The Discovery and Identification of α_1 Macroglobulin, as an Inhibitor of the Acute Phase Reactant, Galβ1$\rightarrow$4GlcNAcα2$\rightarrow$6 Sialyltransferase

by

Phyllis Gwen Harder

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Department of Chemistry
University of Manitoba
Winnipeg, Manitoba, CANADA
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The Discovery and Identification of α_1 Macroglobulin, as an Inhibitor of the Acute Phase Reactant, Galβ1$\rightarrow$4GlcNAcα2$\rightarrow$6 Sialyltransferase

BY

PHYLLIS GWEN HARDER

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

DEDICATION

I will lift up mine eyes unto the hills,
from whence cometh my help,
My help cometh from the Lord,
which made heaven and earth.

Psalm 121: 1,2

PROLOGUE

I would like to take this opportunity to express my deepest thanks to God, for walking with me every step of my journey, and for carrying me when so often I have been to weak.

It has been a precious privilege to have been blessed with this home, the plentiful opportunities, and so many special people. To Isaac and Martha Harder, my parents, who loved me, who showed me great patience throughout this difficult journey of mine, thank you. You have been with me since my birth, guiding me, and supporting me, giving me all that you had, so that I could go forward. Thank you Mom and Dad. Angela Pappas (nee Harder), my sister, my closest childhood friend, thank you for always standing by me. Hilton Harder, my brother, thank you for showing me patience, and for giving me strength. Miranda Harder, my sister, thank you for your earnest care. Bob Pappas, my brother-in-law, but in heart a true brother, thank you for accepting me just the way I am. Adam Pappas, Julia Pappas, and Elainie Pappas, you have given me unconditional love, joy, and hope, each in your own special way, and for this I thank you.

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In thought of these people, these gifts from God, their courage and faith to walk with me, I weep.

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ABBREVIATIONS

Ac	acetyl group; -$COCH_3$
ACS	aqueous counting scintillant
ACTH	adrenocorticotropic hormone
Ara	arabinose
α_1 AGP	α_1 acid glycoprotein
$\alpha_1 I_3$	α_1 inhibitor III
Bis	N,N'-methylene bis-acrylamide
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CDP-choline	cytidine 5'-diphospho-choline
Cer	ceramide
Ci	curie
CMP	cytidine 5'-monophosphate
CMP-NeuAc	cytidine 5'-monophospho-N-acetylneuraminic acid
CRP	C reactive protein
DEAE	diethylaminoethylene
DMSO	dimethylsulfoxide
Dol	dolichol
dpm	disintegrations per minute
ECL	enhanced chemiluminescence
EC	enzyme classification
EDTA	ethylenediamine tetra-acetate, disodium salt
FCS	fetal calf serum
FITC	fluorescein isothiocyanate

Fuc	fucose
g_{av}	average gravitational force
G_{M1}	G = Ganglioside; M= Monosialic acid; 1 = Gal-GalNAc-Gal-Glc-Cer
G_{M2}	G = Ganglioside; M= Monosialic acid; 2 = GalNAc-Gal-Glc-Cer
G_{M3}	G = Ganglioside; M= Monosialic acid; 3 = Gal-Glc-Cer
Gal	galactose
GalNAc	N-acetylgalactosamine
GDP	guanidine diphosphate
Glc	glucose
GlcA	glucuronic acid
GlcNAc	N-acetylglucosamine
HSF	hepatocyte stimulating factor
IFNγ	interferon γ
Ig	immunoglobulin
IL-1	interleukin-1
IL-6	interleukin-6
k_{cat}	catalytic constant (turnover number)
K_m	Michaelis-Menten constant
λ	wavelength (lambda)
LPS	lipopolysaccharide
mA	milliamp
Man	mannose
min	minute
*M*r	relative molecular mass

NCBI	National Center for Biotechnology Information
NeuAc	N-acetylneuraminic acid
nm	nanometer
PDGF	platelet derived growth factor
pI	isoelectric point
PMA	phorbol myristate acetate
PMSF	phenylmethane sulfonylfluoride
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Temed	N,N,N',N'-tetramethyl ethylenediamine
TGF	transforming growth factor
TNFα	tumor necrosis factor α
Tris	tris[hydroxymethyl]aminomethane
U	units
UDP	uridine diphosphate
UDP-Gal	uridine diphosphate galactose
UV-VIS	ultraviolet-visible light spectral region
V	volt
V_{max}	maximum velocity
W	watt
Xyl	xylose

ABBREVIATIONS OF THE AMINO ACIDS

Amino Acid	Three-letter Abbreviation	One-letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Gutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

ABSTRACT

The regulation of Galβ1-4GlcNAcα2-6 sialyltransferase as an acute phase reactant was studied in the rat liver FAZA cell culture system. In these studies rat serum 10 hours post-injury was used as a potentially complete and physiologically balanced source of all possible acute phase response regulators. Addition of this rat serum to FAZA cells resulted in a complete loss of intracellular and secreted sialyltransferase activity, without any effect on cell viability. Pure Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) activity was also completely inhibited in the presence of this serum.

Based on the potential that an endogenous sialyltransferase inhibitor had been discovered, the nature of the inhibition was investigated, with the ultimate objective being to identify the inhibitor. Serum from normal rats that had sialyltransferase inhibitory activity was put through a series of separations to purify the inhibitory component(s). The purification process included size exclusion and anion exchange chromatography, and isoelectric focusing. A standard inhibition assay was developed and used to monitor inhibition throughout the purification process. The final purified inhibitor preparation was analyzed by gel electrophoresis, and the separated polypeptides were analyzed for their N-terminal amino acids. Using the sequences obtained the polypeptides were identified, and the final inhibitor preparation was found to contain two macromolecular components, rat C reactive protein and rat α_1 macroglobulin. Pure rat C reactive protein did not inhibit sialyltransferase activity and therefore it was eliminated as a

possible independent sialyltransferase inhibitor. Since pure rat α_1 macroglobulin was unavailable, monospecific antibodies to rat α_1 macroglobulin were used to determine if their interaction with the purified inhibitor preparation would block sialyltransferase inhibition. Antibodies to the heavy, or α polypeptide chain of α_1 macroglobulin blocked sialyltransferase inhibition in a dose-dependent manner. The sialyltransferase inhibitor was thereby identified as rat α_1 macroglobulin. The final purified inhibitor preparation was also tested on pure galactosyltransferase, with no affect on enzyme activity, indicating that the inhibitor, α_1 macroglobulin may be specific for the sialyltransferase family of enzymes, and potentially for Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) in particular.

INTRODUCTION

I. The Discovery of a Potential Sialyltransferase Inhibitor

Life is an intangible and powerful entity. Its major goal is to exist, and to continue existing. To achieve this goal all living organisms have innate, and mainly uncontrollable systems, designed for self-preservation, and survival. Self-preservation involves a multitude of mechanisms. Firstly, there are characteristic features and mechanisms which aim to protect the organism. If the protective barriers either fail or are violated, the integrity of the organism is maintained by back-up defense mechanisms. Additionally, many organisms have memory systems of various types and complexities, designed to minimize future violations. The host defense response is a common phrase used to describe these mechanisms. Its purpose is to protect, to minimize or contain any trauma, to repair any damage, to heal, and in many situations, to remember so that the organism is guarded and prepared for confrontation with similar traumas in the future (see Appendix A, I).

The host defense response involves local and systemic responses (see Appendix A, II). The response varies according to the organism, and the type and magnitude of the trauma. The acute phase response is a part of the systemic response to injury that occurs in animals. It involves a variety of biochemical and physiological changes (Fey & Fuller, 1987; Gordon & Koj, 1985; Koj, 1974; Koj, 1986; Kushner, 1982; Kushner *et al*, 1982; Kushner, 1988; Schreiber, 1987), which influence and are influenced by concurrent changes in the neurological, endocrine, and immunological systems.

A defining feature of the systemic acute phase response is the major change in the levels of proteins, produced in the liver, and subsequently secreted into the circulatory system (see Appendix A, III). The plasma proteins which undergo these significant changes in response to injury are usually referred to as acute phase reactants (Koj, 1974; Pepys, 1989). The collective changes in the plasma levels of the acute phase reactants help to contain and minimize the trauma, to re-establish homeostasis, to remove damaged tissue, to repair and heal (see Appendix A, III).

There are many acute phase reactants, each of which is unique in its structure and function. For a list of examples of acute phase reactants see Appendix A, IV. The timing and magnitude of response of any given reactant varies with respect to the type and degree of trauma, and with respect to species. Appendix A, V indicates the response of acute phase reactants to injury with examples of the changes of concentrations that occur in the circulation. Despite their differences some generalizations have been possible based on broad similarities in function. The acute phase reactants can be grouped by function to include regulators of the inflammatory and immune responses, inhibitors of proteolytic activity, scavengers, and those that function in the repair and resolution of the trauma (See Appendix A, VI). The changes in synthesis and secretion of the individual acute phase reactants required to facilitate the appropriate acute phase response involves orchestration of all of the individual reactants, and the components and mechanisms involved in their regulation.

The major focus on the acute phase response has been on the determination of the mechanisms involved in the regulation of synthesis of specific reactants. Various factors released in response to trauma are intimately involved in the regulation of the acute phase response. Some of these regulatory factors include hormones, such as glucocorticoids (for example, cortisol), cytokines and growth factors. Interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor α (TNFα), interferon γ (IFNγ), transforming growth factors (TGF), and platelet derived growth factors (PDGF) are some of the cytokines and growth factors identified to be involved in the regulation of the synthesis of the acute phase reactants (Andus *et al*, 1991; Gauldie, 1989; Gauldie *et al*, 1989; Heinrich *et al*, 1990; Whicher *et al*, 1989). Although the regulation of any specific reactant is unique, a model has been suggested (see Appendix A, VII) and provides a basis for investigations of the regulatory mechanisms of any given specific acute phase reactant.

Gal β1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) has been identified as a novel acute phase reactant (Kaplan *et al*, 1983), and it is on this molecule that this entire work pivots. For a review of sialyltransferase as an acute phase reactant see Appendix B. It is a unique reactant, since it is an enzyme which exists in the Golgi in a membrane-bound form. Other acute phase reactants are not membrane-bound, and have no known catalytic functions. During the acute phase response increased levels of sialyltransferase are released from the liver, resulting in elevated levels of the enzyme in the circulatory system. The mechanisms responsible for this acute phase behavior are however, not clearly understood.

In general, direct information on *in vivo* mechanisms can most effectively be obtained by using isolated systems that are intended to mimic the *in vivo* situation, but that are controlled having fewer unknown variables. To study the regulation of the acute phase response numerous types of systems have been used, including liver slice, primary hepatocyte, and established liver cell line systems. While liver slice, and primary hepatocyte systems have the merit of perhaps most closely resembling a physiological functioning liver, they also have a variety of disadvantages. Some of these disadvantages include their short life span, and the difficulty in creating the systems in a reproducible manner, as is necessary to carry out a series of experiments, and to make valid comparisons of the information obtained between those individual experiments. Therefore significant efforts have been put into the development of isolated systems which overcome these difficulties, and have resulted in the creation of many established cell lines currently used to study the regulation of the acute phase response.

Established cell lines have the advantage that they can be maintained over long periods of time, presumably with a minimal deviation in physiology from the tissue of origin. The process used to create cell lines does however result in deviations from the normal tissue from which they are derived. The choice of a particular cell line for a particular study is therefore critical. The cell line should be stable, and as minimally deviant with lengthy periods of culturing time as possible, so that valid comparisons throughout the course of long term studies can be made. It should also, ideally, be one that

most closely mimics the *in vivo* situation that it is intended to represent.

The liver cell lines used for investigations into the acute phase response most commonly used are of human origin, for example the Hep G2 cell line. Most studies on sialyltransferase, as an acute phase reactant have however been done in the rat and a liver cell line derived from the rat would therefore be a more appropriate choice. Of the rat liver cell lines available, the FAZA cell line (Malawista & Weiss, 1974; Darlington, 1987; Woloski & Fuller, 1988) had been observed to very stable over time, with minimal deviation in morphology, growth rate, and responses to various stimuli. Additionally, stimulation of the acute phase response had been observed in the FAZA cell line by the increased production and secretion of various reactants. For example, studies had shown that fibrinogen synthesis and secretion in the FAZA cell line could be stimulated by treatment with monokines, hepatocyte stimulating factor (HSF), phorbol myristate acetate (PMA), and dexamethasone (Ott *et al*, 1984; Evans *et al*, 1987; Otto *et al*, 1987; Fuller *et al*, 1988). The synthesis of the acute phase reactant α_1 Inhibitor III ($\alpha_1 I_3$) had also been shown to be regulated by IL-6 in FAZA cells (Abraham *et al*, 1990). Based on the evidence that the FAZA cells were stable and expressed normal liver-specific function, coupled with the observation that the acute phase reactants, fibrinogen and $\alpha_1 I_3$ could be stimulated, the FAZA cell line was chosen as the model system to investigate the regulation of sialyltransferase activity following injury.

