for 4 days at 4°C. The precipitate was centrifuged down at 14,000g and the serum was removed.) The final sera dilutions were 1/10,000 and 1/5000 respectively. The inhibitors were allowed to equilibrate for 6 hours. One plate was used for each inhibitor.

The plates were washed 5 times with water, then 100 µL of 1/2000 diluted Kirkegaard Perry peroxidase labelled affinity purified goat antibody to rabbit IgG (H+L) was added and allowed to incubate for 3 hours.

The plates were washed 5 times with water, then 50 µL 1 mg/mL ABTS in phosphate-citrate buffer (0.2M pH 4.0 with 0.01% H_2O_2) was added. After 30 minutes, aqueous KCN (0.27 µM, 5 µL) was added to stop the reaction, and the absorption was read at

410 nm relative to the absorption at 570 nm on a Dynatech MR 600 microplate reader. All manipulations were done at room temperature.

Calculations were done as follows. Samples in edge wells were excluded. Values for three samples were averaged. Blanks contained allyl-GlcNAc copolymer 42 and had a background absorbance of 0.020 and 0.150 for anti-NeuAc/TT (#308) and anti-NeuAc/BSA (#305) antisera respectively.

$$\%\text{Inhibition} = \frac{A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})}}{A_{(\text{no inhibitor})}} \times 100$$

where $A_{\text{no inhibitor}}$ is the absorbance of the sample after correction for the control. $A_{\text{with inhibitor}}$ is the absorbance of the sample with inhibitor after correction for the control.

Chapter 7

Nuclear Magnetic Resonance Studies

7.1 Introduction

Sialic acid is a salt at neutral pH. In our study, crystallization was difficult, if not impossible, and good elemental analyses frequently were not obtained. For this reason heavy emphasis was placed on n.m.r. spectroscopy to verify the structure and purity of the compounds prepared.

N.M.R. analysis was also used to probe the conformation of NeuAc derivatives. This was done in combination with computer modelling, and may provide valuable insight into antibody-NeuAc interaction at a molecular level. Current theories of the forces in these interactions include hydrogen bonding with precise orientation, hence an exact spatial model is a prerequisite for a detailed analysis.

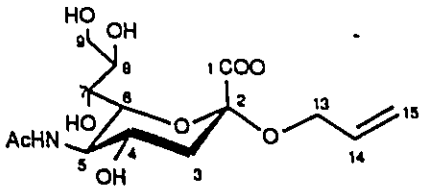
This chapter begins with a survey of ^{13}C and ^{1}H n.m.r. results for the derivatives, and then proceeds to a detailed analysis of pKa of NeuAc and analogues. Finally, the conformation of the GM_3 oligosaccharide lactone (**2b**) is discussed and a model is proposed.

7.2 ^{13}C NMR

^{13}C n.m.r. spectra were obtained on either a Varian XL-300 or Varian Gemini-200 using standard pulse sequences. Samples were 10 to 20 mg in D_2O with an external dioxane referenced to 67.39 ppm. The XL-300 had accuracy better than ±0.1 ppm but the Gemini-200 was found to vary by as much as ±0.3 ppm.

In some instances ADEPT spectra were taken to help assign aglycon resonances, but in most cases assignments were made by a comparison with published assignments (Schauer 1982, Sabesan 1986, Berman 1984).

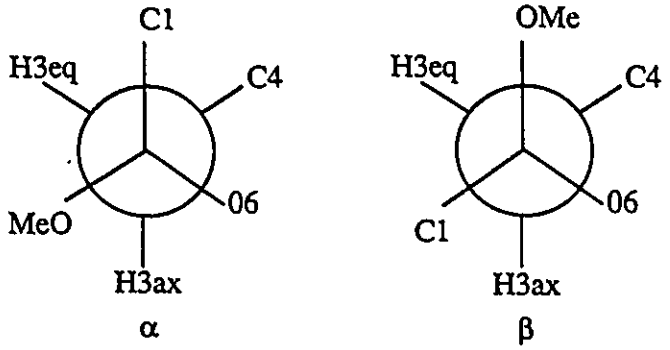
The ^{13}C n.m.r. spectra were particularly useful in verifying chemical modification since the spectra, especially for NeuAc, tend to vary little from compound to compound (Sabesan 1986). For example, the acetamido carbonyl does not vary greatly, and was 176.3 ± .4 for twenty different compounds. Likewise, the allyl function, which was in many of the compounds, varied little and appeared at 119.4±.3 and 134.5±.5 ppm for C15 and C14 respectively. There was a greater variability for the carboxyl C1, which is sensitive to pH and esterification (Schauer 1982). In this thesis it was found that the ester generally appeared at



171.2 ppm and the carboxylate was at 174.6 ppm with some variation.

7.2.1 Anomeric Configuration

Since NeuAc does not have an anomeric hydrogen, the ^{1}H coupling cannot be used to determine the anomeric configuration. It was also found that the ^{13}C chemical shift of the anomeric carbon could not be used since there was no clear trend. Therefore, to verify the α or the β configuration, gated decoupled spectra were taken (p.166 Schauer 1982, Hori 1988). In the α case, coupling between C1 and H3ax, which are diaxial, is expected to be large. In fact, a



coupling constant of 5.7 Hz was observed at C1 for compound **7b**. On the other hand, no coupling (or very small < 1 Hz) was observed at C1 of compound **9b**, which is consistent for the β configuration since C1 bisects H3ax and H3eq at 60°. This experiment also led to a different assignment for the reported (Jennings 1977) carbonyl ^{13}C resonances in the methyl glycoside.

7.3 ^{1}H NMR

The ^{1}H n.m.r. spectra were taken in D_2O unless otherwise stated, on a Varian XL-300 using 3 to 5 mg samples referenced to internal acetone at 2.225 ppm or DMSO at 2.723 ppm at 25°C.

The initial assignments were made on the basis of an homonuclear shift correlated spectrum (HOMCOR) of compound **8b** and were subsequently verified by comparison with

the literature (Schauer 1982). Accuracy was within ±0.003 ppm for most resonances. Other spectra were assigned by comparison with the clearly identifiable coupling patterns associated with each hydrogen in the NeuAc molecule (Berry 1985). Sialyllactose matched well with published identities (Platzer 1989).

7.3.1 Anomeric Configuration

As has been discussed in section 7.2.1, the anomeric configurations of two compounds were confirmed by a gated decoupled experiment. These two compounds, **7b** and **9b**, show very different signals for H3eq at 2.753 and 2.339 ppm respectively, corresponding to the α and β anomers. It is a general observation that H3eq is shifted downfield in the α configuration (Schauer 1982). This is true for all compounds in this thesis except those with the β configuration and the carboxy reduced compound **18**.

7.3.2 Ester vs. Acid

The methyl ester appears at 3.87 ±.02 ppm for all compounds except the ester of the 2-deoxy derivative **27a** where it appears at 3.80 ppm. This signal was particularly important in confirmation of the ester hydrolysis after reductive amination (section 4.2.1.2). It was clearly present in the spectra of polyacrylamide copolymers synthesized with methyl ester monomers **7a** and **16a** (data not shown).

Upon hydrolysis of the ester, H3eq tends to be deshielded by ≈ .03 ppm and H3ax is shielded by up to 0.16 ppm. One might expect shielding to occur with the presence of a negative charge, hence H3eq may be experiencing anisotropic effects. An alternate explanation based on charge density calculations is considered in section 7.5.2.

7.3.3 Coupling Constants

The coupling constants are useful in providing an indication of conformation, but are limited by the resolution obtained on a 300 MHz instrument. The data appear in table 3-1 (p.63).

The coupling constants do not change for all NeuAc derivatives, indicating the molecule remains in the $^5C_2$ ring conformation.

The glycerol side chain (C7 to C9) coupling constants also do not change where they can be determined, except for two cases. In the de-N-acetylated compound **19**, $J_{H7\text{-}H8}$ is 11.0 Hz compared to 9.0±.2 for all other compounds. The result may be due to a change in

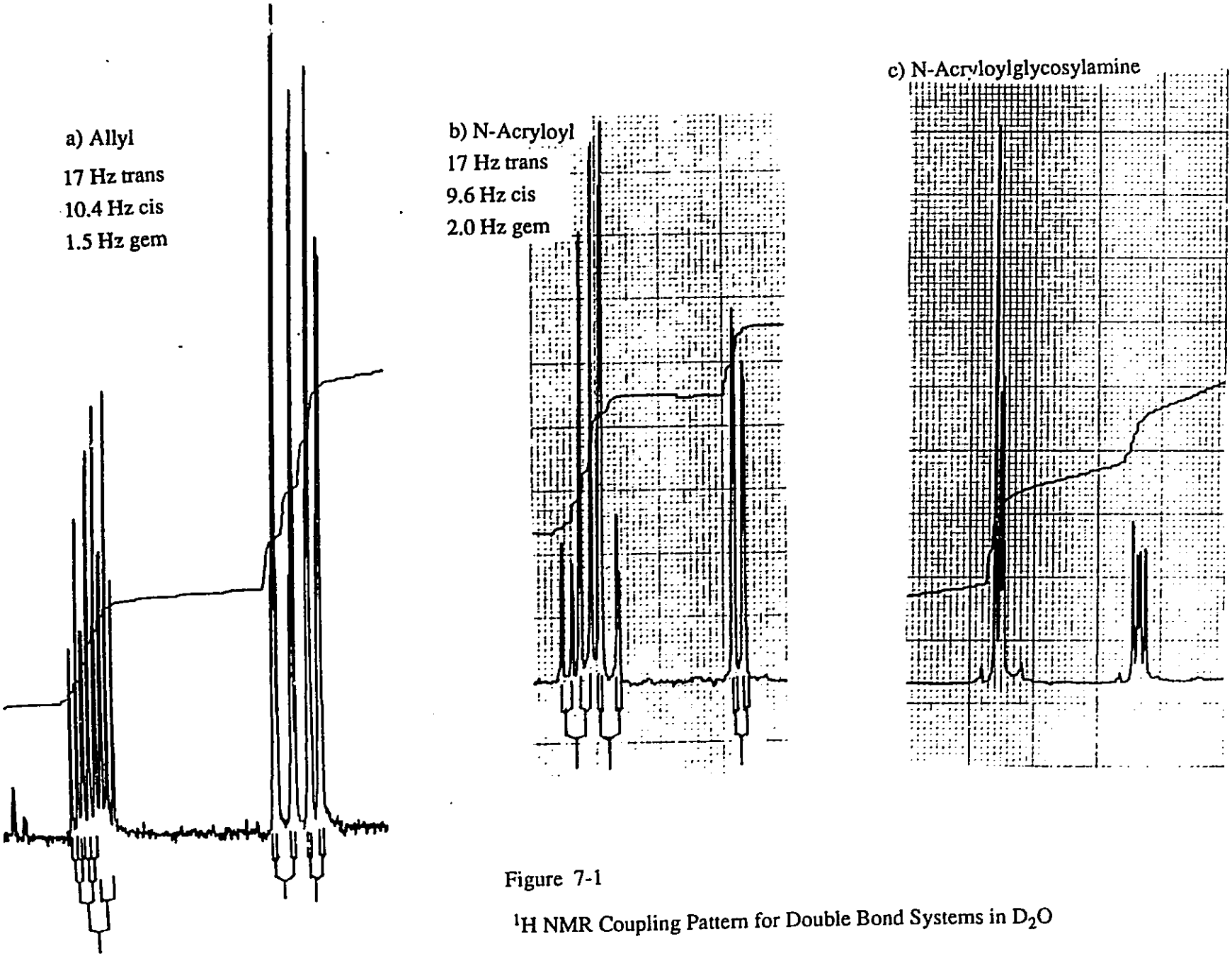


Figure 7-1

^1H NMR Coupling Pattern for Double Bond Systems in D_2O

electronegativity from the loss of the acetamido rather than conformational change, though some groups predict hydrogen bonding between C7 hydroxyl and the acetamido NH (Schauer 1982). The other case is J_{H6-H7}, and is considered with respect to molecular modelling in section 7.4.