Using the FAZA cell line as an isolated system, studies were designed to investigate the regulation of sialyltransferase as an acute phase reactant. The initial studies of sialyltransferase activity in FAZA cells and that secreted into the culture medium involved monitoring the enzyme activity under basal conditions. Cells were then challenged with dexamethasone, a synthetic glucocorticoid, which had been shown to regulate the gene expression of Galβ1-4 GlcNacα2-6 sialyltransferase (Wang *et al*, 1989). Cellular and secreted sialyltransferase activity were each observed to increase approximately six-fold in response to dexamethasone or PMA challenge, as detailed in Appendix C (Harder *et al*, 1990). Additionally, the FAZA cell system was comparable in its increase in sialyltransferase activities to that observed in rat liver and serum 48 hours after injury. Sialyltransferase activity in rat liver increased from 7 to 22 pmole sugar transferred/minute/mg of liver, and in the circulation increased from 5 to 25 pmole sugar transferred/minute/ml of rat serum, 48 hours after injury (Kaplan *et al*, 1983). In the FAZA cell system 48 hours after dexamethasone administration, cellular sialyltransferase activity increased from 4 to 23 pmole sugar transferred/minute/mg cellular protein, and the secreted activity increased from 17 to 100 pmole/min/mg cell protein (Harder *et al*, 1990). The six-fold increase in cellular and secreted sialyltransferase activity was very similar to the five-fold increase seen *in vivo*. Based on the similarity to the *in vivo* response, the FAZA cell line seemed to be an appropriate system for continued studies on the physiological regulation of sialyltransferase during the acute phase response.

Further investigation into the physiological mechanisms of sialyltransferase stimulation were attempted using natural factors already known to be involved in the regulation of other acute phase reactants. All studies were done in the presence and absence of dexamethasone, since many acute phase reactants require glucocorticoid for a full response, and some are dependent on its presence for any response (Baumann *et al*, 1989). Various regulators were tested, including cytokines synthesized and secreted from stimulated human monocytes, and those from the mouse cell lines J774A.1 and P388D1. Additionally, commercially available regulators were used, including IL-1, IL-6, IFNγ, and TNFα. Each was tested separately over broad ranges of concentrations. Additionally, varying combinations of all of the cytokines were tested. Under the conditions used, addition of individual cytokines, or combinations of them, caused no detectable stimulation of sialyltransferase activity by the FAZA cells.

Despite the fact that the physiologic regulators did not result in a detectable increase in sialyltransferase activity, the FAZA cells had responded positively to dexamethasone and PMA. It therefore seemed reasonable to assume that given the appropriate conditions they would still be useful for studying the regulation of sialyltransferase. The routine culture conditions included fetal calf serum (FCS) as a requirement for cell adhesion and viability. The FCS was however, also a potential source for components which could mask or interfere with stimulation or detection of a response. It was postulated that its removal might result in a detectable sialyltransferase response to the acute phase response mediators.

Since serum-free formulations for hepatoma cell lines were not commercially available, one specifically tailored for the FAZA cell line was developed. Under the serum-free conditions developed, the FAZA cells responded to dexamethasone and PMA just as when they were cultured in the presence of FCS. The FAZA cells cultured under serum-free conditions were challenged with the various known acute phase response regulators (IL-6, TNFα, IFNγ, etc.). Broad ranges of concentrations of each cytokine, and variable combinations of the cytokines, in the presence and absence of glucocorticoid, as had been previously performed on the cells cultured in the presence of FCS, resulted in no detectable stimulation of sialyltransferase. The development and use of the serum-free culture conditions did not provide further insight into the regulation of sialyltransferase as an acute phase reactant.

Studies on the regulation of sialyltransferase in the FAZA cells were continued based on several possibilities. These included the possibility that components not yet identified as acute phase regulators may be required, or that the regulators tested were not necessarily in the correct proportions, or that since they were not purified rat proteins they were not physiologically compatible to the FAZA cells. To test these possibilities the best conceivable solution was to use rat serum itself as the physiologically complete source of any potential sialyltransferase regulators. At 10 hours after injury in the rat, many of the known regulatory factors are already nearing peak levels in the serum, and sialyltransferase, although elevated, is not yet at its peak level. Therefore a time of 10 hours post-injury, was chosen in an attempt to provide as rich a source of the factors

which regulate the acute phase response, and at the same time to minimize the sialyltransferase levels, which would cause higher than usual background levels. Rat serum was collected 10 hours after injury, and used as this potentially complete and physiologically compatible source of sialyltransferase regulators. The FAZA cells were challenged by the addition of 10 hour post-injury rat serum, at a ratio of 10 µl of rat serum per ml of cell culture medium under normal (basal) conditions, and also when cultured in the presence of either dexamethasone or PMA. The effect on sialyltransferase synthesis and secretion was monitored. Whenever this rat serum was added there was absolutely no detectable sialyltransferase activity either intracellularly, or secreted, with no loss in cell viability. In every case where rat serum had been added, the detection of sialyltransferase activity by the incorporation of [^{14}C]NeuAc into asialo α_1 AGP, revealed nothing greater than background levels of radioactivity. The anticipated stimulation of sialyltransferase by the rat serum was not observed. Furthermore, not only were normal basal sialyltransferase activity levels abolished, but so were those that would have normally occurred on challenge with either PMA or dexamethasone. Since basal, and stimulated sialyltransferase activities by PMA or dexamethasone occur through different regulatory mechanisms, the loss of activity could not be attributed to interference with the mechanisms of sialyltransferase activation. It was the measured sialyltransferase activity which itself was abolished. Based on the potential significance of this finding, i.e., that rat serum may contain a non-toxic, naturally occurring, endogenous sialyltransferase inhibitor, the

studies on the regulation of synthesis of sialyltransferase as an acute phase reactant were set aside.

To continue the investigation into the cause of the observed loss of sialyltransferase activity, the experiments challenging the FAZA cells with the 10 hour post-injury rat serum were repeated and found to confirm the previous results. Tests were then performed using commercial sialyltransferase by simply monitoring its activity in the presence and absence of serum containing inhibitory activity prepared from normal rats. In all cases when rat serum was added, no sialyltransferase activity could be detected. Galβ1-4GlcNAcα2-6 sialyltransferase was completely inactive in the presence of the inhibitory rat serum. The loss of activity was therefore further confirmed to be independent of the FAZA cells, and to potentially result from the action of a direct endogenous sialyltransferase inhibitor in rat serum.

Natural inhibitors of any glycosyltransferases have rarely been found, and none specific for sialyltransferase have ever been identified. Since it was clearly possible that a natural sialyltransferase inhibitor had been found, it was essential that this possibility be investigated. The question now posed, "Had a potential sialyltransferase inhibitor had been discovered, and if so, could it possibly be identified?", is the focus of this thesis.

II. The Identification of the Sialyltransferase Inhibitor

The existence of carbohydrates has been well known for centuries. Although early biochemical studies of the mucins began in the eighteenth century (Gottschalk, 1966; Gottschalk, 1972) it has only been in the last fifty years that the significance of carbohydrates and their conjugates have begun to be appreciated. Even today, standard biochemistry texts often only provide glimpses of this diverse and complex field of study. Texts on carbohydrates (glycans) and glycoconjugates are plentiful, yet few, if any, provide a clear overview of this area of study. The study of glycans and glycoconjugates remains to be recognized as a science of its own, with standard texts, and a designated identity in the institution. Until such time, individual organization is required to understand how any specific carbohydrate studies fall into the current context of glycoconjugate chemistry and biology.

The abundance and necessity of carbohydrates in life are well known. Carbohydrates are vital, and exist in a variety of forms. They exist in free and stored forms, providing fuel for life. They can provide structure, and influence a wide variety of functions, imparted either as pure carbohydrates or in various conjugations with other organic groups, such as proteins and lipids. As information on carbohydrates and glycoconjugates has been gathered, various classifications have been made based on structure, function, and location. This has lead to significant overlap, with specific glycoconjugates falling into multiple categories.

Classifications have been created based on structure types, on the biosynthesis of structures, on functions, on the mechanisms

which impart the particular function, and on occurrence in location, time, and development. Examples of some of the categories created include monosaccharides, disaccharides, oligosaccharides, polysaccharides (e.g. glycogen and polysialic acid), mucins, milk sugars, blood group antigens, N-linked glycoproteins including high-mannose structures, bi- tri- and tetra- antennary, complex and hybrid structures, O-linked glycoproteins, proteoglycans, glycosaminoglycans, glycolipids, and neural compounds such as cerebrosides, globosides and gangliosides. These classifications have been useful as the study of glycans and glycoconjugates has progressed. They can however be classified much more simply into three groups: glycans; glycan-lipid conjugates; and glycan-protein conjugates.

The glycan group would include for example any mono, di, tri, tetra, oligo, or polysaccharides. The many diverse monosaccharides that exist, and the possible permutations and combinations of them makes this category complex, yet relatively simple in comparison to the other two categories. A few of the more common mammalian monosaccharides that exist in the glycan category, and that are found in the glycan portion of protein and lipid glycoconjugates are shown in Appendix D, I. Since the glycans available for conjugation with either protein or lipid vary widely in type and complexity, the glycan-protein and glycan-lipid conjugates are even more diverse and complex.

The glycan-lipid conjugates would include for example, glycosphingolipids, cerebrosides, and gangliosides. The conjugation in these compounds is typically between glucose, or galactose in a β-

glycosidic linkage at the 1-hydroxyl of the monosaccharide, and any of the various ceramides. The structures of a ceramide, a cerebroside, and several gangliosides are shown in Appendix D, II. The cerebroside shows an example of the linkage between a galactose and a ceramide. The gangliosides show the linkage that occurs between a glucose and a ceramide. They also indicate the further glycosylation to form G_{M1}, G_{M2}, and G_{M3}. The variations in the ceramide structures, as well as the glycans to which they are conjugated create numerous potential structures.

The glycan-protein conjugates would include for example glycosaminoglycans, proteoglycans, mucins, blood group antigens, and all the N- and O- linked glycoproteins. The diversity in the possible types and structures of proteins, and the multiple types of linkages that can occur between the glycan(s) and protein(s), make this category perhaps the most complex of the three groups. Linkages between glycan and protein can either be of the N-linked type, or the O-linked type. The N-linked glycoconjugates are those formed by the conjugation between N-acetylglucosamine (GlcNAc) and the amino functional group of asparagine amino acids which are usually in the asparagine-X-threonine (serine) sequon, where X is any amino acid. The O-linked glycoconjugates are those formed between GlcNAc, N-acetylgalactosamine (GalNAc), galactose (Gal), mannose (Man), xylose (Xyl), or arabinose (Ara) and the hydroxyl functional group on the amino acids, serine, threonine, hydroxylysine, or hydroxyproline. Appendix D, III shows examples of the N- and O- glycosidic linkages that can occur between glycans and proteins.

Beyond the linkage between the glycan and either the lipid or the protein, subsequent additions of glycans can be made through a variety of linkages. The types of linkages vary and can be quite complex. Appendix D, II indicates several examples of linkages in the glycan portion of the gangliosides. Here in addition to the glucose linked to the ceramide, further glycosylation occurs, with the addition of Gal, GalNAc, and Gal, and an α2,3 linked NeuAc attached to the terminal Gal of G_{M3}. These structures are only a few examples of the types of glycan-lipid conjugates which can occur. Many other glycan-lipid conjugates exist, and are created by further glycosylation, in straight and branched chains, and by the addition of other functional groups.

The glycosylation of proteins beyond the core sugar attached to the protein can vary significantly. The production of N-linked structures are used here as an example to illustrate the complex sequential reactions required to create various glycan-protein conjugates. The biosynthesis of these structures are shown in Appendix D, IV. Glycans are first transferred to dolichol, in the dolichol cycle. The oligosaccharide created is subsequently transferred to the protein at asparagine residues in the asparagine-X-threonine (serine) sequon. Once attached to the protein backbone, the oligosaccharide undergoes trimming reactions with sequential removal of the terminal Glc residues by glucosidases to create high-mannose structures. After removal of the terminal glucose residues various high-mannose structures can be created by the action of mannosidases. Subsequent to these trimming reactions, further processing of the various high-mannose structures can occur. The

processing reactions include sequential transfers of GlcNAc to the core mannose structures, followed by further addition of monosaccharides by glycosyltransferases. The final structures produced can be the various high-mannose types, or the complex and hybrid types. Several complex structures of the N-linked type are shown in Appendix D, V and are only intended as examples since many other types of glycan-protein conjugates exist.

In all glycoconjugates, whether they be lipid or protein, the molecules are often sectioned, into what are termed the core structures, intermediate or backbone structures, and the terminal structures. A very simple example of the core, backbone, and terminal structures of glycoconjugates is given in Appendix D, VI where polylactosaminoglycans create the glycan-protein (N-linked and O-linked types) and glycan-lipid. The core structure is that portion of the glycoconjugate where attachment to either the protein or lipid occurs. The intermediate or backbone structure is the glycan structure attached to the core structure. While the types of core structures are limited, the intermediate or backbone structures vary significantly. The terminal structures are the very outer portions of the glycoconjugates. These termini are perhaps slightly more variable than the core structures, but much less variable than the intermediate structures. The terminal structures are most often sialic acids, found at the ends of straight and branched chains, as well as to various monosaccharides throughout the chains and branches. The uniqueness of the sialic acids, and their placement in terminal positions of glycoconjugates make them significant candidates for impact on molecular interactions.