Typical 300 MHz spectra in D_2O for the allyl and acryloyl moieties are shown in figure 7-1. It is interesting to note that the ABX coupling pattern for the N-acryloylglycosylamines in D_2O is more recognizable in DMSO-d6 (data not shown).

7.4. Molecular Modelling

Much of the information about the conformation of NeuAc (Schauer 1982) has been refined (Poppe 1989, Christian 1987, Sauter 1989, Wright 1980, Veluraja 1980).

In this thesis, molecular modelling was performed using MM2(1982) calculations in the program TRIBBLE obtained from Dupont de Nemours, Inc. and modified by Herman (1987).

MM2(1982) is a widely used method for the evaluation of conformational energies (Allinger 1977). The force field is parameterized well for hydroxyl containing compounds and will reproduce anomeric effects (Narskov 1984). Electrostatic effects are not calculated, but are taken into account by dipole interactions. It is suggested (Dabrowski 1989) that MM2 calculations are more reliable than Hard Sphere Exo-Anomeric (HSEA) (Bock 1983) due to the excessively high energy terms of the latter.

Energy minimization of **1a** gave results essentially the same as previous molecular modelling (Veluraja 1980) and X-ray crystallography (Flippen 1973).

Table 7-1 shows the calculated dihedral angles for the methyl glycosides **8b** (α) and **9b** (β) and compares the experimental coupling constants with coupling constants calculated from the model dihedrals.

Table 7-1 Calculated Dihedral Angles from MM2(1982)

	Dihedral Angle (MM2)		Coupling Constant Calculated ($10\cos^2\phi$ Hz)		Coupling Constant Experimental (±.2 Hz)	
	ϕ	ϕ				
	8b(α)	**9b(β)**	**8b**	**9b**	**8b**	**9b**
H_{3ax}-H_4	172°	174.5°	9.8	9.9	11.7	11.3
H_{3eq}-H_4	57°	60°	3.0	2.5	4.6	4.7
H_6-H_7	58°	64°	2.8	1.9	1.9	1.0
H_7-H_8	180°	179	10.0	10.0	9.1	9.1
H_8-H_9	61°	58°	2.4	2.8	2.6	2.7

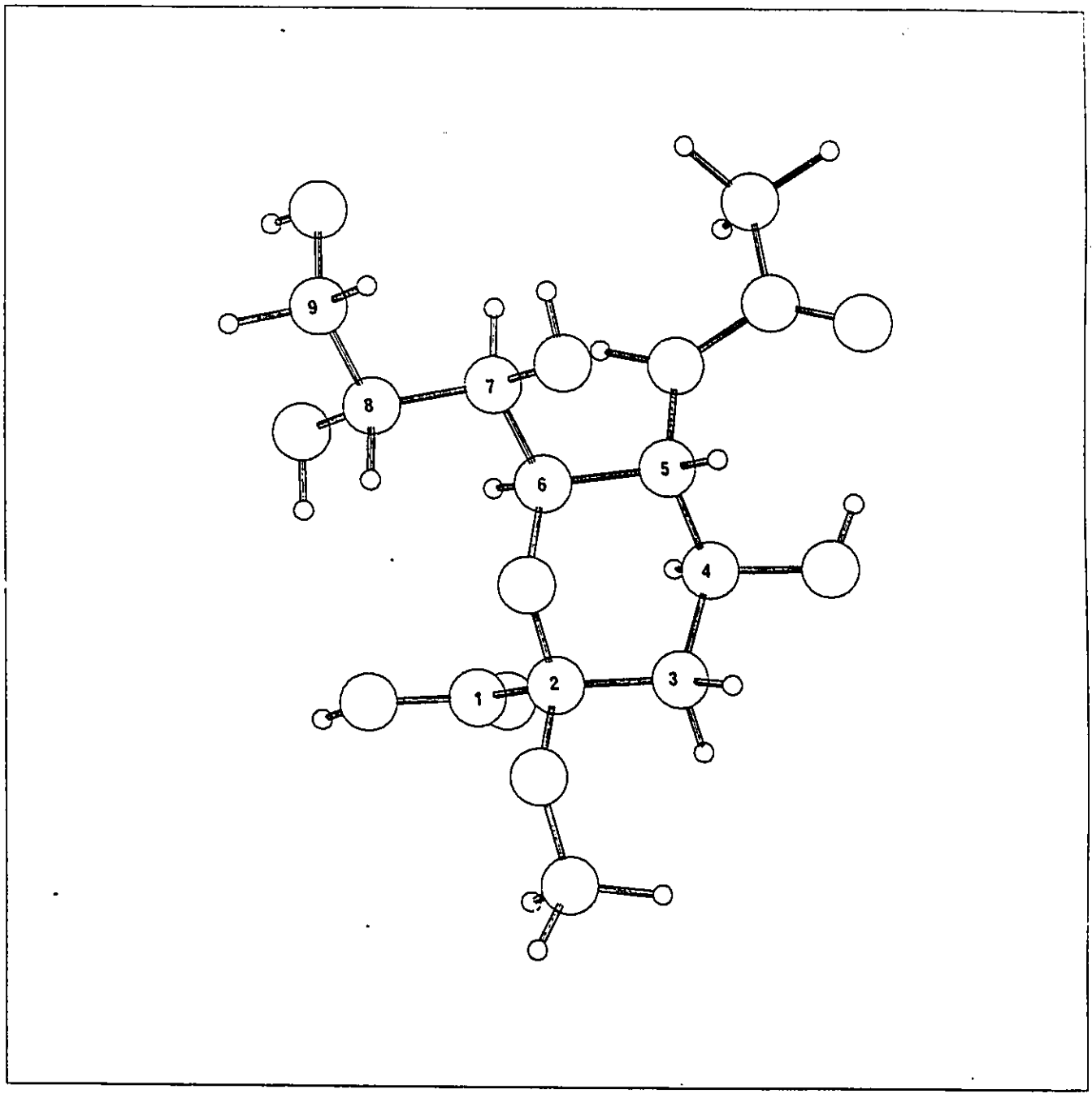


Figure 7-2a MM2 Energy Minimum Conformation of α-NeuAc (**8b**)
(Courtesy Lee Herman 1987)

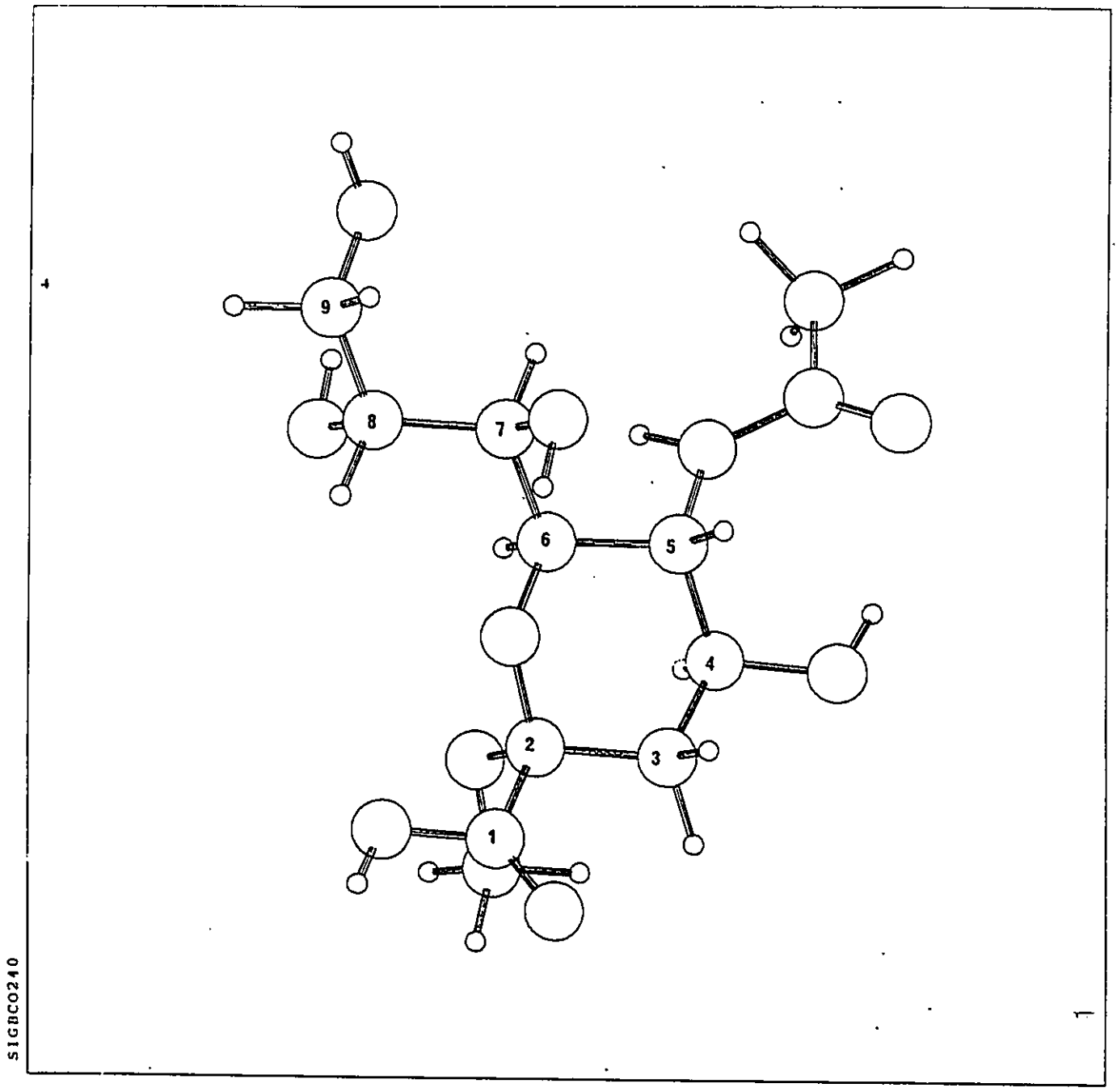


Figure 7-2b MM2 Energy Minimum Conformation of β-NeuAc (9b)
(Courtesy Lee Herman 1987)

The coupling constants for compounds **8b** and **9b** are almost identical, except for $J_{H6\text{-}H7}$ which shows a difference larger than the experimental error. The modelling quantitatively predicts this change in angle. This change in angle could be the result of hydrogen bonding postulated between the C8 hydroxyl and the carboxyl in the α configuration based on the large change in the C8 ^{13}C n.m.r. resonances (Jennings 1977). Figure 7-2 shows α and β NeuAc as minimized by MM2(1982). Note that in the α anomer the C8 hydroxyl proton is pointed toward the carboxyl. MM2(1982) is able to estimate hydrogen bonding on the basis of dipole-dipole interactions (Herman 1987). By rotating the C8 hydroxyl, the energy of the interaction was estimated to be 4.1 kCal/mol. Similarly, an energy of 1.4 kCal/mol was calculated between C4 hydroxyl and the acetamido carbonyl. These models did not show any interaction between the C7 hydroxyl and the acetamido function.

7.5 pKa Measurements

The outstanding feature of NeuAc which differentiates it from so many other carbohydrates is the fact that it is an acid. The negative charge that it imparts to its environment is largely responsible for its properties and function.

The effect of pH on the n.m.r. spectra of sialic acids has been used to make assignments of peaks (Jennings 1977, Berman 1984). These data could be used to measure pKa, but the values reported in the literature were obtained by potentiometric titration (Scheinthal 1968, Schneir 1962, Heyworth 1957, Trucco 1954) and there has been no consistent value reported for the α-glycoside (Schauer 1985).

As part of a study of the binding of NeuAc to serotonin, the ^1H n.m.r. spectra of NeuAc versus pH were taken in order to reproduce the results obtained by Berry (1985). These data were then expanded and used to calculate pKa. To complement these results, molecular modelling and MNDO calculation were undertaken in collaboration with Dr. Lee Herman and have given some insight into the stereoelectronic effects of the carboxyl in NeuAc.

7.5.1 Experimental pKa Values and Theoretical Considerations

The experimental results for the ^1H n.m.r. titration of the α-methyl glycoside (**8b**) and the β-methyl glycoside (**9b**) of NeuAc are shown in figure 7-3. The data for each proton were fit to equation 7-1 (see section 7-7) using least squares, and gave calculated values for K_a (the acidity constant) and δ_{hs} (the chemical shift of the acid). The data were also fit to the equation using non-linear least squares (SAS 1984) and gave essentially the same results.

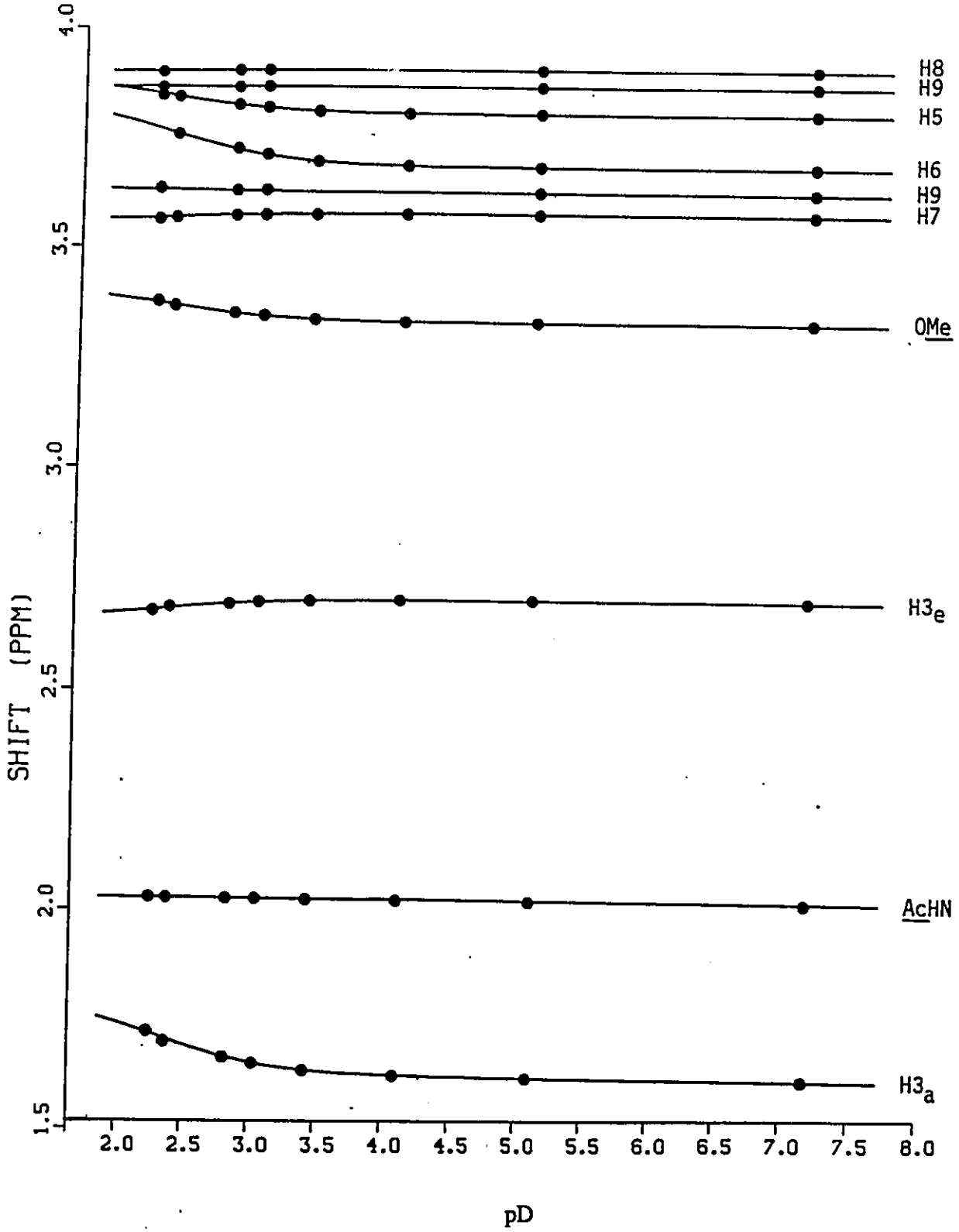


Figure 7-3a ^{1}H NMR Titration of α-NeuAc (8b) in D_2O

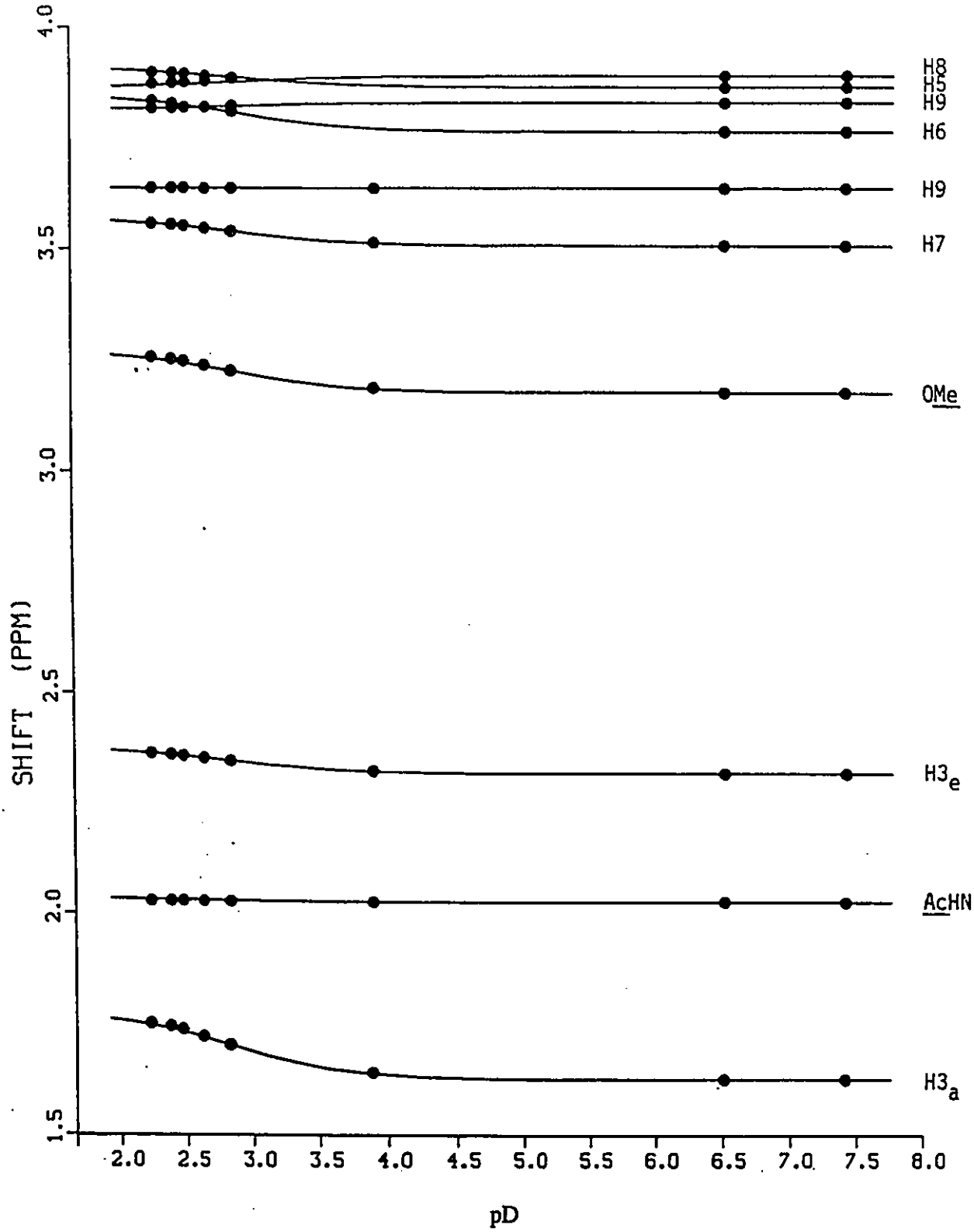


Figure 7-3b ^1H NMR Titration of β-NeuAc (9b) in D_2O

In this same manner, pKa values were obtained for different compounds and the results are summarized in table 7-2.

Table 7-2 Experimental pKa Values and Calculated Heat of Deprotonation for Selected Compounds

Compound		pKa[a]	pKa (lit.)	ΔH deprotonation (kCal/mole)[b]
1a	NeuAc	2.5 ±.2	2.6[c]	-14.4
2a	NeuAc(2-3)lactose	1.94±.04	2.08[d],2.2[e]	
8b	αNeuAcOMe	1.87±.02		-18.1
9b	βNeuAcOMe	2.37±.07		-16.2
24b	αHeptuloside	2.3 ±.1		
27b	α-2-deoxyNeuAc	2.77±.05		

[a] Calculated from nmr titration data. See section 7.7 for details. [b]Based upon MNDO calculations. See section 7.7 for details.[c] (Schienthal 1968), [d](Trucco 1954), [e] (Heyworth 1957)

Each proton in the n.m.r. of a compound was used to calculated K_a, and the average value is reported in the table. In the least squares analysis, the correlation coefficient was generally greater than 0.94 and standard deviations for K_a were less than 5%. Since this was quite low, the error values reported in table 7-2 are based on the standard deviations from the average.

The ΔH values do not give absolute estimates of pKa since there are no estimates of entropy, but the trend is in the correct direction. As the ΔH decreases, the pKa decreases.

It is interesting to note that compound **9b** and **24b** have similar pKa values. As discussed in section 7.4, in the β anomer there is no hydrogen bonding between C8 hydroxyl and the carboxyl, and likewise in **24b** the glycerol side chain has been removed, also eliminating C8. This implies that 0.4 pKa units of acidity is due to intramolecular hydrogen bonding, and this is consistent with other examples (March 1985).

A comparison of **1a** and **9b** shows the effect of the hemi-ketal versus ketal, and the latter, being more electron withdrawing is slightly more acidic. This is also confirmed by the MNDO calculations which predict a greater ΔH. Furthermore, compound **27b**, which is not a ketal at all, has the highest pKa, even though it is in the α configuration.

These results may be summarized by three factors which result in the high acidity of NeuAc:

i) the electron withdrawing effect of the ketal,

ii) intramolecular hydrogen bonding between C-8 hydroxyl and C-1 carboxyl in the α configuration, and

iii) stereoelectronic effects predicted by MNDO calculations, possibly involving σ* antibonding orbital overlap between C-1 and one of the ring oxygen lone pairs.