Sialic acids are a unique family of sugars (for a comprehensive review see Schauer, 1982a), that includes both the N-glycolic and N-acetyl derivatives of neuraminic acid (see Appendix E, I). They are distinct in structure, being acidic and having a nine carbon basic structure to which various substituents can be attached at C4, C5, C7, C8, or C9. The basic structure of the sialic acids, and a list of some of the naturally occurring substituted forms are given in Appendix E, I. Sialic acids are ubiquitous in life, found in everything from bacteria to man. Since sialic acids are ubiquitous, and are usually in terminal positions, they exert a significant influence on the biological properties of the macromolecules to which they are attached (Schauer, 1982b; Pilatte *et al*, 1993; Reuter *et al*, 1982; Roth, 1993; Troy, 1992; Varki, 1992). They form various linkages with themselves, and with other monosaccharide residues in glycans, glycan-lipid, and glycan-protein conjugates, making them intimately involved in varying degrees of complexity within any organism (see Appendix E, II). Within specific organisms they are found in a variety of forms and locations, intracellularly, on cellular surfaces, and extracellularly in fluids and in structural environments. They are important in development, cell migration, and a variety of molecular interactions within organisms, and between organisms.

The creation of glycoconjugates containing sialic acids is achieved by the action of sialyltransferases. The sialyltransferases, EC 2.4.99.1 - 2.4.99.11 (Nomenclature Committee, IUBMB, 1992), include all enzymes which catalyze the transfer of sialic acid from its activated form, CMP-NeuAc, to varying acceptors. The acceptors are most often the terminal positions of the glycan chains on proteins

and lipids (Broquet *et al*, 1991; Corfield & Schauer, 1982b; Schauer, 1982a). The enzymes are quite specific, recognizing the terminal sugar on the acceptor, the anomeric linkage between the terminal sugar and the preceding sugar, as well as the backbone and core of the acceptor glycan structure. The attachments of sialic acids occur between the C2 position of the sialic acids in an alpha linkage to the acceptor sugars at their C3, C4, C6, C8 or C9 positions producing α2-3, α2-4, α2-6, α2-8, and α2-9 linkages. The most commonly found acceptor sugars in glycans and glycoconjugates to which sialic acids are transferred are Gal, GlcNAc, GalNAc, or NeuAc. While the α2-3, α2-4, α2-6, α2-8, and α2-9 linkages have all been found to exist, the most common of these are the α2-3 and the α2-6 types. More recently the α2-8 type has been observed in numerous polysialic acid-containing glycoconjugates (Troy, 1992). The α2-3 and the α2-6 linkages are still however, the major types found in all glycoconjugates. Examples of the molecular α2-3 and the α2-6 linkages between the terminal NeuAc and an accepting Gal residue are shown in Appendix E, III. These α2-3 and the α2-6 linked sialic acid-containing glycoconjugates are created by the action of the various α2-3 sialyltransferases, and the α2-6 sialyltransferases, with an example of the latter reaction shown in Appendix E, IV. The linkages formed by the various sialyltransferases depend on the availability of the CMP-NeuAc donor, and the various types and quantities of acceptors. The physiological occurrence of terminal sialic acids and the specific types of linkages to glycoconjugates also depends on the availability and activity of the various specific sialyltransferases.

The activity of the sialyltransferases which catalyze the transfer of sialic acids to glycoconjugates, and the uniqueness and impact of the sialic acids themselves, together affect the physiologic roles of the sialic acid-containing glycans and glycoconjugates. Sialylation affects intra- and intermolecular interactions, intra- and intermembrane interactions, fluid phases and the interaction of fluid phases with structural components, and the structural components themselves. Sialylation affects physiology at every level of complexity, from molecular to cellular, to tissue, to the organism, to the interactions between organisms. The physiological significance of sialylation varies with the complexity of the organism, and the state of the particular organism. There are many systems in which sialylation is known to have significant physiological impact, both in health and disease. For example, increased sialylation is known to correlate with metastatic potential (Bernacki, 1977; Collard *et al*, 1986; Nicolson, 1982; Yogeeswaran & Salk, 1981). It has also been shown that the enzyme Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) is expressed in larger amounts in cells transformed with the *ras* oncogene (Le Marer *et al*, 1992), while Galβ1-3GalNAcα2-3 sialyltransferase (EC 2.4.99.4) is decreased (Delannoy *et al*, 1993), indicating that not only the activity of sialyltransferases, but that their specificities may also be key factors in the resultant physiologic state.

A greater understanding of how and why altered states of sialylation occur, both in health and disease, may therefore provide insight into a variety of biological processes. The use of specific enzyme inhibitors is one of the most valuable tools with which to

face these challenges. The isolation of an inhibitor with sialyltransferase specificity would therefore be a valuable tool to study the biological significance of terminal sialic acids on glycoproteins, and perhaps to further investigate the development of potential therapeutics for a variety of states where sialylation is abnormal.

Inhibitors of glycosylation are valuable tools to study the correlation between various altered oligosaccharide structures and functions, and any consequential changes in physiology. Well known glycosylation inhibitors include sugar analogues, nucleotide analogues, antibiotics, and inhibitors of the complex carbohydrate biosynthetic pathway (Elbein, 1987; Elbein, 1991; Schwarz & Datema, 1982). The main focus on glycosyltransferase inhibitors has been on synthetic compounds, for example sugar analogues, since they can be tailored for specific investigations with one aim being to develop clinically useful compounds.

Naturally occurring proteins, which act as inhibitors of glycosyltransferases exist, but are less well studied, since they are essentially discovered, and not deliberately created. Their potency, lack of toxicity, and their specificity, make them potentially very valuable tools, either for the direct use as glycosyltransferase inhibitors, or as models for the creation of new synthetic compounds. Examples of some of these glycosyltransferase inhibitors include inhibitors of glucosyl and galactosyltransferases (Constantino-Ceccarini & Suzuki, 1978), of fucosyltransferase (Martin *et al*, 1990), and of N-acetylgalactosaminyltransferases (Quiroga *et al*, 1984; Quiroga & Caputto, 1988; Quiroga *et al*, 1990). Natural

glycosyltransferase inhibitors are rare, and the identities of those already discovered have not been reported.

With respect to sialyltransferases specifically a few naturally occurring inhibitors have been reported. Some small endogenous proteins (*M*r range of 14,000 to 22,000) have been found that inhibit GalNAc α2,6 sialyltransferase in calf brain (Duffard & Caputto, 1972; Van den Eijnden & Schiphorst, 1988), and lactosylceramide α2,6 sialyltransferase in rat, chicken and bovine brain, as well as in other rat and bovine organs (Albarracin *et al*, 1988). The mechanisms, specificities, and identities of these sialyltransferase inhibitors have however, not been reported. Since a natural sialyltransferase inhibitor had potentially been found here, the nature of the inhibition was investigated with the ultimate goal being to identify the sialyltransferase inhibitor.

The sialyltransferase inhibitor found here was discovered as a result of the studies on the regulation of Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) as an acute phase reactant (see Introduction, Part I. The Discovery of a Potential Sialyltransferase Inhibitor). The discovery of a natural sialyltransferase inhibitor, especially one with specificity for the Galβ1-4GlcNAcα2-6 rat liver enzyme, was an important finding since it might provide insight into the regulation and purpose of the enzyme in the circulation under normal conditions as well as during the acute phase response. Furthermore, it could be a valuable tool for studies on the significance of sialylation in physiology and pathophysiology. Therefore, the new aim, and the focus of this thesis, was to

determine the nature of the inhibition, and to attempt identification of the sialyltransferase inhibitor.

Described here are the results of the most successful attempts at purification of the inhibitor as required to determine the identity, and also a variety of the properties of the inhibitor determined throughout the purification process. The rat serum containing sialyltransferase inhibitory activity was fractionated until near purity. The final preparation was determined to contain two proteins. These were identified as rat C reactive protein (CRP) and rat α_1 macroglobulin by N-terminal amino acid sequence analysis. Tests with purified rat CRP indicated that independently, it could not inhibit sialyltransferase. Furthermore, antibodies monospecific for rat α_1 macroglobulin blocked sialyltransferase inhibition in a dose dependent manner. Based on the experimental evidence, and the known properties of the macroglobulin family of proteins, it is concluded that rat α_1 macroglobulin can function to inhibit Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) purified from rat liver.

MATERIALS

CMP-[4,5,6,7,8,9-^{14}C]-NeuAc (247 mCi/mmol) and UDP-[1-^{3}H(N)]-Gal (11.32 Ci/mmol) were from New England Nuclear Corp., Mississauga, ON, Canada. Sephadex G100 and Sephadex DEAE A25 were from Pharmacia Biotech Inc., Baie d'Urfe, PQ, Canada. Heparatinase, chondroitinase ABC, hyaluronidase (from *Streptomyces hyalurolyticus* and bovine testes), pronase, Tris base, glycine, FITC-casein, leupeptin, pepstatin A, PMSF, Sepharose CL 4B, alcohol dehydrogenase, β-amylase, apoferritin, thyroglobulin (porcine), CRP (human and *Limulus polyphemus*), ampholine pH 3.5-5.0 and 5.0-7.0, CMP-NeuAc, and UDP-Gal were from Sigma Chemical Company, St. Louis, MO, U.S.A. BSA (fraktion V), galactosyltransferase EC 2.4.1.38 (3.5 Units/mg protein), and Galβ1-4GlcNAcα2-6 sialyltransferase EC 2.4.99.1 (8 Units/mg protein) were from Boehringer Mannheim Canada, Laval, PQ, Canada. Difco bactotrypsin, and SDS were from BDH, Inc., Toronto, ON, Canada. Centricon units and YM10 membranes were from Amicon Canada Ltd., Oakville, ON, Canada. Immobilon-P membranes (PVDF) were from Millipore (Canada) Ltd., Mississauga, ON, Canada. ACS scintillant mixture, anti rabbit Ig horseradish peroxidase whole antibody (from Donkey), ECL western blotting kit, and hyperfilm were from Amersham Life Science, Oakville, ON, Canada. Bromophenol blue was from Matheson Coleman & Bell, Cincinnati, Ohio, USA. Coomassie blue G-250 was from Pierce Chemical Company, Rockford, IL, U.S.A. EDTA, nitrocellulose, Whatman 3MM filter paper, and 2.5 cm diameter Whatman No. 1 filter paper discs were from Fisher Scientific,

Edmonton, AB, Canada. Acrylamide, Bis, 2-mercaptoethanol, Temed, Tween-20 E1A grade, high and low molecular weight standards for gel electrophoresis, and Bio-Rad reagent, were all from Bio-Rad Laboratories Ltd., Mississauga, ON, Canada. Rat α_1 AGP, asialo α_1 AGP and asialoagalacto α_1 AGP were prepared as previously described (Kaplan *et al*, 1983). Asialo inhibitor was prepared using the same method of acid hydrolysis at 80°C for 1 hour. All other chemicals were of reagent grade.

The following were kind gifts: pure rat C-reactive protein from Dr. G. Nelsestuen (Department of Biochemistry, University of Minnesota, MN, U.S.A); antibodies that were monospecific for the heavy (α) and light (β) chains of rat α_1 macroglobulin from Dr. P.C. Heinrich, (Institut für Biochemie, Aachen, Germany) and Dr. T. Geiger (Ciba-Geigy, Ltd., Basel, Switzerland); and pure rat methylamine α_1 macroglobulin from Dr. L. Sottrup-Jensen (Dept. Mol. Biol., University of Aarhus, Denmark).

METHODS

I. Enzyme Assays

Galβ1-4GlcNAcα2-6 sialyltransferase and galactosyltransferase were assayed based on methods previously described (Kaplan *et al*, 1983; Lammers & Jamieson, 1988; Woloski *et al*, 1986). The standard mixture for sialyltransferase assay contained 250 μg rat asialo α_1 acid glycoprotein as acceptor, 5 nmole, 20 nCi CMP-[^{14}C]NeuAc (4 nCi/nmole), 1.2 mmole Tris, pH 7.0, 50 μg BSA and 0.1 or 0.2 mU pure sialyltransferase in a total volume of 80 μl. For assay of galactosyltransferase the mixture was as described above, except that 2 mmole $MnCl_2$ was present, 250 μg rat asialoagalacto α_1 acid glycoprotein was the acceptor, and 5 nmole, 20 nCi UDP-[1-^{3}H(N)]-galactose (4 nCi/nmole) was the labeled donor, and the enzyme was 1 mU pure galactosyltransferase. Incubation was for 2 hr at 37°C. At the end of incubation samples were immediately transferred to ice and 70 μl volumes were spotted on 2.5 cm diameter circles of Whatman No. 1 filter paper, washed as described before (Lammers & Jamieson, 1988) and counted using ACS cocktail in a liquid scintillation counter. In all assays, conditions were such that product formation was linear with time and amount of enzyme protein used. One unit of sialyltransferase activity is that amount of enzyme that transfers 1 mmole NeuAc from CMP-NeuAc to asialo α_1 acid glycoprotein per minute at 37°C under the described conditions (Weinstein *et al*, 1982). One unit of galactosyltransferase activity is that amount of enzyme that transfers 1.0 mmole Gal from UDP-Gal to

GlcNAc per minute at 37°C under the described conditions (Verdon & Berger, 1983).

II. Enzyme Inhibition Assays

When sialyltransferase or galactosyltransferase inhibition was monitored the enzyme was suspended in 50 µl of 1 mg BSA per ml 20 mM Tris, pH 7.0. Enzymes were mixed with up to 10 µl aliquots of test samples pre-equilibrated in 20 mM Tris, pH 7.0 and incubated for 2 hours at 37°C. The aliquots of test samples from the various purifications, either individual fractions or pooled fractions, contained or were adjusted to contain inhibitor quantities capable of near complete inhibition of the enzyme. These high quantities of inhibitor relative to sialyltransferase were used to ensure that no inhibition would be undetected. After incubation the radioactive sugar nucleotide donor and macromolecular acceptor were added and assayed as described above for sialyltransferase or galactosyltransferase. Controls were samples of pure enzymes mixed with buffer rather than inhibitor samples and were incubated in parallel. The controls are taken as uninhibited samples, and inhibition for test samples is expressed as a percentage relative to these controls.