Table 7-3 MNDO Calculated Charge Density Changes Compared to Experimental N.M.R. Resonance Changes

Hydrogen	Charge Density difference[a] (e)	Chemical Shift difference[b] (ppm)	Calculated Chemical Shift difference[c] (ppm)
βNeuAc **1a**			
H3-ax	+0.0102	+0.078	+0.182
H3-eq	+0.0134	+0.122	+0.239
H4	+0.0120	n.r.	
H5	+0.0120	+0.031	+0.214
H6	+0.0791	+0.084	+1.408
H7	+0.0246	+0.061	+0.438
H8	-0.0250	-0.010	-0.445
H9	+0.0087	+0.017	+0.155
H9'	+0.0050	+0.000	+0.089
$NHCOCH_3$	+0.0080	+0.006	+0.142
βNeuAcOMe **9b**			
H3-ax	+0.0100	+0.155	+0.178
H3-eq	+0.0123	+0.059	+0.218
H4	+0.0009	+0.052	+0.016
H5	+0.0058	+0.048	+0.103
H6	+0.0132	+0.088	+0.234
H7	+0.0245	+0.066	+0.436
H8	-0.0261	-0.022	+0.465
H9	+0.0087	n.r.	+0.128
H9'	+0.0042	+0.004	+0.075
$NHCOCH_3$	+0.0079(ave)	+0.012	+0.141
OCH_3	+0.0179(ave)	+0.097	+0.459
αNeuAcOMe **8b**			
H3-ax	+0.0451	+0.174	+0.802
H3-eq	-0.0042	-0.049	-0.075
H4	-0.0241	n.r.	-0.429
H5	+0.0177	+0.080	+0.315
H6	-0.0214	+0.154	-0.381
H7	+0.0183	-0.021	+0.326
H8	-0.0053	-0.007	-0.094
H9	+0.0117	+0.006	+0.208
H9'	+0.0107	+0.000	+0.191
$NHCOCH_3$	+0.0082(ave)	+0.002	+0.146
OCH_3	+0.0252(ave)	+0.076	+0.449

n.r. = not resolved

[a] Based upon MNDO calculations. See section 7.7 for details.

[b] Calculated from nmr titration data. See section 7.7 for details.

[c] Calculated by multiplying the difference in charge density by the Shielding Constant (17.8 ppm/e) (Karplus 1970).

7.5.2 Chemical Shifts and Calculated Charge Density

The most immediate difference between the two curves in figure 7-3 is the fact that H3eq moves in the opposite direction between α and β anomers. This effect was quite interesting, and we have explored it in detail, as well as the other anomalies, such as the effect at H8.

The changes in resonance for a few selected compounds are reported in table 7-3. Also reported are the calculated change in charge density.

The direction of the change was correctly predicted, vis-a-vis H8 in βNeuAc **1a** and H3eq in αNeuAc methyl glycoside **8b** even though solvent effects and anisotropy were not included in the calculations. Furthermore, the ability to predict the correct direction of the change was found to be dependant on which of the two oxygens in the carboxylate was protonated in the calculation. For example, in βNeuAc **1a**, X-ray data (Flippen 1973) gives the ring oxygen - carboxyl/hydroxyl dihedral angle to be 132° and at this angle the MNDO calculations show changes in charge density consistent with the n.m.r. data. When the other oxygen is used as the site for protonation, giving a dihedral of 310°, H3ax was predicted to have an opposite change in charge density.

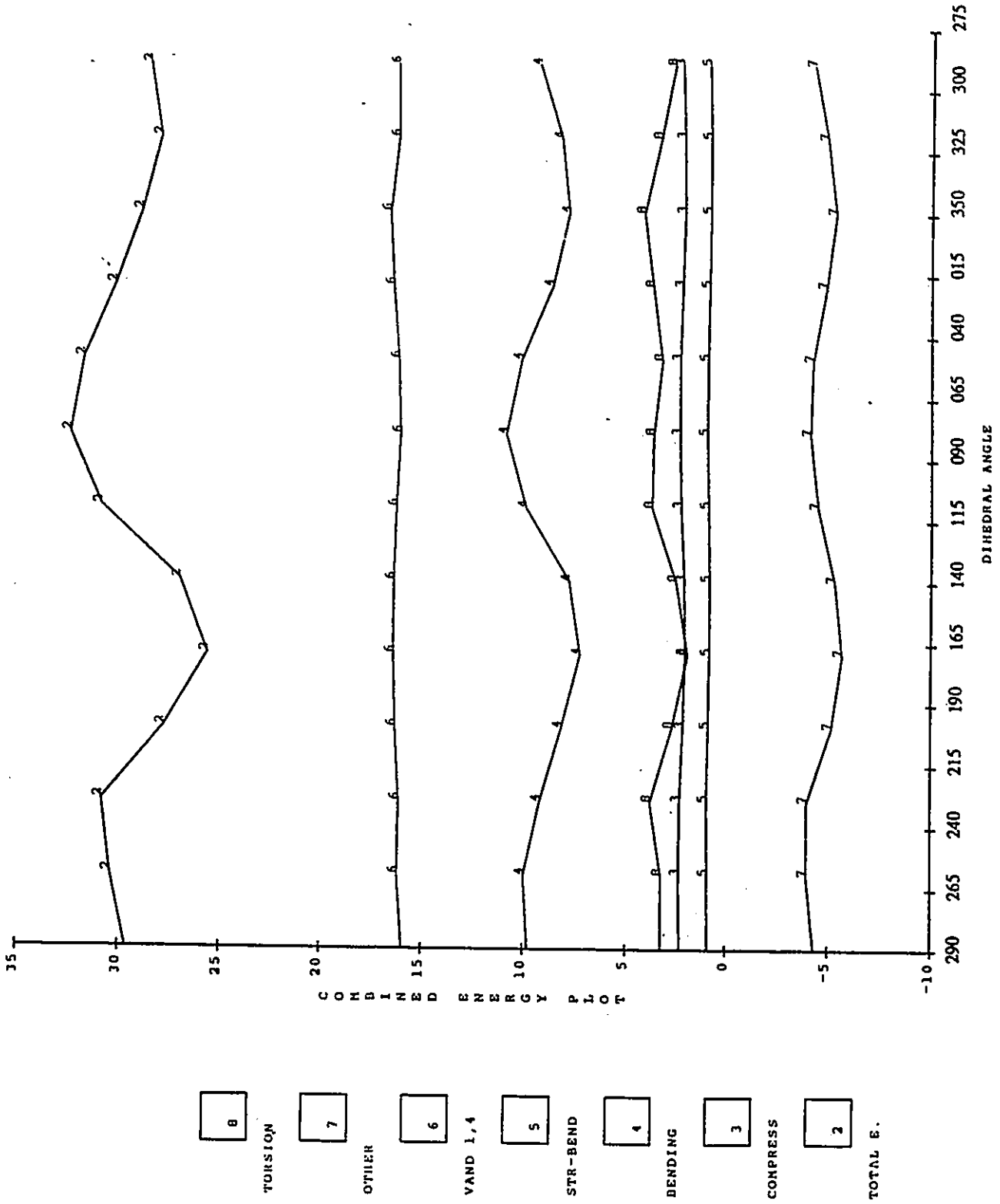
For the αNeuAc methyl glycoside **8b**, MM2(1982) calculated the Karplus diagram energy profile for the carboxylic acid dihedral as shown in figure 7-4. The dihedral angle of the ring oxygen and the acid hydroxyl has two energy minima, one at 165° and the other at 325°. Changes in charge density at H3eq are predicted correctly only when the carboxyl is protonated at 160±10° dihedral from the ring oxygen.

The MNDO calculated differences in charge density were not able to predict the observed differences in chemical shift for H6 or H7 in the α methyl glycoside **8b**. Interestingly, the C6-H6 bond is perpendicular to the carboxylate, and should not experience a large electrostatic effect from the negative charge (Jackman and Sternhell 1969), yet H6 has one of the largest observed chemical shift changes. Evidently there is either a solvent effect or an anisotropic effect which is causing this difference.

7.5.3 Discussion

The stereoelectronic effects of the carboxyl are quite profound in the NeuAc molecule. The available preliminary MNDO calculations are no. conclusive, but they warrent discussion

Figure 7-4 Karplus Dihedral Energy Diagram for O_{ring}-C2-C1-OH in α-NeuAc (8b) Calculated by MM2



because of the interesting phenomena they predict.

The ^1H n.m.r. of the β glycoside shows deshielding upon protonation of the anion, as would be expected for a reduction in charge. But in the α glycoside, H3eq is actually shielded, a result similar to that of esterification (see section 7.3.2). In an attempt to rationalized these results, MNDO calculations were performed to obtain differences in charge density at the different protons. These were then used to calculate chemical shift differences. This simple method could not account for the magnitude of the observed differences, or even the upfield or downfield trends observed at some protons (H6 and H7 in **8b**); however, the conformation of the carboxylate was found to play a role in the MNDO charge density calculations at both H3 protons. The experimental results were modelled with a slight improvement depending on the conformation chosen. It remains to be seen whether this phenomenon is general.

The pKa values were also measured for NeuAc and several analogues. On the basis of this, the high acidity of NeuAc was explained by three effects which stabilize the negative charge: induction, hydrogen bonding, and stereoelectronic effects.

The negative charge of the carboxylate strongly influences the n.m.r. spectra, but it has not been shown that these results also effect the chemical properties of NeuAc derivatives. The final conclusion is that NeuAc is an unusually strong organic acid, and as such resists soft type interaction which could result in complexes. This is consistent with the proposed biological role for NeuAc as an immunogenic shield.

7.6 Sialyllactose Lactone

The existence of ganglioside lactones involving NeuAc was described twenty years ago by Wiegandt (1966) and Gross (1975). The structures have been elucidated in GM_3 (Yu 1985), GD_{1a} (Acquotti 1987), and GD_{1b} (Fronza 1989). Other possible lactones involving the NeuAcα2-8NeuAc linkage (Lifely 1984) will not be considered here.

We used this lactone as a tool to isolate the GM_3 oligosaccharide **2a** from bovine colostrum (see section 2.3 and Roy 1989) and we were intrigued by several questions raised by this molecule. We wished to determine the site of lactonization since there were two hydroxyls on galactose which were in the correct geometry. We also wished to determine the conformation of the lactone ring. This would clarify the relative orientation of Gal and NeuAc in the lactone, and could provide evidence for the cross reactivity observed in anti-GM_3 antibodies. Finally, we wished to determine if this lactone could exist *in vivo*. Since there are no X-Ray crystallographic data available for the molecule, n.m.r. provides the best method for elucidating the structure of this molecule.

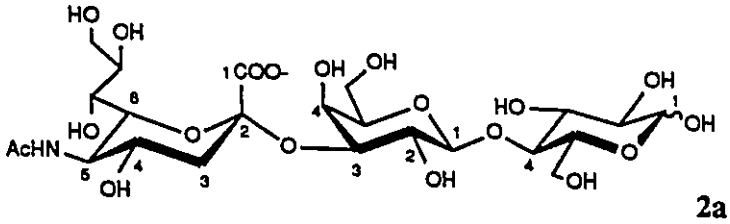
The following is a summary of the n.m.r. data collected to answer the above questions and a discussion of the results as they relate to a computer model for the lactone.

7.6.1 Assignment of ^1H NMR and Site of Lactonization

The lactone (**2b**) of NeuAcα2-3Galβ1-4Glc was obtained in high purity from colostrum as described in Chapter 2. The lactone was formed by treatment of **2a** with glacial acetic acid which may have resulted in hydrolysis of the glycosidic bonds, but the integrity of the molecule was confirmed when base hydrolysis of **2b** resulted in a compound which had all the physical and spectroscopic properties of **2a**.

Full n.m.r. characterization of **2a** and the lactone **2b** was done in DMSO-d6:D_2O 49:1. The small amount of D_2O was added to exchange with hydroxyl protons which otherwise gave broad peaks in the ^1H n.m.r. spectra. The assignments were made using homo- and hetero-nuclear shift correlated 2D n.m.r. (HOMCOR and HETCOR) experiments. The 2D spectra for **2b** are shown in figures 7-5a and b.

In order to assign the ^1H 2D spectrum, one resonance from each sugar unit must be identified. The HETCOR spectra were used in conjunction with assignments made by Berman (1984). For example, Gal-C1 is the furthest downfield (103.92 ppm) of the four anomeric



2a

NeuAc Gal Glc

carbons in compound **2a** in D_2O. An examination of the spectrum in figure 7-5b for **2b** in DMSO-d6 also shows four anomeric carbons. The furthest downfield is at 99.8 ppm, and appears to be coupled to a (triplet) in the ^1H n.m.r. spectrum at 4.57 ppm. Since NeuAc does not have an anomeric hydrogen, this must be either Gal-H1 or Glc- H1. In the HOMCOR spectrum in figure 7-5a, this resonance is coupled to another at 4.70 ppm (H2) which is coupled to another at 3.96 ppm (H3). This last resonance is very distinctive (Sabesan 1986) for Gal-H3 due to glycosylation at the Gal-3 hydroxyl. Hence three very important resonances in **2b** have been identified. Once a few resonances were identified, complete assignment could be made, as shown in figure 7-6. Similarly, the assignments for **2a** were made, and are reported in table 7-4 below.

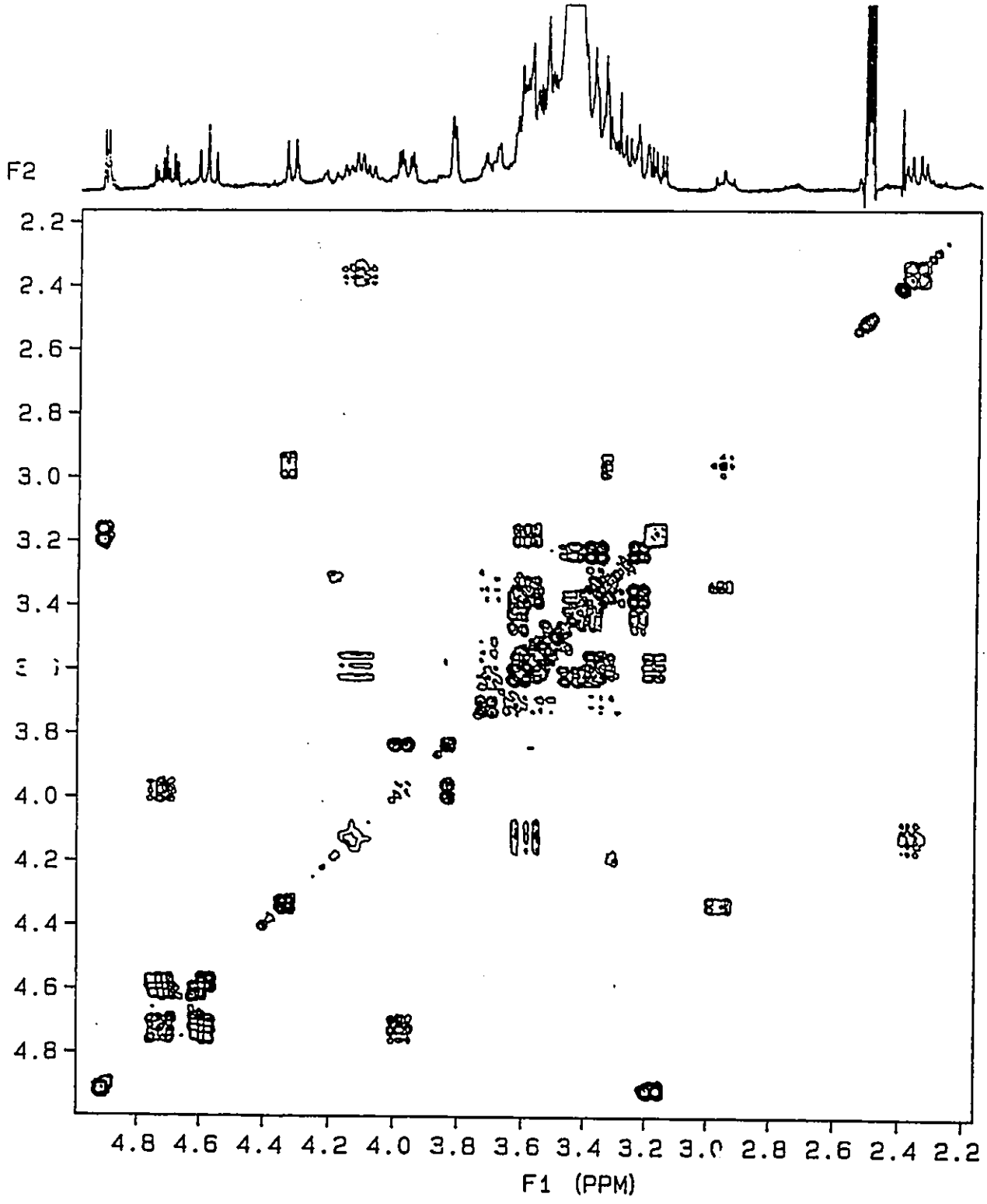


Figure 7-5a

HOMCOR of Sialyllactose lactone 2b in DMSO-d6

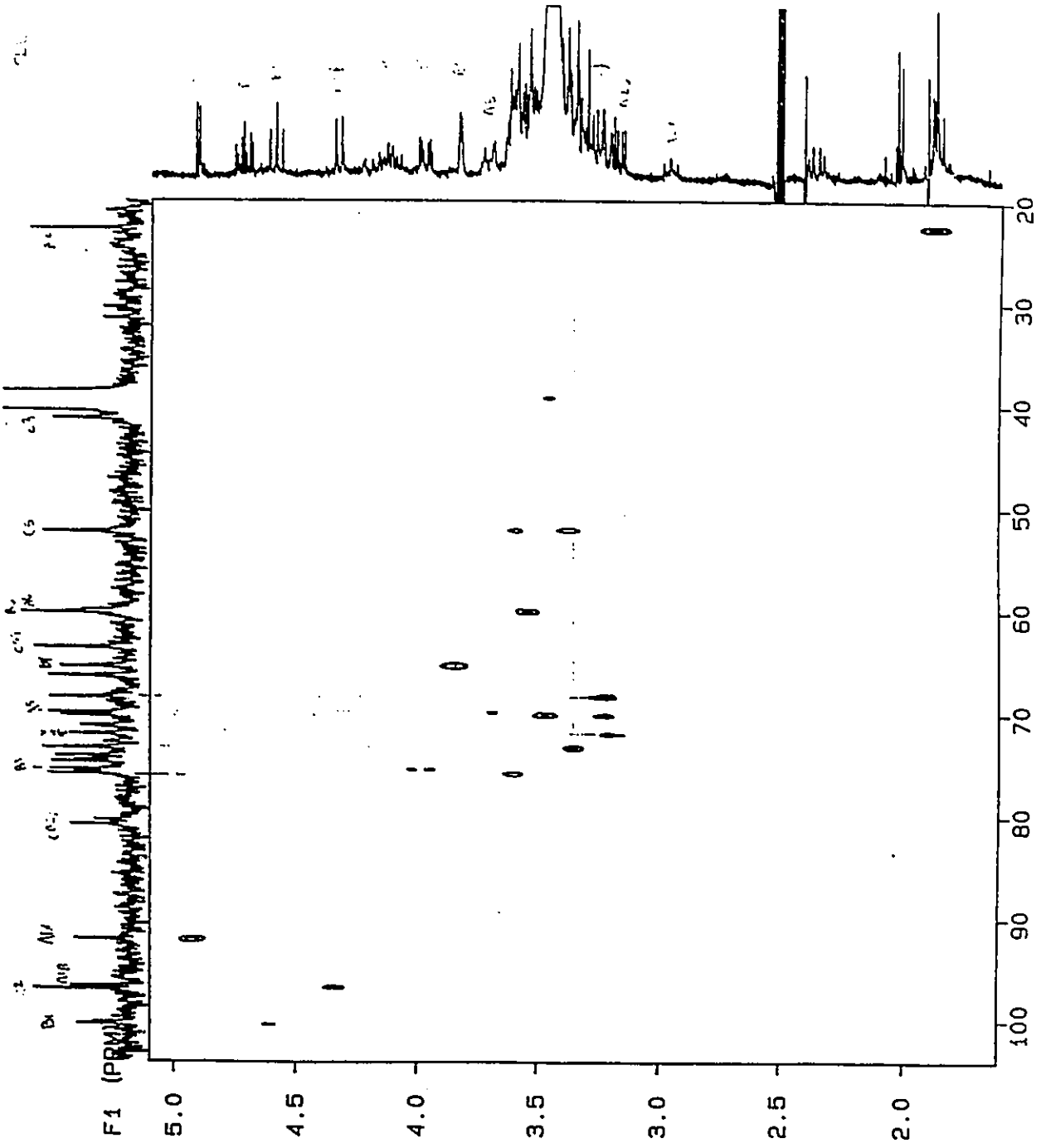


Figure 7-5b HETCOR of Sialyllactose lactone **2b** in DMSO-d6

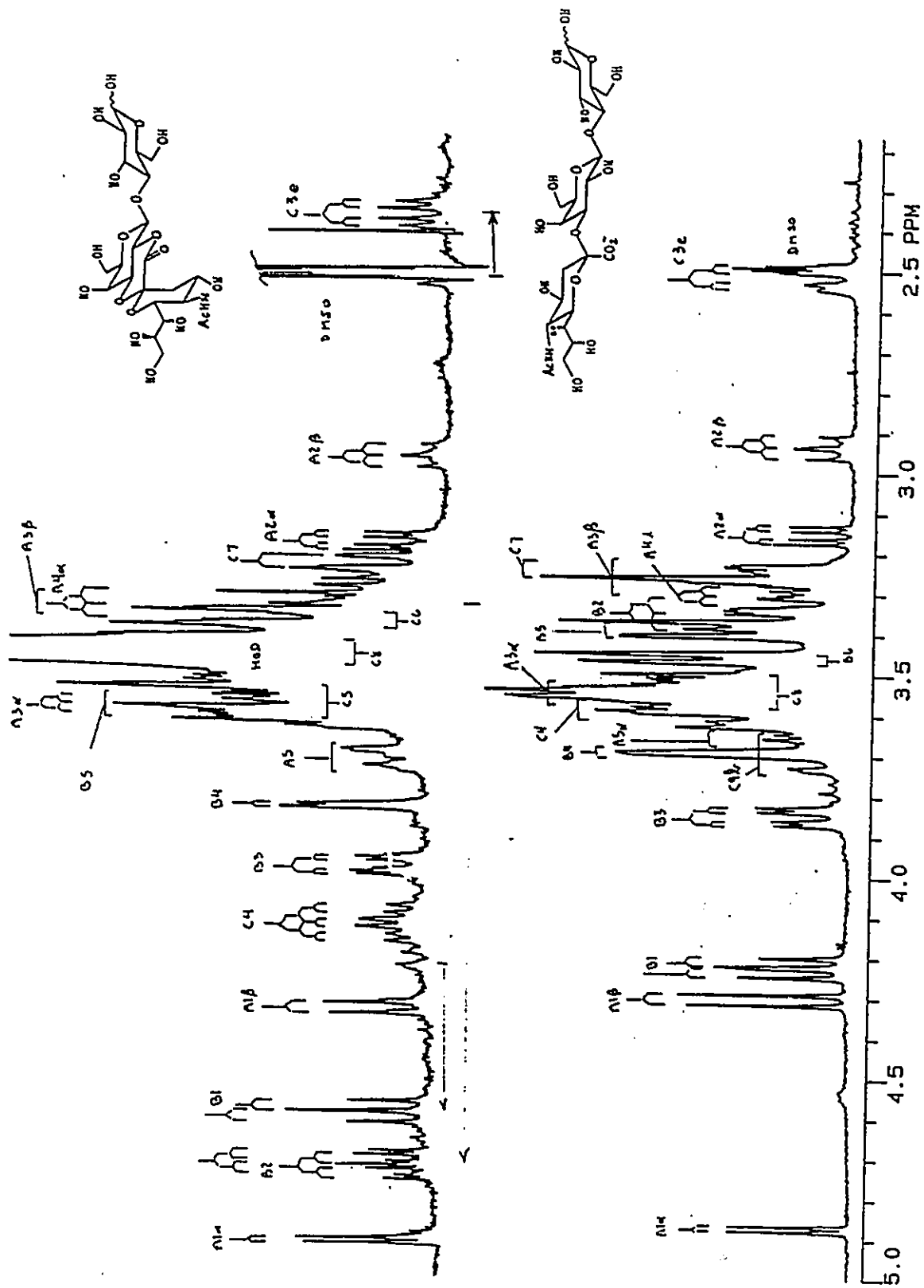


Figure 7-6 Assignment of ^1H NMR Resonances of Siallylactose 2a and Lactone 2b

A ≡ Glc B ≡ Gal C ≡ NeuAc

Table 7-4 ^{1}H n.m.r Chemical Shifts (ppm) for NeuAcα2-3Galβ1-4Glc **2a** and Lactone **2b** in DMSO-d6 at 25°C, 300 MHz.