III. Isolation of the Sialyltransferase Inhibitor from Rat Serum

The actual methods attempted for the isolation of the sialyltransferase inhibitor were quite varied. Typical examples of the most successful methods are described here. Blood was obtained

from 150-200 g male Long Evans Hooded rats by partial decapitation, severing the jugular veins. Serum was prepared by allowing clot formation for approximately 1 hour at room temperature. It was then centrifuged at 4000 x g_{av} to remove any remaining cell debris, as previously described (Ashton *et al*, 1970). Serum samples containing inhibitory activity towards sialyltransferase were pooled. For the results shown a 40 ml pool of serum (48.2 mg/ml) containing approximately 1930 mg of protein was applied to a water-jacket cooled (4°C) column of Sephadex G100 (90cm x 7cm) using a peristaltic pump. Elution was performed by pumping 20 mM Tris, pH 7.0 in an upward flow at a rate of 40 ml per hour. Fractions of 7 ml in volume were collected and kept at 4°C. Absorbance at 280 nm, for detection of protein, was determined for ten fold dilutions of each fraction. Fractions were also monitored for the presence of sialyltransferase inhibitor using 10 µl aliquots from each fraction and 0.1 mU sialyltransferase in the standard inhibition assay. The fractions containing inhibitory material and intended for use in further purification were pooled giving a total volume of 184 ml (3.55 mg/ml) and a total of approximately 653 mg protein. From this pool 182 ml were reduced in volume to approximately 15 ml using a YM10 membrane (*M*r cut off 10,000) in an Amicon concentrator, and then dialyzed exhaustively against 20 mM sodium phosphate buffer, pH 8.0, in preparation for further fractionation.

The 15 ml dialysate (43.1 mg/ml) containing 646 mg protein, was applied to a column of Sephadex DEAE A25 (45cm x 5cm) equilibrated with 20 mM sodium phosphate buffer, pH 8.0. The column was eluted by downward flow, with a stepwise gradient of

20 mM sodium phosphate buffers of decreasing pH and increasing NaCl concentration. This procedure was based on earlier work for the isolation of serum glycoproteins (Ashton *et al*, 1970). The buffers used were: I. pH 8.0; II. pH 6.6, 70 mM NaCl; III. pH 6.0, 100 mM NaCl; IV. pH 5.0, 300 mM NaCl; and V. pH 5.0, 800 mM NaCl. The column was eluted at 45 ml per hour and 7 ml fractions were collected at 4°C. Absorbance at 280 nm was determined, and buffers were changed when readings fell to approximately 0.1 units. Fractions containing protein were pooled, concentrated, and dialyzed exhaustively against 20 mM Tris, pH 7.0 to remove high salt which inhibits sialyltransferase activity (Sticher *et al*, 1988). After dialysis each protein pool was assayed for sialyltransferase inhibition using 10 µl aliquots of each peak and 0.1 mU sialyltransferase in the standard inhibition assay. All of the inhibitory activity was found in the Peak IV pool. After dialysis and concentration to 35 ml (1.9 mg/ml) the pool contained a total of 68 mg of protein.

The concentrated and dialyzed Sephadex DEAE A25 Peak IV pool was further fractionated by applying a 2 ml (1.9 mg/ml) aliquot containing 3.9 mg of protein to a column of Sepharose CL 4B (150cm x 2.25cm). Elution was with 20 mM Tris, pH 7.0 in a downward flow at approximately 25 ml per hour. Fractions of 1.5 ml volume were collected, monitored for absorbance at 280 nm and for sialyltransferase inhibition using 10 µl aliquots from the fractions and 0.2 mU sialyltransferase in the standard inhibition assay. Protein and sialyltransferase inhibitory activity eluted as a single peak which were pooled giving a total volume of 8.6 ml (0.3 mg/ml) and a total of approximately 2.6 mg protein. Material intended for

further purification by isoelectric focusing was dialyzed against deionized water.

Purification by isoelectric focusing was based on a method used in previous work (Jamieson *et al*, 1972) using a water-jacket cooled (4°C) 120 ml column, a pH range of 3.5 to 7.0 in a sucrose gradient, and 2% (v/v) ampholine. A 6 ml aliquot of Sepharose CL 4B purified inhibitor, dialyzed against deionized water, containing approximately 1.8 mg of protein was added with the sucrose gradient. Focusing was at a constant voltage of 1800 V. The initial current was typically in the range of 12 to 20 mA and the final current was approximately 2 mA. Focusing was stopped after 4 to 5 days. The column was emptied by collecting 1 ml fractions. Each was monitored immediately for absorbance at 280 nm and for pH. To remove ampholytes and sucrose and to equilibrate in 20 mM Tris, pH 7.0 each fraction was washed exhaustively (3 to 5 times with 2.5 ml of 20 mM Tris, pH 7) by Amicon centricon ultrafiltration (*M*r cut off 30,000). The final volume per fraction was adjusted to 0.75 ml and pH values were checked to ensure that exchange into the pH 7.0 buffer was successful. Fractions were then monitored for absorbance at 280 nm and assayed for sialyltransferase inhibition using 10 µl aliquots of each fraction and 0.2 mU sialyltransferase in the standard inhibition assay. Fractions containing inhibitory activity, i.e., those focusing between pH 4.2 to 4.5, were pooled giving an initial volume of 20 ml and a protein quantity of 0.45 mg. This pool was concentrated by centricon ultrafiltration (*M*r cut off 30,000) and stored until required at -20°C.

IV. Inhibitor *M*r Determination

The Sepharose CL 4B (150cm x 2.25cm) column used for purification was also used to estimate the *M*r of the sialyltransferase inhibitor. The column was calibrated with approximately 2.5 mg of each marker protein in 0.5 ml 20 mM Tris, pH 7. Elution was performed with 20 mM Tris, pH 7 and 1.5 ml fractions were collected. The marker proteins included thyroglobulin, *M*r 669,000; apoferritin, *M*r 443,000; β-amylase, *M*r 200,000; and alcohol dehydrogenase, *M*r 150,000. A calibration curve was constructed (log *M*r versus elution volume). The inhibitor *M*r was determined from the calibration curve using the average elution volume of the inhibitor from five separate runs, based on the described technique for *M*r determination (Andrews *et al*, 1965).

V. Definition and Determination of Inhibitor Activity

One unit of inhibitory activity is defined as that quantity of the inhibitor preparation required to cause 50% inhibition of 0.1 mU α2,6 sialyltransferase following incubation for 2 hours at 37°C. Titration of each inhibitor pool was performed initially over broad ranges of inhibitor quantities. Titrations over the narrower 50% inhibition range were then performed and used to determine the units of inhibitor activity. Protein was determined by the Bio-Rad method (Bradford, 1976). The specific activities were calculated from the experimentally determined units of inhibitor activity and protein quantities for the initial serum and the various pools throughout the purification process.

VI. Effect of Hydrolysis of the Inhibitor on its Activity

The effect of acid hydrolysis was done with inhibitor purified by Sephadex G100. Inhibitor was incubated for 1 hour at 80°C with 0.05 M sulfuric acid, followed by cooling and addition of 0.1 M sodium hydroxide to neutralize the acid. The control was inhibitor treated identically, except that water was added in place of the acid and base. The effect of acid hydrolysis of the inhibitor was then monitored using 0.1 mU sialyltransferase in the standard inhibition assay, and compared to the mock-treated inhibitor activity.

For proteolytic hydrolysis, the effect of trypsin and pronase treatments of the inhibitor were investigated. Bactotrypsin was mixed with Sephadex G100-purified inhibitor at weight ratios of up to 15 parts trypsin to 1 part inhibitor, at pH 7. The mixtures were incubated for up to 6 hours at 37°C, and were then boiled to destroy tryptic activity. The trypsin-treated samples were assayed for inhibitory activity using 0.1 mU sialyltransferase and compared to controls containing no inhibitor. Pronase digestions were carried out in Tris pH 8, 1 mM $CaCl_2$, for up to 120 hours at 37°C using DEAE-purified inhibitor. Initially 1% (w/w) pronase was added to the inhibitor. At each 24 hour interval thereafter an additional 0.5% (w/w) pronase was added. At the end of the incubation samples were boiled to destroy pronase activity. The pronase-treated samples were then assayed for inhibitory activity using 0.1 mU sialyltransferase and compared to controls containing no inhibitor.

The effect of polysaccharide hydrolyzing enzymes was monitored on DEAE-purified inhibitor. Heparatinase (up to 0.3 Units per 20 µl DEAE Peak IV concentrated material) digestions were

carried out in 20 mM Tris, pH 7. Chondroitinase ABC (up to 0.3 Units per 20 µl of DEAE Peak IV inhibitor) digestions were carried out in 20 mM Tris, pH 8. Hyaluronidase, (from *Streptomyces hyalurolyticus* and from bovine testes, with up to 30 Units per 20 µl of DEAE Peak IV inhibitor) digestions were carried out at pH 5 in 20 mM sodium acetate buffer. Digestions were carried out at 37°C for up to 4 hours followed by boiling to inactivate the hydrolytic enzymes. The samples were then assayed for inhibitory activity using 0.1 mU sialyltransferase and compared to controls containing no inhibitor.

In all experiments attempted for hydrolysis of the inhibitor, an additional control was always included to ensure that the heat-inactivated hydrolytic agent(s) was not interfering with the sialyltransferase inhibitor activity. These controls were identical to the test samples, except that inhibitor samples were treated with heat-inactivated hydrolytic agent(s) rather than the active hydrolytic agent(s).

VII. Protein and Carbohydrate Analysis of the Inhibitor

Protein was assayed by the Bio-Rad method, with BSA used to prepare the standard calibration curve. Briefly, the assay involves the addition of 0.1 ml of sample to 5 ml diluted (1 part reagent to 4 parts deionized water) Bio-Rad protein assay dye reagent, followed by measurement at 595 nm in a spectrophotometer (Bradford, 1976). The absorbance values for the test samples are used to determine the weight of protein from the calibration curve. Carbohydrate was analyzed by the resorcinol method (Svennerholm, 1957) which

involves mixing the samples or the standard mannose solutions with the resorcinol reagent, boiling for 30 minutes, diluting with water and measuring absorbance at 428 nm. The absorbance values for the test samples are used to determine the weight of carbohydrate from the standard mannose calibration curve.

VIII. Determination of Whether the Inhibitor is Hydrolyzing the Nucleotide Donor

Inhibitor isolated from the Sephadex G100 column was tested for the presence of hydrolase activity that could potentially destroy CMP-NeuAc in the inhibition assay system. Inhibitor and 20 nCi CMP-[^{14}C]NeuAc (5 nmole) were mixed and incubated for 2 hours at 37°C. The mixtures were examined for the presence of free [^{14}C]NeuAc by chromatography as before (Kaplan *et al*, 1983). Samples were spotted onto (47cm x 56cm) Whatman 3MM paper, with 5 cm between each sample. The chromatogram was run in a closed chamber with the solvent ethyl acetate: pyridine: water (12:5:4, by volume), in a descending manner for 7 hours. After the chromatogram was dry, it was cut into strips (5 cm width, 2.5 cm on either side of each sample spot) along the length of the paper, parallel with each sample lane. Strips were then cut crosswise in 0.5 cm segments, placed in 4 ml of ACS cocktail, and monitored for radioactivity. Controls included were 20 nCi CMP-[^{14}C]NeuAc (5 nmole), and [^{14}C]NeuAc prepared by hydrolysis of 20 nCi CMP-[^{14}C]NeuAc (5 nmole), with 0.05 M sulfuric acid at 80°C for 1 hour.

IX. Determination of Whether the Inhibitor is Destroying the Macromolecular Acceptor

To determine whether the inhibitor was destroying the acceptor, rat asialo α_1 acid glycoprotein was treated with DEAE Peak IV purified inhibitor. Approximately 0.5 mg of protein from the inhibitor preparation in 20 µl of 20 mM Tris, pH 7, and 1.25 mg of acceptor in 100 µl water, were mixed and incubated for 18 hr at 37°C. The control was acceptor incubated with buffer, rather than the inhibitor preparation. The inhibitor-treated acceptor, and control buffer-treated acceptor mixtures were then each fractionated on a Sephadex G100 column (22cm x 0.8cm). Elution was with 20 mM Tris, pH 7, and 10 drops per fraction were collected. The fractions were monitored for absorbance at 280 nm, relative to a 20 mM Tris, pH 7 buffer blank. After fractionation, the protein peaks containing the acceptor were pooled, volume adjusted so that appropriate protein concentrations were achieved, and the pools were monitored for their ability to act as acceptors in the standard sialyltransferase assay. The control for this sialyltransferase assay used untreated acceptor of equivalent quantity to that of the buffer-treated acceptor, and of the inhibitor-treated acceptor.

X. Determination of Whether the Inhibitor is Itself Acting as an Acceptor in the Sialyltransferase Assay

Experiments were also performed to determine if the inhibitor was acting as an acceptor. The standard sialyltransferase assay was used with the inhibitor or asialo inhibitor, substituted for the usual acceptor. The inhibitor was the Sephadex DEAE, peak IV dialyzed

and concentrated material. Following incubation of enzyme, donor, and the various potential acceptors, the assay mixtures were fractionated on Sephadex G100 (24cm x 0.8cm) using 20 mM Tris, pH 7 as eluent. Fractionation was performed to separate any free CMP-[^{14}C]NeuAc from that incorporated into the potential acceptor protein. Fractions of 10 drops were collected, added to 10 ml of ACS cocktail, and monitored for radioactivity. Controls were parallel treatments run in the presence and absence of asialo α_1 acid glycoprotein acceptor, as well as pure CMP-[^{14}C]NeuAc, fractionated as above, and monitored for radioactivity.