Hydrogen	2a	2b	Difference
NeuAc			
H3ax	1.66	1.49	-.17
H3eq	2.51	2.34	-.17
H4	3.56	4.10	+.53
H5	3.52	3.53	+.01
H6	3.39	3.35	-.04
H7	3.24	3.21	-.04
H8	3.55	3.45	-.10
H9	3.57		
NAc	1.85	1.86	+.01
Gal			
H1	4.22	4.57	+.35
H2	3.34	4.70	+1.36
H3	3.85	3.96	+.11
H4	3.69	3.81	+.12
H5	3.39	3.56	+.17
H6			
αGlc			
H1	4.87	4.89	+.02
H2	3.15	3.16	+.01
H3	3.54	3.57	+.03
H4	3.27	3.33	+.06
H5	3.67	3.69	+.02
βGlc			
H1	4.30	4.31	+.01
H2	2.94	2.94	.00
H3	3.27	3.29	+.02
H5	3.67	3.69	+.02

A comparison of the ^{1}H n.m.r. of **2a** and **2b** indicate that many resonances are shifted slightly downfield, as would be expected from the change from a charged species to a neutral species. The largest changes (>0.2 ppm) occur in NeuAc and Gal. Gal-H2 is by far the most effected, changing from 3.34 ppm in **2a** to 4.70 ppm in **2b**, a deshielding of 1.36 ppm. Gal-H1 is also deshielded by 0.35 ppm. These results indicate that lactonization occurs at Gal-2 hydroxyl. It is expected that Gal-H3 would also be deshielded as much as Gal-H1, since both are adjacent to the site of lactonization, but this is not observed. The reason for this is unclear.

NeuAc also experiences strong effects, such as the deshielding of H4 (0.53 ppm) and shielding of H3eq and H3ax (0.17 ppm each). In order to explain these results, the effect of esterification on the ^{1}H n.m.r. of NeuAc may be estimated by the examination of a model compound. The methyl ester of NeuAc α-methyl glycoside **8a** did show changes in the ^{1}H

n.m.r compared to the acid (**8b**). Esterification resulted in a downfield shift at H3ax of 0.174 ppm and an upfield shift at H3eq of 0.038 ppm, and these results were rationalized on the basis of charge density calculations (see table 7-3). NeuAc-H4 was shifted downfield by 0.08 ppm (data not shown) in the methyl ester compared to the acid. These results do not concur with the lactone, and hence esterification alone can not explain the changes in chemical shift of NeuAc observed upon lactonization.

The results reported by Yu(1985) for the lactone of GM_3, which shares the same structure as **2b**, agree to a large extent to what has been reported here. Due to a large downfield shift of 1.42 ppm at Gal-H2, they also conclude the site of lactonization is at the Gal-2 hydroxyl. Similar changes are reported for the NeuAc resonances, however; they differ from our results in that they found deshielding (0.15 ppm) at H3ax. They have speculated that "these lactonization effects are probably due to reorientation of the NeuAc-1 carboxyl group after ring formation." In order to explore this possibility further, we undertook the study of the conformation of this lactone.

7.6.2 Conformation of the Lactone

The populations of the three favored conformations of NeuAcα2-3Gal were recently estimated on the basis of nOe data (Poppe 1989). In particular, a nOe between Gal-H3 and NeuAc-H3eq was observed. We also performed nOe difference experiments on **2a** and observed the same effect, indicating that the disaccharide NeuAcα2-3Gal and the trisaccharide **2a** have a similar conformation around the NeuAc-Gal linkage. When we repeated these nOe difference experiments on the lactone **2b**, we did not observe any nOe between Gal-H3 and NeuAc-H3eq, despite the fact that the chair conformation describe by Yu(1985) for the lactone should have brought Gal-H3 to within 1Å of NeuAc-H3eq.

To examine the possible conformations of the lactone, we undertook molecular modelling calculations using the program "Alchemy". This simplified version of MM2 minimizes the conformational energy on the basis of equations for bond stretching, angle bending, torsional deformation, van der Waals interactions and out-of-plane bending. It does not include dipole-dipole interactions.

The lactone was made by first placing NeuAc and Gal in roughly the correct conformations previously reported (Poppe 1989). Then Gal-2 hydroxyl was brought close to the NeuAc-1, and the ring was fused. (This does not reflect the conformers predicted by Poppe (1989) since these have the Gal-2 hydroxyl quite far from the NeuAc-1 carboxyl group.) After

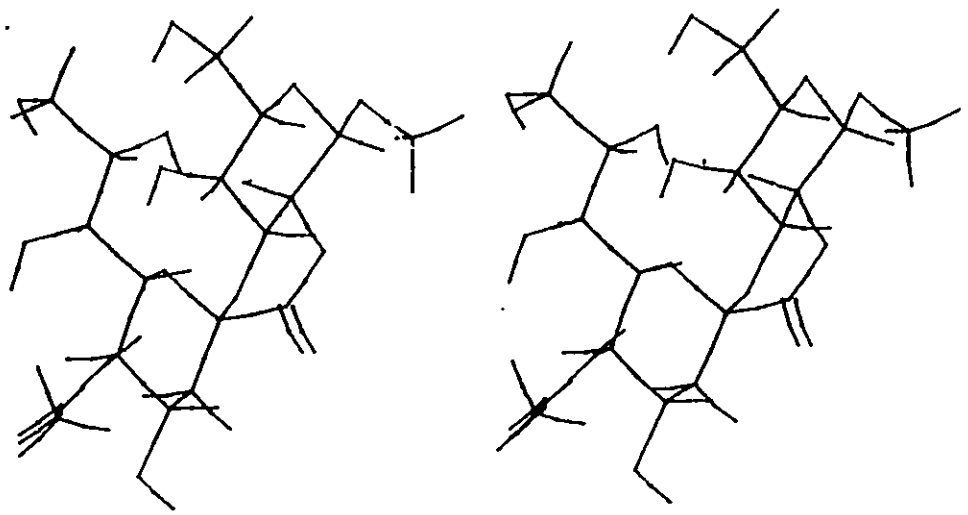


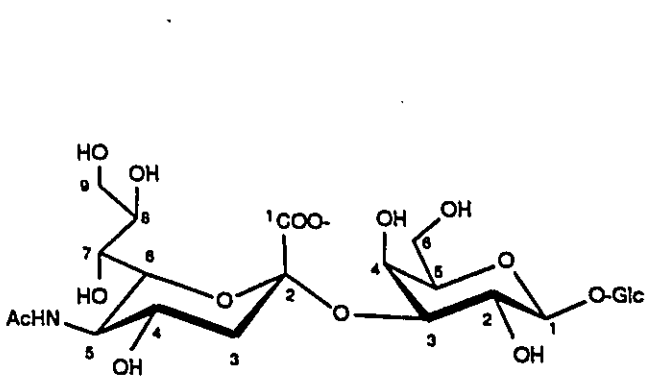
Figure 7-7 Stereo-View of the Boat Conformation of Sialyllactose Lactone 2b Predicted by Alchemy^{R}.

Alchemy II

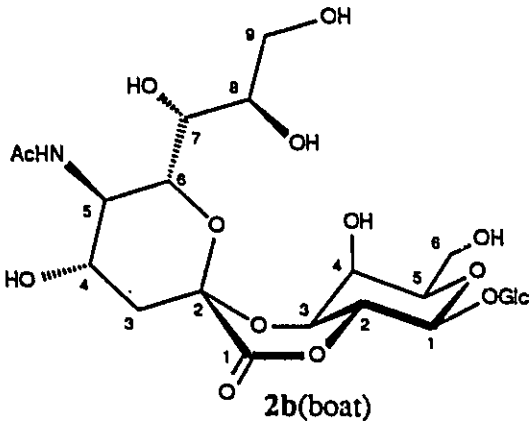
TRIPOS Associates

SIALYL LACTOSE LACTONE

energy minimization, a boat conformation was always obtained, and this is illustrated in figure 7-7. In order to make a computer model of the chair conformation predicted by Yu (1985), the bond angles of the lactone ring had to be preset before energy minimization. The program predicted a higher energy for the chair conformation. The results of the calculations are summarized in table 7-5.



2a



2b(boat)

Table 7-5 MM2(1982) Predicted Conformation of the NeuAcα2-3Gal Lactone, with Experimental Evidence.

	Distance[a] (Angstroms)		
Item[b]	NeuAcα2-3Gal **2a**	Lactone **2b**	
		Boat	Chair
$H3_{gal}$-$H3_{eq}$	2.64	3.83	1.94
$H3_{gal}$-$H3_{ax}$	3.37	4.30	2.90
$H2_{gal}$-$H6_{NeuAc}$		3.58	5.40
$H2_{gal}$-$H8_{NeuAc}$		2.48	5.87
Observed nOe			
$H3_{gal}$-$H3_{eq}$	yes		
$H3_{gal}$-$H3_{ax}$			
$H2_{gal}$-$H6_{NeuAc}$			
$H2_{gal}$-$H8_{NeuAc}$		yes	

[a]Calculated using Alchemy. [b]The numbering scheme can be seen in the diagram above.

Also reported in table 7-5 are the results of nOe experiments. The chair model for the lactone has a distance of 1.94 A between Gal-H3 and NeuAc-H3eq, but as described above, no nOe was observed. The boat model predicted a distance of 2.48 A between Gal-H2 and NeuAc-H8, hence we looked for an nOe between these nucleii. Indeed, an nOe was observed, and work is now underway to confirm this result using a higher field spectrometer.

This result is similar to that obtained with the corresponding lactone in Gd_{1b} (Fronza 1989). They also observed an nOe between Gal-H2 and NeuAc-H8 and have described three possible conformations for the boat lactone, but they could not predict which would be preferred. Our computer model matches the $B_{2Gal,2NeuAc}$ conformation they describe.

The Alchemy model does not include dipole moments in the calculation of minimum energy. Dipole energies are included in the MM2(1982) calculation (Herman 1987), and with MM2 (results not shown), it was impossible to find a stable chair conformation for the lactone. A possible explanation for this comes from an examination of the angle of the anomeric oxygen with respect to the ring oxygen.

Table 7-6 Calculated Dihedral Angles of NeuAcα2-3Galβ1-4Glc
Dihedral Angle NeuAcO6-C2-O2-GalC3

2a[a]	**2b** Boat[b]	**2b** Chair[b]
60% -85° 40% -153°	66°	176°

[a] From (Poppe 1989). [b] From Alchemy models.

It is apparent in table 7-6 that the almost linear angle of the acetal in the chair form of the lactone would result in a very strong exo-anomeric effect which would prohibit this conformation. Since we could not find a stable chair conformation using MM2(1982) we do not have an estimate of the energy difference.