XI. Effect of Addition of Protease Inhibitors

Sephadex G100 purified inhibitor (10 µl aliquots) were mixed separately with each protease inhibitor solution (10 µl aliquots). Protease inhibitor solutions included PMSF in ethanol, leupeptin in water, pepstatin A in DMSO, and EDTA in water. Each were present at a final concentration of 0.1 mM, after mixing with inhibitor preparation. Each mixture of protease inhibitor plus Sephadex G100-inhibitor was incubated at 37°C for 2 hr followed by addition of 0.1 mU sialyltransferase for the standard inhibition assay. Controls used to calculate the percent inhibition were identical samples except that the G100 purified inhibitor was replaced by buffer. Additional controls were included to monitor the effect of the protease inhibitors themselves on sialyltransferase, and to monitor the effect of the solvents used to dissolve the protease inhibitors on sialyltransferase, and inhibition of sialyltransferase by the G100 purified material.

XII. Determination of Proteolytic Activity in Inhibitor Preparations

Inhibitor purified by Sepharose CL 4B and by isoelectric focusing, were each assayed for proteolytic activity using the FITC-casein hydrolysis method (Twining, 1984) with some modifications. Standard curves using bactotrypsin were run at the same time as the inhibitor preparations. Standard curves from 0 to 1 ng of trypsin per tube, and from 0 to 100 ng of trypsin per tube were prepared, each in a final volume of 50 µl. Each contained 10 µl of various trypsin concentrations in deionized water, 20 µl of assay buffer (100 mM Tris, pH 7.8, 10 mM $CaCl_2$), 10 µl of the substrate FITC-casein (5 µg FITC-casein/µl of 50 mM Tris, pH 7.2), and 10 µl of 50 mM Tris, pH 7.2. The blanks contained 10 µl of deionized water, instead of trypsin. Varying amounts of inhibitor preparations were run concurrently with the standard curves. These assay mixtures were identical to the standard curve mixtures, except that the inhibitor preparations replaced the tryptic solutions. Samples and standard curves were incubated at 37°C from 1 to 24 hours, depending on the tryptic activity. One hour at 37°C, allows 10 ng of trypsin to be easily detected, and longer incubations allow for even more sensitive protease detection. Incubation at 37°C allows for the enzymatic hydrolysis of the FITC-casein and release of the fluorescent label from the protein. After incubation the protein is precipitated by the addition of 120 µl of 5% trichloroacetic acid (TCA). The samples are vortexed, and allowed to stand at room temperature for at least 1 hour prior to microcentrifugation to pellet the precipitated protein. For convenience, samples were sometimes left to stand for up to 24

hours at 4°C. 60 µl of the supernatant, containing any protein-free fluorescent label, is transferred to fresh tubes containing 1 ml of 0.5 M Tris, pH 8.5, with thorough mixing. The relative fluorescence is determined using an excitation λ of 490 nm and an emission λ of 525 nm, on a Gilford Fluoro IV instrument. In some cases, where the concentration of protease was high, dilution of the 60 µl of assay supernatant with up to 3 ml of buffer was required. Inhibitor samples were diluted with 1 ml of buffer, and were allowed to undergo reaction for varying lengths of time, for up to 24 hours, since no activity could be detected after 1 to 4 hours. For instrument readings, the relative fluorescence of deionized water was 0, and that for the blanks were measured and subtracted from the standard and test fluorescent readings. Blanks for the standards contained no trypsin, but were volume adjusted with water. Blanks for the test samples contained 20 mM Tris, pH 7 in place of the inhibitor preparations. Fluorescence readings for blanks diluted with 1 ml of buffer were approximately 4.7 - 4.9, and 1.6 - 1.7 for those diluted with 3 ml of buffer.

XIII. Kinetic Analysis

For kinetic analysis of the effect of the inhibitor on α2,6 sialyltransferase activity, the standard sialyltransferase assay was used, except that a fixed amount of 8 nmole, 40 nCi, CMP-[^{14}C]NeuAc (5 nCi/nmole) was added to each mixture that contained acceptor of varying quantities, from 15 to 250 µg per assay mixture. The activity of 0.2 mU sialyltransferase was monitored over the varying acceptor quantities, in the absence and presence of varying inhibitor

quantities. Inhibitor (2.5, 5.0, or 10 µl) from the Sepharose CL 4B purified pool was used, and the final volumes of each mixture were adjusted by the appropriate addition of buffer. Incubations were for 30 minutes at 37°C, and CMP-NeuAc consumption was limited to less than 2%. Michaelis-Menten, and double reciprocal plots were prepared from the rate determinations (pmole sugar transferred per minute) and the known quantities of Gal acceptor sites.

Kinetic analysis was also performed using the standard sialyltransferase assay but in the presence of 0.78 mM Gal acceptor sites and over a range of donor concentrations, including 0.013, 0.038, 0.025, 0.050, and 0.10 mM CMP-[^{14}C]NeuAc. Radioactivity was maintained at 5 nCi/nmole for each mixture. Assays were performed in the absence and presence of varying inhibitor quantities, as above. In these assays CMP-NeuAc consumption was limited to less than 3%. Michaelis-Menten, and double reciprocal plots were prepared from the rate determinations (pmole sugar transferred per minute) and the known quantities of CMP-NeuAc.

CA-Cricket Graph III version 1.5 (Computer Associates) software was used for the graphic preparations. The Michaelis-Menten plots were prepared using the power curve fit function, and double reciprocal plots were prepared using the linear curve fit function.

XIV. Gel Electrophoresis and Protein Sequence Analysis

Polyacrylamide gel electrophoresis was performed using the Bio-Rad Mini-Protean II electrophoresis system based on the previously described method (Laemmli, 1970), but discontinuous stacking gels were omitted, and instead, continuous running gels were used. Electrophoresis was performed on the inhibitor prepared by isoelectric focusing with 25 µg and 45 µg of protein loaded for non-reducing and reducing gels, respectively. For non-reducing conditions 4.8% acrylamide gels were used, and for reducing conditions 7.5% acrylamide gels were used. The inhibitor preparations were suspended in sample buffer, such that the final concentrations were 2% (w/v) SDS, 10% (v/v) glycerol and 0.1% (w/v) bromophenol blue in 0.0625 M Tris, pH 6.8 for non-reducing conditions. For reducing conditions the samples were suspended in sample buffer as above, except that 2-mercaptoethanol was present to a final concentration of 5% (v/v). The running buffer contained 0.2 M glycine, and 0.1% (w/v) SDS, in 0.025 M Tris, pH 8.1. Prior to sample loading, gels were pre-run at 100 V for at least 30 minutes, spent running buffer discarded and fresh running buffer was added. Samples were loaded and electrophoresis was performed at 150 V for approximately 30 minutes. Two types of standard mixtures were used. The high *M*r standard mixture contained 2 µg each of myosin (*M*r 200,000), β-galactosidase (*M*r 116,250), phosphorylase b (*M*r 97,400), BSA (*M*r 66,200), and ovalbumin (*M*r 45,000). The low *M*r standard mixture contained 2 µg each of phosphorylase b, BSA, ovalbumin, soybean trypsin inhibitor (*M*r 21,500), and lysozyme (*M*r 14,400). All samples were boiled for 10 minutes prior to loading on

the gels. For protein detection acrylamide gels were stained with 1% Coomassie blue.

When N-terminal sequences were to be determined, gels were not stained or fixed, but were electroblotted onto Immobilon-P (PVDF) membranes at 100 V for 1.75 hr at 4° C. The membranes were stained with 0.1% Coomassie blue. Coomassie blue positive bands were excised and N-terminal sequence analysis performed with an Applied Biosystems Model 470 Gas-Phase Sequencer at the Microsequencing Center at the University of Victoria, BC, Canada. Transfer, staining and analyses were carried out as described (LeGendre & Matsudaira, 1988). The resulting sequences were used in a search for identity with the BLAST program (Altschul *et al*, 1990) at the National Center for Biotechnology Information (NCBI), Bethesda, MD, USA.

XV. Effect of C Reactive Protein on Sialyltransferase Activity

Pure human, *Limulus polyphemus* or rat CRP (0.3 - 0.6 μg), were tested as potential sialyltransferase inhibitors. The standard inhibition assay with 0.2 mU sialyltransferase was used, but the usual inhibitor preparations were replaced with the CRP solutions. Controls used to determine percentage inhibition, contained 20 mM Tris, pH 7 buffer in place of the CRP solutions.

XVI. Effect of Methylamine α_1 Macroglobulin on Sialyltransferase Activity

Methylamine α_1 macroglobulin was tested over a range of 0.1 to 10 µg for the potential to inhibit sialyltransferase. The standard inhibition assay was used with 0.2 mU sialyltransferase, and methylamine α_1 macroglobulin added in place of the usual inhibitor preparations. Controls used to determine the percentage inhibition, contained 20 mM Tris, pH 7 buffer in place of the methylamine α_1 macroglobulin solutions.

XVII. Immunological Methods

Antibodies specific towards the heavy (α) and light (β) chains of α_1 macroglobulin were prepared as described (Geiger *et al*, 1987). Varying volumes were prepared by dilution of these antibodies with deionized water in a total volume of 10 µl. The 10 µl antibody aliquots were mixed with 10 µl aliquots of inhibitor and incubated for 2 hours at 37°C. The inhibitor was that isolated by isoelectric focusing, adjusted with 20 mM Tris, pH 7 buffer to contain approximately 0.5 µg of protein in each 10 µl aliquot. After incubation of antibodies with inhibitor, 0.2 mU sialyltransferase in 40 µl of 1 mg BSA/ml 20 mM Tris, pH 7 buffer, was added and incubated for 2 hours at 37°C, followed by the standard sialyltransferase assay. Controls used to calculate the percentage inhibition consisted of samples in which inhibitor and antibodies were absent. Controls were also run in which inhibitor was absent but antibodies were present, to determine if the antibodies themselves could affect sialyltransferase activity. Antibodies to

other rat serum proteins, including anti- albumin and anti- α_2 macroglobulin, were also monitored for their effect on sialyltransferase inhibition, as additional controls. The volumes of all controls were adjusted by the addition of 20 mM Tris, pH 7 so that they would be equivalent to the test samples.

RESULTS

I. Discovery of a Sialyltransferase Inhibitor in Rat Serum

Rat serum, used as a potential source of physiological mediators of the acute phase response, was found to cause a complete loss of sialyltransferase activity in early FAZA cell culture studies on the acute phase response. A potential sialyltransferase inhibitor had been discovered. Investigation into the possibility that the rat serum contained component(s) capable of inhibiting sialyltransferase began quite simply by monitoring its effect on commercial enzyme activity. The inhibitory effect rat serum was first observed to have on commercial sialyltransferase is summarized in Table 1. The activity of 0.5 mU sialyltransferase, as measured by the quantification of the radioactivity transferred from the donor to the acceptor, was 99.5% inhibited by the addition of 45 µl of rat serum. Since it was unnecessary to use 0.5 mU sialyltransferase in order to achieve reliable quantities of transferred radioactivity (dpm), the quantities used were subsequently reduced to 0.1 - 0.2 mU as described in the standard inhibition assay (see Methods). Tests were also carried out with reduced volumes of serum. It was found that as little as 1 µl of rat serum could cause 95% inhibition of 0.1 mU sialyltransferase, indicating that the inhibitory component in the rat serum was very potent. Owing to the potency, and the potential that a specific, natural sialyltransferase inhibitor had been discovered, characterization of the type of inhibition and isolation of the inhibitor were undertaken concurrently.

Table 1: Galβ1-4GlcNAcα2-6 Sialyltransferase Inhibition by Rat Serum

Commercial sialyltransferase was assayed using 5 nmole (20 nCi) CMP-[^{14}C]NeuAc donor and 250 µg asialo α_1 AGP acceptor and incubating for 2 hours at 37°C. The control samples contained water in place of the rat serum. For the test samples 45 µl of rat serum (prepared as described in Methods) was included. The average dpm represents the amount of radioactivity transferred from the donor to the acceptor, acid precipitated and counted. The sialyltransferase activity in the absence of rat serum is set as the 100% control. The ratio of the activity in the presence of rat serum to the activity of the control is used to calculate the percentage of control, and the percent inhibition. Additionally, 45 µl of the rat serum itself was monitored for sialyltransferase activity and had virtually no activity, with 66 dpm measured per assay mixture.

	Average dpm per assay mixture	% of Control	% Inhibition
Control	14,917	100	0
Rat Serum	72	0.5	99.5

II. Isolation of the Sialyltransferase Inhibitor from Serum

Isolation of the sialyltransferase inhibitor required multiple attempts using various methods, with the results of the most successful purification sequence presented here. Rat serum containing inhibitor was fractionated first by size on a Sephadex G100 column, as shown in Figure 1. The absorbance at 280 nm was monitored and indicated two protein peaks. Fractions were also assayed for sialyltransferase inhibition. The elution profile shown is from 350 to 1000 ml, since no inhibitory activity or protein was detected below or above these volumes. All of the inhibitory activity was found in the first protein peak. The fractions in the first protein peak were therefore pooled and prepared for further purification, as described in Methods.