7.6.3 Rate of Hydrolysis

In order to determine if the lactone could exist *in vivo*, the stability of the lactone in aqueous solution was studied. Attempts to characterize the lactone **2b** in D_2O were only partially successful because the lactone hydrolyzes back to the acid at a quick rate. The location of the H3eq resonances of the lactone and acid in D_2O (results not shown) are different by 0.11 ppm, and hence ^1H n.m.r. spectroscopy can be used to follow the rate of hydrolysis. The rate follows first order kinetics, and the half lives are reported in table 7-7.

Table 7-7 Half Life for NeuAcα2-3Galβ1-4Glc Lactone **2b**

Buffer	Lactone half life[a]
D_2O	43 hrs.
50 mM acetate pH 5.0	4.5 hrs.
50 mM phosphate pH 7.2	4.1 hrs.

[a] Measured at 20°C.

The presence of buffers seems to have a catalytic effect since the half life in pure D_2O (at acidic pH due to the NeuAc) is much longer. When the lactone was placed in phosphate buffer at 37°C, it was completely hydrolyzed in 30 minutes. This result is particularly relevant because the lactone has been postulated to exist *in vivo* (Gross 1975, Riboni 1986). The GD_{1b} lactone apparently involves C9 hydroxyl of NeuAcα2-8NeuAc, and not NeuAcα2-3Gal (Acquotti 1987). It is doubtful that this lactone could exist under physiological conditions unless the lactone of the ganglioside is stabilized.

7.6.4 Discussion

The biological consequences of changing a highly charged species into a neutral species could have some interesting biological effects. It has been postulated that the immunogenicity of GM_3 is due to the lactone form, which gives rise to antibodies which cross react with the acid form of GM_3 (see section 1.2.5.4). The results presented here would exclude this hypothesis as the conformation of the lactone is boat-like, bringing the NeuAc and Gal molecules close together. This does not resemble any of the three conformations of the parent compound NeuAcα2-3Gal as predicted by Poppe (1989). Or this would indicate that the antibodies are only recognizing part of the trisaccharide.

An interesting feature of GM_3 is its apparent "crypticity" under certain circumstances. If the lactone did form *in vivo*, it would present a fairly large hydrophobic surface extending from NeuAc-H3 to Gal-H5. This large hydrophobic region could probably result in submerging the ganglioside into the lipid bilayer. This would then act to stabilize the lactone against hydrolysis. As we have noted, the lactone would not last long otherwise.

The fact that the lactone of GM_3 has a different ^1H n.m.r. than the oligosaccharide is an interesting phenomenon. It is too early to draw any conclusion from a difference as small as shielding or deshielding at NeuAc-H3ax, but this information may be useful in the accumulation of evidence. It is interesting to speculate the possible causes. A slightly different conformation of the lactone would make such an effect, or the ceramide moiety may be involved, and this could be an indication of a folding over of the glycosphingolipid. This type of information will be useful as the role of GM_3 in cell-cell interactions is refined.

7.7 Experimental Methods

Spectroscopy

^{1}H n.m.r. spectra were recorded on a Varian XL-300 in D_2O with acetone (2.225 ppm) or DMSO (2.745 ppm) as internal references for the pKa studies. The siallylactose lactone studies were performed in DMSO-d6:D_2O 49:1 unless otherwise noted. The spectra were obtained with a 3000 Hz width (0-10 ppm), 90° pulse width and 10 s cycle time. .

pH Measurement

pH was recorded using a Fischer Accumet pH meter standardized with Fischer buffers pH 4.00 and 7.00 at 25°C.

Conformation, ΔH and Charge Density Calculations

Molecular mechanics and MNDO calculations were performed using TRIBBLE (Herman 1987). The program was run on a Digital VAX 750 and I/O was done on a Tektronix 4014 or PC with Tektronix emulator.

MM2(1982) molecular mechanics were used to obtain conformations of the molecules as described in section 7.3.5. These results were consistent with the experimental coupling constants.

MNDO calculations were then performed on the molecule in the conformation obtained by MM2(1982) giving heat of formation and charge density at each atom as output. One Self Consistent Field (SCF) was specified as the condition for terminating calculations. The hydrogen atom on the carboxylic acid was then deleted, and the calculations were redone specifying a negative charge in the system. The differences in ΔH and charge density between the acid and anion were then recorded.

Treatment of Chemical Shift vs. pH Data

Data for the chemical shifts versus pH for NeuAc derivatives were treated as follows. A modified form of the Hill equation (Zimmerman 1988) was used which assumes the ^{1}H n.m.r. resonance of the acid and conjugate base are different, but that the dynamic equilibrium between the two species is faster than the n.m.r. time scale, and hence the resulting resonance is a weighted average of the two resonances. The weighting is directly proportional to the concentration of the species. It may be shown that:

$$\frac{\delta_{obs}-\delta_s}{[H^+]} = K\delta_{hs} - K\delta_{obs} \qquad (7\text{-}1)$$

where δ_{obs} is the observed resonance.
δ_s is the resonance of the anion.
δ_{hs} is the (unknown) resonance of the acid.
$K=1/K_a$ where K_a is the acid constant.

Hence by plotting the change in chemical shift divided by the proton concentration versus the change in chemical shift, pKa may be determined by the logarithm of the slope. The chemical shifts of the acid may also be determined.

The pH measurements were done in D_20, but the buffers used to standardize the pH meter were in H_2O. Therefore, the pKa values obtained were corrected for this difference by the following equation obtained from Marshall (1978):

$$pD = pH_{measured} + 0.41 \qquad (7\text{-}2)$$

$$pK_a^D = 1.02 \times pK_a - 0.42 \qquad (7\text{-}3)$$

Conclusion and Prospects

The object of this thesis was to study the antigenicity and immunogenicity of sialic acid. By doing so, products were prepared which could be used to screen for antibodies directed against tumor-associated antigens, and which could be used to elicit antibodies (by immunization) with specificity to sialic acid which could then cross-react with tumor cells.

The preparation of these products involved the isolation of starting material from readily available sources. The isolation procedures were simple and required a minimal amount of equipement, though they were somewhat lengthy. Sialic acid was then available as a monomer and as a trisaccharide.

A variety of derivatives of sialic acid were prepared, mostly according to literature procedures modified to improve yield or work-up. New molecules which were prepared, or new procedures which were developed are presented in appendix 2.

Other new derivatives were prepared to allow reaction to form polyvalent macromolecules. Both protein conjugates and polyacrylamide copolymers (neoglycoproteins and pseudopolysaccharides) were prepared with special attention to the number of sialic acid residues conjugated per molecule. This feature, termed "clustering", is of critical importance in biochemical phenomena such as antibody binding. A model study with the lectin WGA revealed this effect, and the postulate of the micro-dynamic effect of multi-ligand binding was discussed. An interesting aspect of the syntheses of the polyvalent macromolecules was the fact that a ceiling was found in the incorporation of sialic acid. This maximum could only be surpassed if the methyl ester was used in the syntheses. This indicates (probably) a repulsive effect due to the negative charge of the acid, and again shows the importance of a micro-dynamic environment. These results will undoubtedly be useful in understanding cell surface phenomena.

The protein conjugates were used to immunize rabbits, and antibodies were obtained with specificty for sialic acid. The aglycon portion of sialic acid was also recognized in the binding site, as determined by inhibition studies. In future attempts to create antibodies with less specificity for the aglycon, a bulky aglycon such as adamantyl might be used in the immunogen.

To get a better understanding of the conformation of sialic acids, n.m.r. studies were

undertaken on the methyl glycosides and the sialyllactose lactone in conjunction with computer modelling. MNDO calculations were deemed to simplistic to be able to determine the relative basicity of the carboxylate oxygens, but MM2 calculations showed preffered conformations which were consistent with the n.m.r. data. This information will also be useful in understanding why some molecules are antigenic in cancer cells, and not antigen in normal cells.

Appendix 1

GENERAL METHODS

Melting points were taken on a Gallenkamp melting point apparatus.

Optical rotation was measured using a Perkin-Elmer 241 Polarimeter at the sodium D line 589 nm and using 10 cm cuvettes at room temperature.

Thin Layer Chromatography was done using Merck Silica Gel Precoated TLC plates 60 F254. High performance thin layer chromatography was done using Merck HPTLC precoated plates Silica gel 60 F254 with concentration zone. The following solvent systems were used:

A-	nPrOH:H_2O 7:3	B-	$CHCl_3$MeOH 7:3
C-	nPrOH:H_2O 8:2	D-	CH_3CN:10% HOAc 8:2
E-	CH_3CN:10% HOAc 7:3	F-	CH_3CN:10% HOAc 6:4
G-	EtOAc:HOAc:H_2O 3:2:1	H-	$CHCl_3$:MeOH 8:2
I	nPrOH:H_2O:NH_4OH 6:2:1	J-	Acetone:H_2O:HOAc 9:1:1

The plates were sprayed or dipped in molybdate reagent and heated at 150°C

Fast Atom Bombardment Mass Spectroscopy (FAB MS) was done using a V.G. 7070 E . Xenon was the neutral fast atom, and the samples were dissolved in glycerol.

Infrared (IR) spectra were taken on a Perkin Elmer 783 Infrared Spectrophotometer. The samples were 1-2 mg in KBr pellets. Standardization was with polystyrene at 1602 cm^{-1}.

Combustion analysis were performed by Guelph Chemical Lab Ltd., Guelph, Ontario.

Desalting was done on a 3 cm by 60 cm glass column (Pharmacia) packed with Pharmacia Sephadex G-10 (N° 17-0010-01) eluted with water pumped by a Pharmacia Peristaltic Pump P3. Detection was with a Waters Differential Refractometer R403 and recorded on a Linear 1200 chart recorder. Fractions were collected in 14 by 1.5 cm test tubes in a LKB 2112 Redirac Fraction Collector.

HPLC was performed on a Perkin-Elmer Series 10 Liquid Chromatograph with a LC 25 RI detector. Integration was performed on a Sigma 15 Chromatography data station. BioRad Aminex

A-28 (11 μ) anion exchange resin and Altex Ultrapack ODS (10 μ) columns were packed locally.

Lyophilization was performed with a Virtis Freezmobile 24.

REAGENTS

Phosphate buffered saline (PBS)

0.01 M PO_4^{-3}
0.15 M NaCl
pH 7.3

7.08 g Na_2HPO_4 anhydrous (50 mM, 141.96 g)
1.40 g Na_2HPO_4 anhydrous (50 mM, 141.96 g)
52.8 g NaCl dissolved in 6 L H_2O

Ninhydrin

2% ninhydrin in 4 M NaOAc buffer pH 5.5
or 2% ninhydrin in 4% aqueous pyridine
Standard: 1 μM $GlcNH_2$.HCl

1 mL sample is added to 1 mL reagent and heated at 100°C for 15 minutes.
Add 5 mL H_2O and read at 570 nm.

Resorcinol (Svennerholm 1957)

2% resorcinol - 2.0 g of resorcinol is dissolved in 100 mL of H_2O. This reagent is stable for months in a refrigerator.
0.1 M copper (II) sulfate - 2.5 g of $CuSO_4.5H_2O$ is dissolved in 100 mL of H_2O
Resorcinol reagent - 10 mL of 2% resorcinol is added to 80 mL of concentrated hydrochloric acid. To this solution is added 0.25 mL of 0.1 M copper (II) sulfate. The solution is diluted to 100 ml with distilled water. The resorcinol reagent should be prepared at least 1 hr before use and is stable for about 2 weeks at 20°-25°C when stored in an amber bottle.
Extraction solvent- 15% n-Butanol, 85% n-Butylacetate

A sample (containing 10-70 μg of sialic acid) is placed in a 16 x 150 mm Pyrex test tube and the sample volume is made up to 2.0 mL with H_2O. Standards of N-acetylneuraminic acid are also run in concentrations of 10 to 100 μg in a total volume of 2.0 mL. Resorcinol reagent (2.0 mL) is

added, and the tubes are placed in a boiling water bath for 15 min. The tubes are cooled to 20°-25°C by p' ·ing them in a water bath. Extraction solvent (4.0 mL) is added ot each tube and the rubes are vigourously shaken. At 20°-25°C, the organic solent layer will separate completely from the aqueous phase. If turbidity remains in the organic phase, the samples should be centrifuged. The organic solvent layer is transferred to a 1.0 cm cuvette, and the absorbance is dedetermined against pure organic solvent in a spectrophotometer at 580 nm.