The inhibitor pool from the Sephadex G100 column was further fractionated, as shown in Figure 2, by stepwise elution from an anion exchange column of Sephadex DEAE A25. Buffers of decreasing pH and increasing ionic strength were used to sequentially elute molecular species according to their affinity for the column. This fractionation resulted in a complete separation of the inhibitor from the other protein peaks. All the inhibitory activity was confined to peak IV, as summarized in Table 2. The inhibitor was apparently acidic in nature, since it was not released from the column until the pH had been dropped to 5, and the ionic strength increased to 300 mM NaCl. This was an important factor used to determine an appropriate pH range to be used later in the purification by isoelectric focusing. The peak IV inhibitor was prepared as described in the Methods, for subsequent purification.

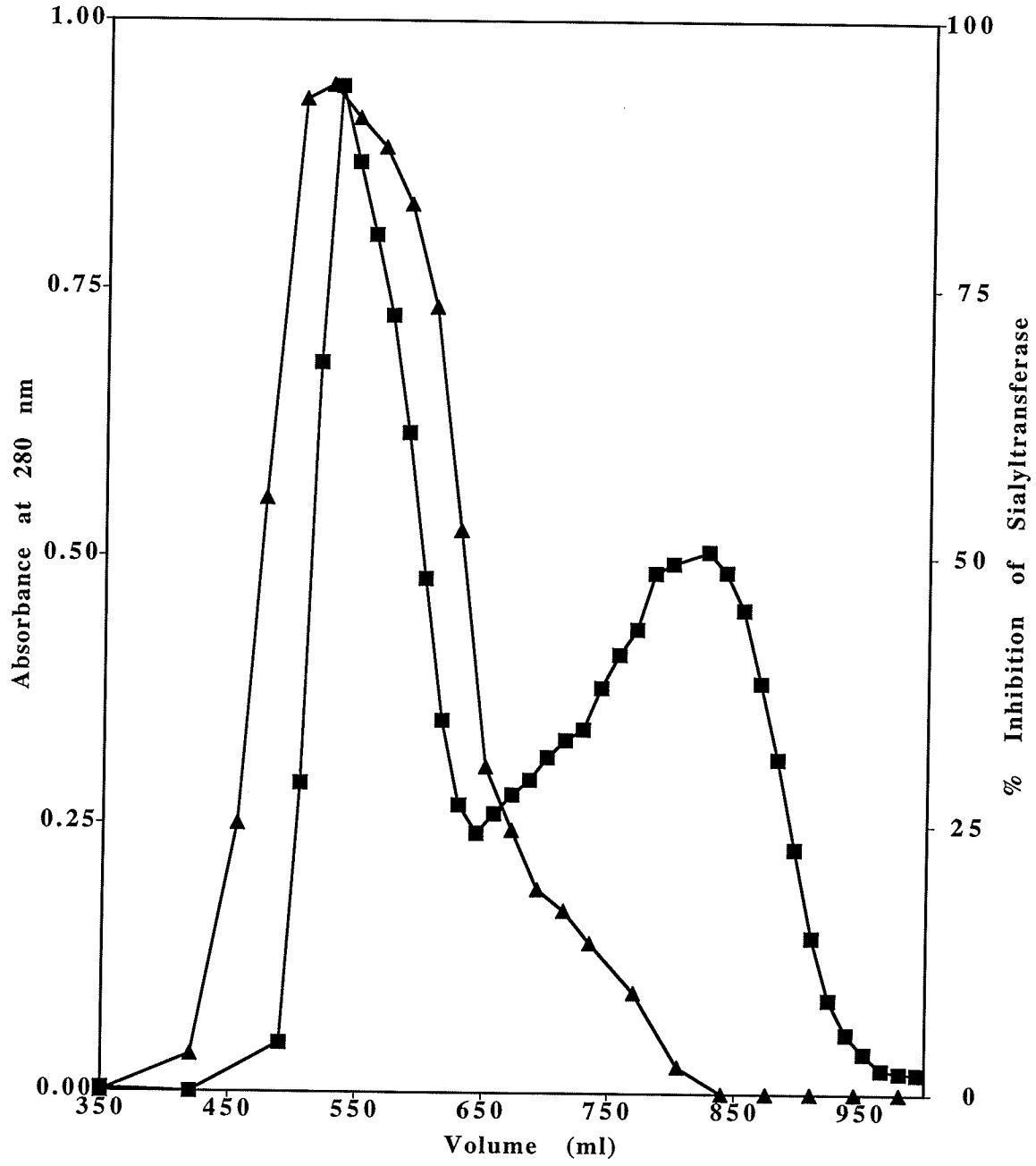


Figure 1: Fractionation of Serum on Sephadex G100
Rat serum containing sialyltransferase inhibitory activity was fractionated on a column of Sephadex G100 (90cm x 7cm). The serum, 40 ml containing approximately 1930 mg of protein was loaded by pump, using reverse flow, followed by the 20 mM Tris, pH 7 elution buffer. Fractions of 7 ml were collected. The absorbance at 280 nm of column fractions diluted 1/10 by volume (■) were monitored. The presence of inhibitor was determined for 10 µl aliquots from column fractions with 0.1 mU pure sialyltransferase using the standard inhibition assay. The percentage sialyltransferase inhibition (▲), is calculated relative to uninhibited controls that contained no column material, but were adjusted to the same volume with 20 mM Tris, pH 7.

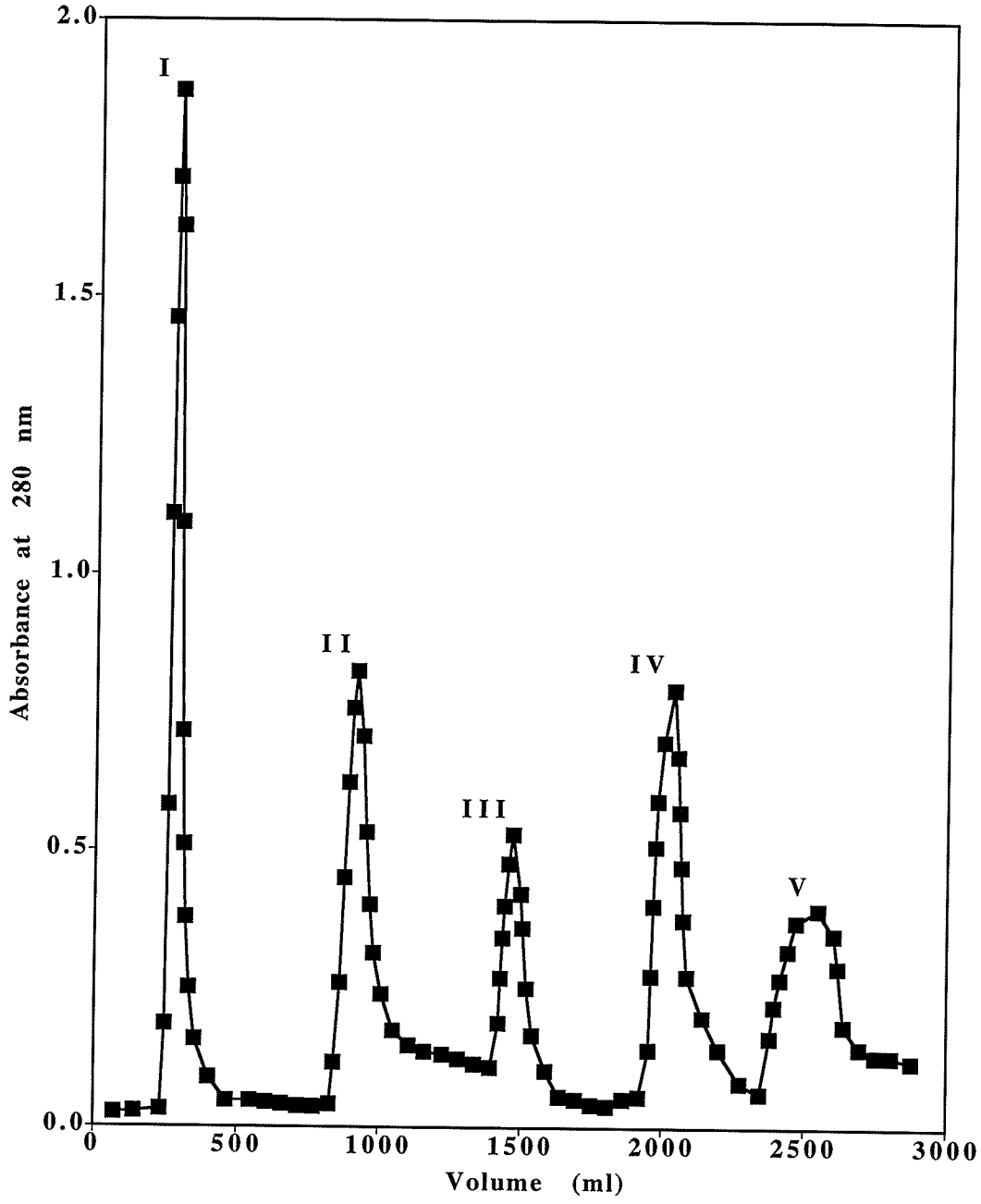


Figure 2: Step-Wise Fractionation of the Sialyltransferase Inhibitor Isolated from the Sephadex G100 Column on Sephadex DEAE A25

Fractions containing the inhibitory activity from the Sephadex G100 column were pooled, reduced in volume, and dialyzed exhaustively against 20 mM sodium phosphate buffer, pH 8. A 15 ml aliquot of this Sephadex G100 material, containing 646 mg protein, was loaded onto the Sephadex DEAE A25 column (45cm x 5cm). Stepwise elution was with 20 mM sodium phosphate buffers I. pH 8; II. pH 6.6, 70 mM NaCl; III. pH 6.0, 100 mM NaCl; IV. pH 5, 300 mM NaCl; and V. pH 5, 800 mM NaCl. Fractions of 7 ml were collected, the absorbance at 280 nm (■) was monitored, and buffers were changed when these values fell to 0.1, or less.

Table 2: Inhibitory Effect of Sephadex DEAE A25 Protein Peaks on Galβ1-4GlcNAcα2-6 Sialyltransferase Activity

Protein peaks eluted at each step were dialyzed exhaustively against 20 mM Tris, pH 7, and concentrated, prior to assay for effect on α2,6 sialyltransferase. Sialyltransferase inhibition was determined using 10 µl aliquots of concentrated material from each peak, and 0.1 mU sialyltransferase in the standard inhibition assay. Inhibition is calculated relative to controls which contained no material from the column, but were volume adjusted with 20 mM Tris, pH 7.

	Step-Wise Elution Buffers	% Inhibition of Sialyltransferase
Peak I	pH 8	0
Peak II	pH 6.6, 70 mM NaCl	0
Peak III	pH 6.0, 100 mM NaCl	0
Peak IV	pH 5, 300 mM NaCl	95
Peak V	pH 5, 800 mM NaCl	0

Samples of the dialyzed, and concentrated Sephadex DEAE peak IV inhibitor were further fractionated on a Sepharose CL 4B column. Figure 3 shows the absorbance readings at 280 nm and the sialyltransferase inhibitory activity peak at approximately the same position. The elution profile shown is from 150 to 450 ml, since no inhibitory activity or protein was detected below or above these volumes. This particular purification step did not result in the dramatic removal of non-inhibitory protein, as had been observed in the purifications using Sephadex G100 and Sephadex DEAE A25. It did however provide some improvement in purity (see Table 3), and because of its *M*r range capacity (60,000 to 2,000,000) it was useful for estimation of the inhibitor size.

The Sepharose CL 4B column was calibrated, using proteins of known molecular weight. The elution positions of the marker proteins were used to construct a calibration curve, as shown in Figure 4. The elution profile shown is from 270 to 420 ml, since this included the elution volumes of all the marker proteins. The inhibitor *M*r was determined from the calibration curve using the elution volumes of the inhibitor from the Sepharose CL 4B column. The inhibitor *M*r was estimated to range from 370,000 to 730,000, with the most potent activity observed at the higher relative molecular mass.