The following compounds were tested with this method and gave the listed relative absorbances (mol/mol) at 580 nm with respect to free NeuAc (**1a**).

Compound	Relative Absorbance	Compound	Relative Absorbance
1a	1.00	**2a**	0.83
3a	0.72	**7a**	0.96
7b	0.88	**8b**	0.76
11	0.85	**12**	0.86
13	0.44	**14**	0.96
15b	1.00	**17**	0.46
18	0.00	**20b**	0.95
19	1.36	**23**	0.93
21b	0.88	**25c**	0.94
24b	1.48	**28b**	0.81
27b	0.00		

Aminoff Sialic Acid

0.04 N $NaIO_4$ in 0.125 N H_2SO_4
2% $NaAsO_2$ in 0.5 N HCl
0.1 M Thiobarbituric acid
in H_2O adjusted to pH 9.2 with 4 N NaOH
DMSO

Add 0.5 mL sample (5-30 μg sample) (50-300 ml) to 0.25 mL $NaIO_4$, Heat at 37°C for 30 minutes. Add 0.2 mL $NaAsO_2$ until yellow colour disappears. Add 2.0 mL Thiobarbituric acid, heat at 100°C for 7 minutes. Cool then add 3 mL DMSO. Read at 549 nm.

Dubois (Phenol-Sulfuric) (Dubois 1956)

5% Phenol (aqueous)

concentrated H_2SO_4

Add 200 µl sample (0-20 µg) to 200 µl 5% Phenol.

Add 1 mL H_2SO_4

Read at 490 nm.

Lowry Protein (Lowry 1951)

Standard: Human γ Globulin, 10 mg/10 ml in 0.1 M NaOH.

A: 2% Na_2CO_3 in 0.1 M NaOH

B: 0.5% $CuSO_4.5H_2O$ in 1% NaK tartrate

(mix 1% $CuSO_4$ + 2% tartrate 1:1 before use)

C: Mixture of 50 ml A + 1 mL B.

D: 2 N Folin Reagent diluted 1:1 with H_2O

Make 100 µL samples in 0.1 M NaOH, Vortex. Add 1 mL Mixture C, Vortex. Wait 10 minutes. Add 100 µL Folin Reagent, Vortex immediately. Wait 30 minutes. Read at 750 nm.

2,4,6 - Trinitrobenzenesulfonic Acid for Amines (TNBS)

0.2 M borate buffer (pH 8.7)

0.2% TNBS

0.5 M hydrochloric acid

To 0.5 mL of a sample solution (0.1 - 0.5) µmol of primary amine or hydrazide were added 0.5 mL each of 0.2 M borate buffer (pH 8.7) and 0.2% TNBS, and the mixture was incubated for 30 minutes at 55°C, and then diluted with 0.5 M hydrochloric acid (2 mL). The absorbance at 340 nm was read with a suitable spectrophotometer. When this method was used to measure hydrazide, the reaction mixture was kept for 30 minutes at room temperature, and then diluted with water (2 mL). The absorbance at 500 nm was measured. 6-Aminohexanoic acid and butanoic hydrazide (both from Aldrich Chem. Co.) were used as the respective standards.

Colorimetric Test for Esters (Hestrin 1949)

1:hydroxylamine - 2 M hydroxylamine hydrochloride

This solution should be stored in the cold.

2:alkali - 3.5 M sodium hydroxide

3:acid - 4.0 M hydrochloric acid

4:iron - 0.37 M iron (III) chloride ($FeCl_3$) in 0.1 M hydrochloric acid

Alkaline hydroxylamine reagent - prepared shortly before use by mixing equal parts

of reagents 1 and 2 above. This reagent is stable for about 3 h at 20°-25°C.

To a sample or standard containing 0.5-4.0 μmole of ester in a volume of 1.0 mL is added 2.0 mL of hydroxylamine reagent. After 5 minutes or longer if necessary, the pH is brought to 1.2 with 1.0 mL of the iron solution. The tubes are shaken vigorously after the addition of each reagent to avoid the formation of gas bubbles in the cuvettes. If gas bubbles are noticeably present or a precipitate forms, the samples should be centrifuged for several minutes at a moderate speed. The samples are read against a blank which contains the above reagents at 540 nm. If the samples are dissolved in buffers, the samples may be made by repeating the above procedure, except that the order of addition of alkaline hydroxylamine and acid is reversed. By reversing the order of addition of reagents, esters do not form any hydroxamic acid.

IMMUNOCHEMICAL METHODS

Staining Precipitin Bands (Coomassie blue)

PBS for washing excess proteins

filter paper (Whatman)

Stain: 0.025% w/v Coomassie brilliant blue R
in MeOH/ H_2O/ HOAc 50:45:5

Destain: H_2O/ HOAc/ MeOH 87:8:5

Wash gel 3 or 5 times with PBS (24-48 h).

Dry gel by covering with a sheet of filter paper for 16 hours at room temp. or 2-6 hours at 40°C.

Immerse gel in stain for 1 h + 30 min (until bands visible).

Destain in 3-4 changes of destain (until background clear).

Dry in air at room temperature.

Appendix 2

Claims to Original Research

1. A new procedure for the acid (resin) hydrolysis of bird mucin was introduced for the isolation of sialic acid **1a**.

2. Sialyllactose isomers isolated from bovine colostrum were separated by a new method involving lactonization of only one of the isomers, NeuAcα2-3Galβ1-4Glc **2a**.

3. The synthesis and characterization of the following new compounds were reported:

   Methyl (2'-oxoethyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate **10** (Methyl ester and acid)

   2'-[N^{ϵ}-(N^{α}-Acetyl-(L)-lysyl)]ethyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*- 2-nonulopyranosidonic acid **14**

   Methyl (3'-(N-acryloyl-2'-aminoethylthio)propyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate **16** (Methyl ester and acid)

   Allyl 3,5-dideoxy-5-hydroxyacetamido-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid **20b**

   Allyl 5-acetamido-9-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid, **23**

   Allyl 5-acetamido-3,5-dideoxy-β-L-*arabino*-2-heptulopyranosidonic acid, **24** (Methyl ester and acid)

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-3)β-D-galactopyranosyl(1-4)1-[(6-acrylamido-1-hexyl)amino]-1-deoxy-D-glucitol, **30**

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-3)β-D-galactopyranosyl(1-4)N-acryloyl-β-D-glucopyra nosylamine, **32**

5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid(2-8)5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-n onulopyranosylonic acid(2-3)β-D-galactopyranosyl(1-4)N-Acryloyl-β-D-glucopyra nosylamine **33**

4. New methodology introduced includes:

A single pot preparation of compound **5** which excludes the use of HCl (gas).

Reductive amination to prepare compound **12**.

Light induced radical synthesis of compound **15**.

A better solvent system for the reduction in the preparation of compound **17**.

Use of acetoxyacetyl chloride in the preparation of the N-glycolyl derivative **20**.

Periodate and borohydride supported on a solid phase for the ease in work up in the preparation of the C-7 analogue of NeuAc, **24**.

Improved phase transfer catalysis conditions and improved yields in the preparation of the aryl glycoside **25c**.

Increased rate of reaction by increasing the temperature in the preparation of glycosylamines **32** and **33**.

5. Sialic acid Protein conjugates **34a**, **34b**, **35**, **36** and **37** were made with varying amounts of sialic acid content.
Sialic Acid Polyacrylamide copolymers **38**, **39** and **41** were made with varying amount of sialic acid content.

Conjugate **34** was made by a novel aldehyde **10** and reductive amination to the ϵ-amino group of protein. This resulted in a "neoglycoprotein." Conjugate **35** was

made following an improved reductive amination procedure.

Conjugates **36** and **37** were made by a new technique which involves a Michael addition of the ϵ-amino group of lysine on the acryloyl moieties in compounds **16** and **32**.

Polyacrylamide copolymers were made by radical polymerization with the new compound **16**. This new monomer resulted in higher yields, and immunochemical studies indicated the improved characteristics of the polymer.

6. Initial studies using sialic acid protein conjugate **34** as an immunogen in rabbits resulted in the production of antibodies with a high specificity for sialic acid. Inhibition studies showed that the nature of the protein carrier influences the specificity of the antibodies produced. A novel method was introduced which uses the sialic acid polyacrylamide copolymers to screen for the antibodies.

7. The pKa of several derivatives of sialic acid were determined using n.m.r titration. These results were compared with preliminary MNDO calculations which indicate some unusual effects due to the axial carboxylate.

8. The characterization of the lactone of NeuAcα2-3Galβ1-4Glc **2b** was made using n.m.r. analysis and computer modelling. The site of lactonization was found to be the galactose C-2 hydroxyl, and the conformation of the lactone ring was determined to be in a boat conformation rather than a chair form claimed in the recent literature.

Appendix 3
Publications Arising from thid Thesis

A. Gamian, M. Chomik, C.A. Laferriere and R. Roy,
"Inhibition of Influenza A virus hemagglutination and induction of interferon by synthetic sialylated glycoconjugates",
Manusript submitted.

René Roy and Craig A. Laferrière,
"Michael Addition as the Key Step in the Synthesis of Sialyloligosaccharide-Protein Conjugates from N-Acryloylated Glycopyranosylamines",
J.C.S., Chem. Comm. (1990), in press.

René Roy, Craig A. Laferrière,
"Synthesis of Protein Conjugates and Analogues of N-acetylneuraminic Acid",
Canadian Journal of Chemistry, 68, 2045-2054 (1990)

René Roy, Craig A. Laferrière, and Heather Dettman,
"Simple isolation of α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc*p* (G_{M3}O.S.) from bovine colostrum through lactonization",
Carbohydrate Research, **186**, C1-C5 (1989)

René Roy and Craig A. Laferrière,
"Synthesis of antigenic copolymers of N-acetylneuraminic acid binding to wheat germ agglutinin and antibodies" ,
Carbohydrate Research, **177**, C1-C4 (1988)

René Roy and Craig A. Laferrière,
"Synthesis of hapten inhibitors for the binding studies of rabbit anti-sialic acid antibodies", and
"Immunochemical specificities of rabbit antibodies to sialic acid-tetanus toxoid conjugate",
Sialic Acids 1988, Proceedings of the Japanese-German Symposium on Sialic acids, R. Schauer, T. Yamakawa (Eds.), Kieler Verlag Wissenschaft + Bildung (1988)

René Roy, Craig A. Laferrière, Andrzej Gamian and Harold Jennings,
"N-Acetylneuraminic acid: Neoglycoproteins and Pseudopolysaccharides",
J. of Carbohydrate Chemistry, **6(1)**, 161-165 (1987)

Patents:

Rene Roy and Craig Laferriere,
"Synthetic Antigens of Sialic acid and derivatives thereof",
U.S. Patent Application # 081,431

Presentations:

International Carbohydrate Symposium, Yokohama, Japan, Aug. 16-23, 1990
"Synthetic Studies Related to Artificial Sialic Acid Conjugates"
R. Roy, C.A. Laferriere, R.A. Pon, J. Boratynski and L. Cousineau

57^{e} Congres de l'ACFAS, Montreal, Quebec, May 15-22 1989
"Etude du site actif d'un anticorps contre l'acide sialique"
R. Roy et C. Laferriere

55^{e} Congres de l'ACFAS, Ottawa, Ontario, May 10-17 1987
"Synthese et serologie de glycoconjugues de l'acide N-acetylneuraminique"
R. Roy et C. Laferriere

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