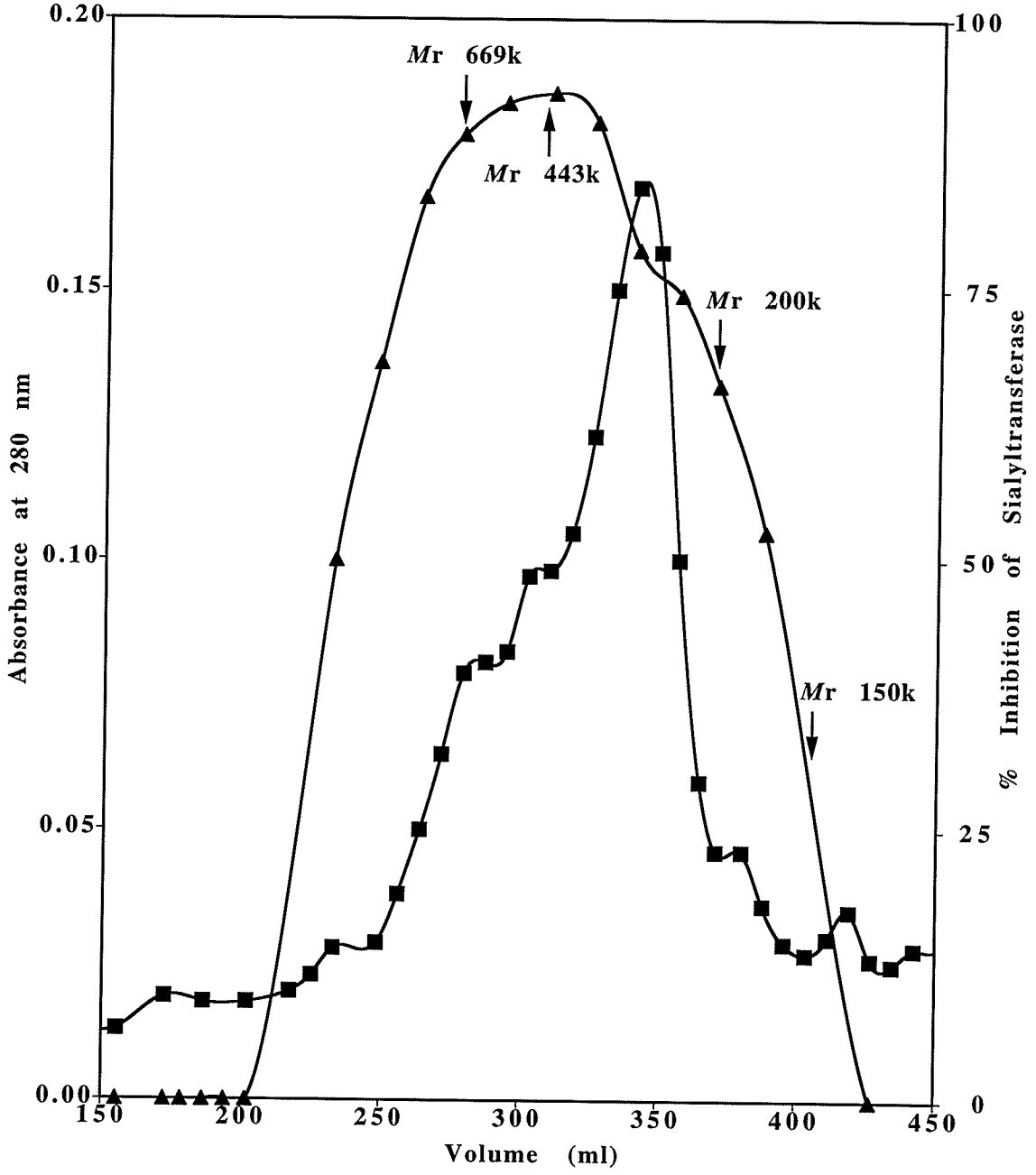


Figure 3: Fractionation of the Sialyltransferase Inhibitor Isolated from the Sephadex DEAE A25 Column on Sepharose CL 4B
Inhibitor from the Sephadex DEAE A25 column (Peak IV) was concentrated, and dialyzed against 20 mM Tris, pH 7, and a 2 ml aliquot, containing 3.9 mg of protein was applied to a column of Sepharose CL 4B (150cm x 2.25cm). The column was eluted with 20 mM Tris, pH 7, and 1.5 ml fractions were collected. The absorbance of each fraction at 280 nm (■) was monitored. The percentage inhibition of sialyltransferase relative to controls (▲), was determined using 10 µl of each fraction, or buffer for the controls, and 0.2 mU pure sialyltransferase in the standard inhibition assay. The column was calibrated with proteins of known molecular weight, thyroglobulin 669,000; apoferritin 443,000; β-amylase 200,000; and alcohol dehydrogenase 150,000, with the elution positions indicated by the arrows.

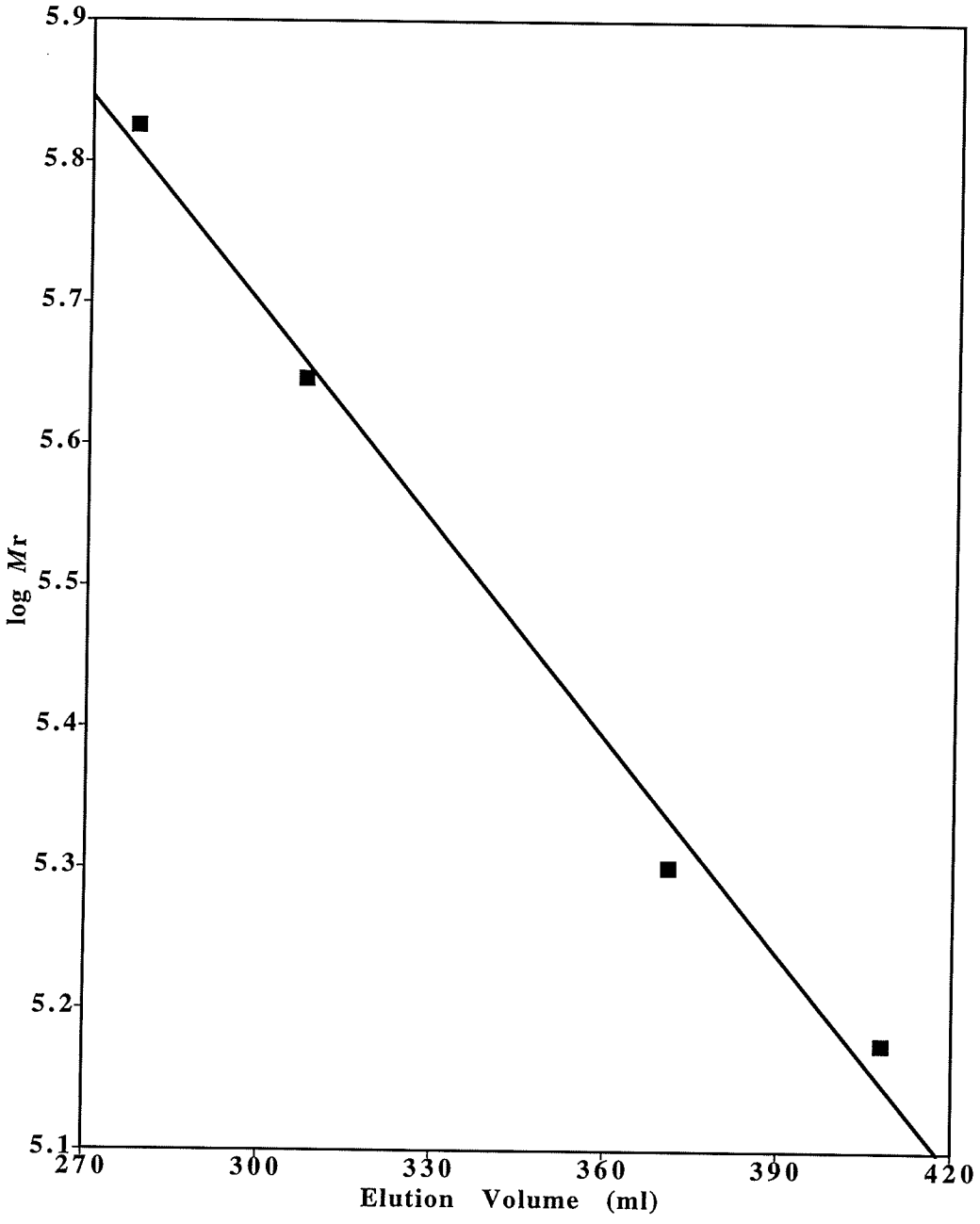


Figure 4: Calibration Curve of the Sepharose CL 4B Column
The Sepharose CL 4B (150cm x 2.25cm) column used for the separation and *Mr* determination of the Sephadex DEAE A25 purified inhibitor was calibrated with standard proteins. Approximately 2.5 mg of each protein in 0.5 ml was applied, and eluted with 20 mM Tris, pH 7. The absorbance of each fraction at 280 nm was monitored, and the volume of each protein peak was identified. The standard proteins were thyroglobulin *Mr* 669,000; apoferritin *Mr* 443,000; β-amylase *Mr* 200,000; and alcohol dehydrogenase *Mr* 150,000. The log *Mr* of each protein versus its elution volume is plotted (■), and the resulting linear equation is used to estimate the inhibitor *Mr*.

Further purification of the inhibitor isolated from the Sepharose CL 4B column was done by isoelectric focusing in a sucrose gradient, over a pH range of 3.5 to 7.0. The choice of pH range was based on the results from the Sephadex DEAE A25 fractionation that indicated the pI may be near 5. After separation by isoelectric focusing the pH and absorbance at 280 nm of each fraction were measured. Each fraction was then exchanged into 20 mM Tris, pH 7 buffer, so that ampholytes, sulfuric acid, sucrose, and sodium hydroxide required to create the gradient, but which interfere in the absorbance at 280 nm and the sialyltransferase assay, would be removed. Figure 5 indicates the absorbance readings at 280 nm and the percentage sialyltransferase inhibition determined for the fractions after exchange into 20 mM Tris, pH 7 buffer. A single peak of sialyltransferase inhibitory activity was observed encompassing two protein peaks as detected by the absorbance readings at 280 nm. A flattened, wide peak of activity, rather than two discrete peaks of activity was observed, since the quantity of inhibitor added relative to the quantity of sialyltransferase was high. The aim of using high quantities of inhibitor relative to sialyltransferase, as had been done in every purification step, was to ensure that any inhibitory activity throughout the purification profile would be detected. If lower quantities of inhibitor would have been used here, in the separation by isoelectric focusing, the results would probably have indicated two peaks of inhibitory activity coinciding with the two protein peaks.

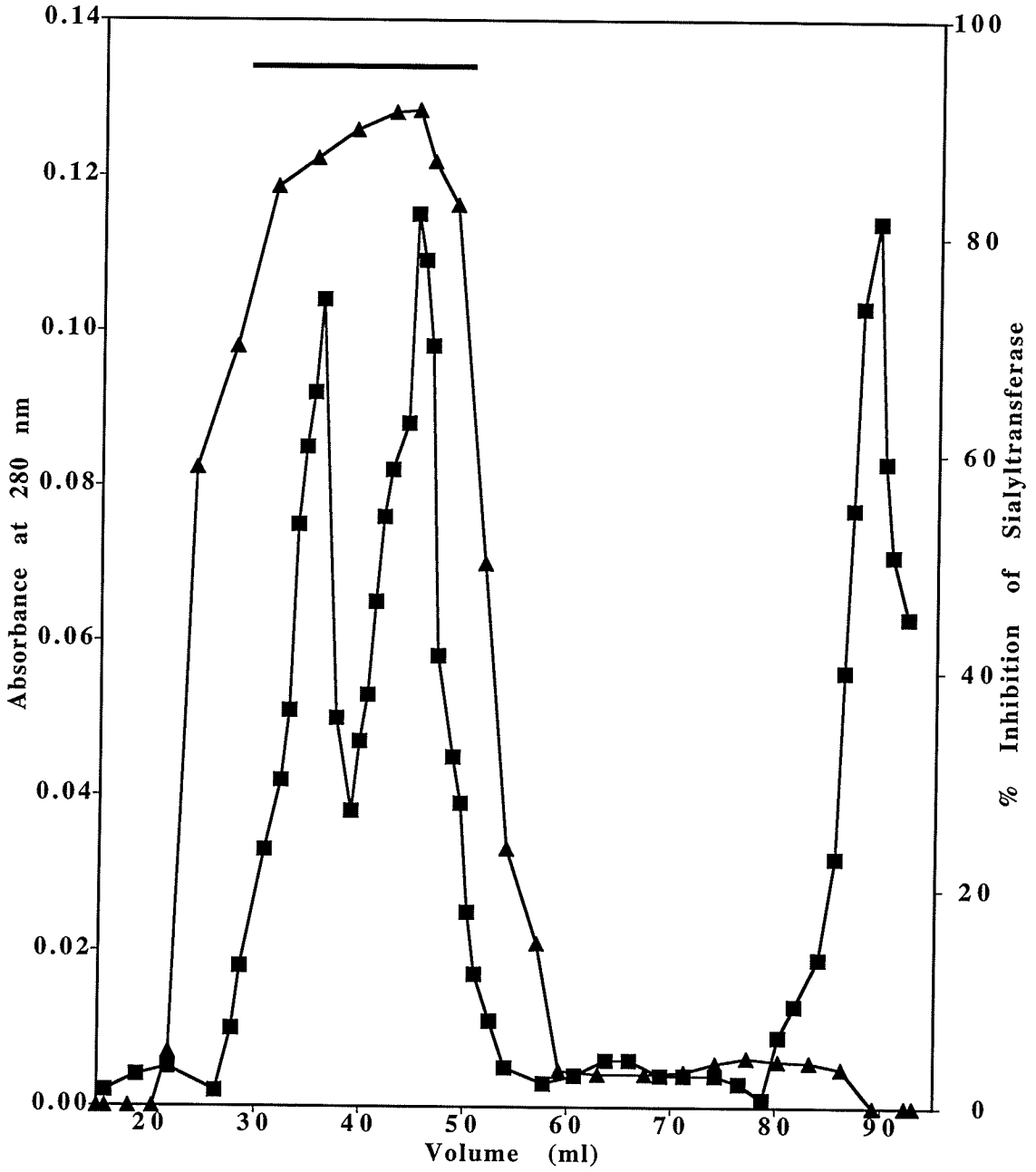


Figure 5: Purification of the Sialyltransferase Inhibitor Isolated from the Sepharose CL 4B Column by Isoelectric Focusing
A 6 ml aliquot of the inhibitor purified by the Sepharose CL 4B fractionation, containing approximately 1.8 mg protein, was included in the sucrose gradient used for focusing. Electrofocusing was in a 120 ml column, over the pH range 3.5 to 7.0, at 1800 W, 4° C, for 112 hours. Fractions of 1 ml were collected, and the pH and absorbance at 280 nm of each fraction were measured. Each fraction was then exchanged into 20 mM Tris, pH 7, to a final volume of 0.75 ml per fraction. The absorbance at 280 nm (■), and the percentage inhibition of sialyltransferase (▲) were determined on these exchanged fractions. For the inhibition assay 10 µl aliquots from each fraction and 0.2 mU pure sialyltransferase were used. The bar indicates the fractions containing inhibitor that were pooled, and used for subsequent analysis.

Initially the two protein peaks that were resolved by isoelectric focusing were examined separately by gel electrophoresis. According to the migration distances of the proteins under non-reducing conditions on SDS-containing gels, two protein bands of the same size were observed. The two protein components did not appear to differ in size, but did appear to differ quantitatively according to the relative intensities of Coomassie blue stain. The first protein peak (pH 4.2) contained greater amounts of the lower *M*r component (band B, Figure 15, I) relative to the higher *M*r component (band A, Figure 15, I). The second protein peak (pH 4.45) contained greater amounts of the higher *M*r component (band A, Figure 15, I) than the lower *M*r component (band B, Figure 15, I). According to the electrophoretic analysis of the two protein peaks isolated by isoelectric focusing, each contained two proteins of the same size in different quantitative proportions.

The original pH profile eluted from the isoelectric focusing column was used to determine the pI values of the two protein peaks seen in Figure 5. The first protein peak occurred at pH 4.20, and the second protein peak at pH 4.45. Since the first protein peak, at pH 4.20, contained greater quantities of the lower *M*r protein, it appeared that the unknown lower *M*r protein would have a pI near 4.20. Since the second protein peak, at pH 4.45, contained greater quantities of the higher *M*r protein, it appeared that this unknown higher *M*r protein would have a pI near 4.45. The greatest peak of inhibitory activity, as determined from multiple focusing runs, occurred over the second protein peak at pH 4.45. Therefore the

major inhibitory protein was believed to be the higher M_r protein and that this protein would have a pI near 4.45.

Although it appeared that the major inhibitory component was the large M_r species, with a pI of 4.45, and was contained mainly in the second protein peak, the two unknown proteins were not completely resolved. As a result of the lack of complete separation of the proteins, it was not possible to conclusively determine which component was causing the sialyltransferase inhibition. The two protein peaks as indicated by the bar in Figure 5 were therefore pooled and used for all subsequent studies.

III. Summary of the Purification Process

The purification of the inhibitory rat serum, as described in the Methods, and shown in Figures 1, 2, 3, and 5, involved four steps. The first purification was by size using a Sephadex G100 column, the second by charge using a Sephadex DEAE A25 column, the third by a higher resolution sizing column of Sepharose CL 4B, and the fourth and final step was by isoelectric focusing. The entire quantity of serum applied to the Sephadex G100 column was not carried throughout the purification process. Each purification utilized a quantity of inhibitory protein in a volume that was suitable for the size and type of column used. For a quick reference, Table 3 summarizes the quantities of protein loaded and recovered at each purification step, and the percentage recovery for each step. Assuming that the entire quantity of protein initially applied to the Sephadex G100 column was carried throughout the purification, the total percent recovery of protein is summarized in Table 4.

Table 3: Summary of the Purification Process

The first purification involved addition of 40 ml rat serum to a Sephadex G100 (90cm x 7cm) column. The second purification used 15 ml of the Sephadex G100 pooled inhibitor applied to a Sephadex DEAE A25 (45cm x 5cm) column. The third purification used 2 ml of the Sephadex DEAE A25 pooled inhibitor applied to a Sepharose CL 4B (150cm x 2.25cm) column. The final purification used 6 ml of the Sepharose CL 4B pooled inhibitor applied to a 120 ml isoelectric focusing column. Protein was determined by the Bio-Rad method (Bradford, 1976).

Purification Step:	Protein Applied (mg)	Protein Recovered (mg)	% Recovery for each Step
Sephadex G100	1929	653	34
Sephadex DEAE A25	646	68	11
Sepharose CL 4B	3.9	2.6	67
Isoelectric Focusing	1.8	0.45	25

Table 4: Efficiency of Protein Purification

The values presented here are based on the data in Table 3, assuming that the entire inhibitor protein in the 40 ml rat serum was carried throughout the purification process. The quantities of protein collected at each step are calculated from the ratios of protein actually loaded and recovered. The % recovery of inhibitory protein for each inhibitor-containing pool is expressed relative to the total quantity of rat serum protein.

Inhibitor-Containing Pool:	Protein (mg)	% Recovery of Total Protein
Rat Serum	1929	100
Sephadex G100	653	34
Sephadex DEAE A25	69	3.6
Sepharose CL 4B	46	2.4
Isoelectric Focusing	12	0.6

IV. Quantitation of Inhibitor Activity

One unit of inhibitor activity is defined as that quantity of inhibitor required to cause 50% inhibition of 0.1 mU α2,6 sialyltransferase using the standard inhibition assay. To determine the number of units of sialyltransferase inhibitory activity samples were initially titrated using a broad range of inhibitor quantities. Titration over narrower ranges of inhibitor quantities, i.e., from 40 to 60% inhibition was then performed to more accurately determine the units of activity. All of the graphs used to determine the activity of each purified pool are not shown. However, a typical titration curve is shown in Figure 6. Here 0.1 mU sialyltransferase is titrated with up to 10 µl of inhibitor purified by isoelectric focusing. In this particular titration 50% inhibition, or 1 unit of inhibitory activity occurred at less than 1 µl of the inhibitor preparation. Similarly, from the titrations over the 40 to 60% inhibition range, the volumes for 1 unit of inhibitory activity were determined for each purified pool. The total number of units recovered is subsequently calculated from the total volume of each pool. Table 5 summarizes the total number of units, and the total protein recovered for each purification step performed as described in the Methods, and as shown in Figures 1, 2, 3, and 5, and are therefore relative to the quantity of inhibitor applied at each individual stage. The specific activities for each purified pool are calculated and included in Table 5. The specific activity of the rat serum initially loaded onto the Sephadex G100 column was also determined and found to be 0.3 U/µg. The efficiency of recovery of inhibitor activity is indicated in Table 6. The specific activity increased with each purification step. The final

inhibitor preparation purified by isoelectric focusing had a specific activity of 18.1 U/μg. On comparison to the initial 0.3 U/μg specific activity for the rat serum, the entire process resulted in an approximate 60-fold increase in the purity of the inhibitor.

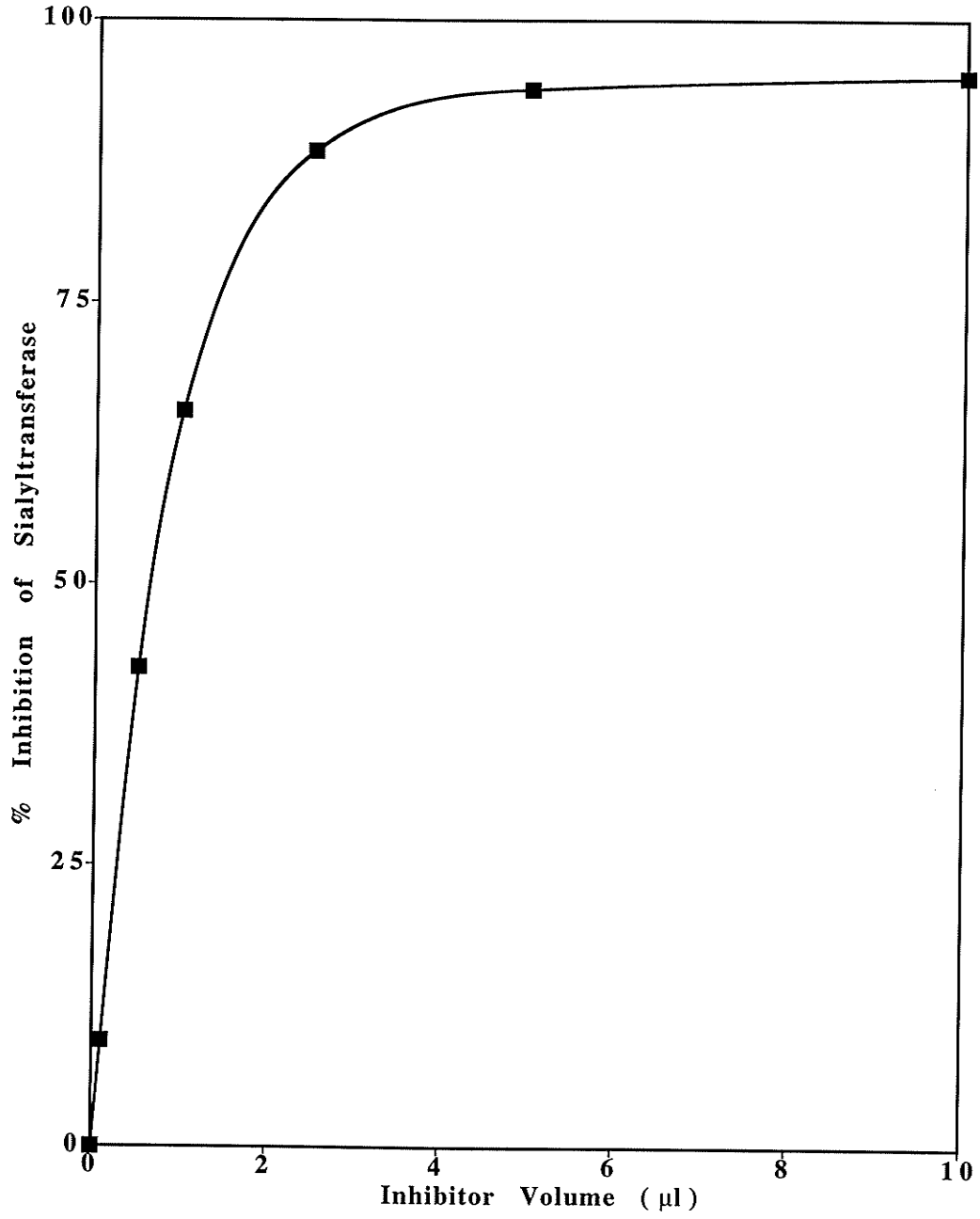


Figure 6: Titration of 0.1 mU α2,6 Sialyltransferase with Inhibitor Purified by Isoelectric Focusing

A portion of the inhibitor pool, as indicated by the bar in Figure 5, purified by isoelectric focusing, was concentrated using Centricon ultrafiltration (*M*r 30,000) to give a solution with a protein concentration of 0.08 mg/ml. This solution was subsequently serially diluted, using 20 mM Tris, pH 7, and 10 µl aliquots were incubated with 0.1 mU sialyltransferase in the standard inhibition assay. The percentage inhibition of sialyltransferase versus volume of 0.08 mg/ml inhibitor solution is indicated (■).

Table 5: Quantitation of α2,6 Sialyltransferase Inhibition

Units of activity were determined by titration of 0.1 mU sialyltransferase with inhibitory material. 1 unit of activity is defined as that quantity of inhibitor which caused 50% inhibition of sialyltransferase activity under the described conditions. Protein concentrations were determined by the Bio-Rad method (Bradford, 1976). The specific activities were calculated from the experimentally determined units of activity and protein for each inhibitory pool recovered throughout the purification process.

Inhibitor-Containing Pool:	Activity (units)	Protein (mg)	Specific Activity (U/μg)
Sephadex G100	2,266,372	653	3.5
Sephadex DEAE A25	583,760	68	8.6
Sepharose CL 4B	27,090	2.6	10.4
Isoelectric Focusing	8141	0.45	18.1

Table 6: Purification Efficiency of Inhibitor Activity

The specific activity of the starting material (rat serum) was calculated from the experimentally determined units of inhibitory activity and protein quantity. The specific activities of the inhibitor-containing pools from each purification step were determined as detailed in Table 5. The fold purification of inhibitor activity is expressed as a function of the original activity in rat serum.

Inhibitor-Containing Pool:	Specific Activity (U/μg)	Fold Purification
Rat Serum	0.3	1
Sephadex G100	3.5	11.7
Sephadex DEAE A25	8.6	28.7
Sepharose CL 4B	10.4	34.7
Isoelectric Focusing	18.1	60.3

V. Properties of the Inhibitor

Throughout the purification process a variety of properties of the inhibitor were investigated. The reported properties were therefore determined for inhibitor preparations of varying purity. The aim of these investigations was to provide insight into the type or mechanism of inhibition, and to provide information which would aid in the identification of the inhibitor.

The inhibitor stability was investigated first, so that any requirements for maintenance of its integrity, during and between the various experiments to be performed, would be met. The results of these experiments are summarized in Table 7. The inhibitor was found to be very stable. It could undergo lengthy standing at room temperature, multiple freeze/thaw cycles, and temperatures as high as boiling, and still be active as a sialyltransferase inhibitor. Lyophilization followed by rehydration also did not affect its ability to inhibit sialyltransferase. The inhibitor was apparently very stable.

Other experiments were not only aimed at determining the stability of the inhibitor but also at understanding the molecular nature of the inhibitor. Attempts to hydrolyze the inhibitor included incubation with sulfuric acid at 80°C for one hour, and incubations with various hydrolyzing enzymes. The results of these experiments are included in Table 7. The inhibitor was resistant to acid hydrolysis, and to proteolysis by trypsin. Since it was unaffected by treatment with trypsin it seemed possible that the inhibitor may not be a protein, or that any potential protein was for some reason protected. It was also resistant to pronase when incubation time was

limited to 24 hours, but when incubation was increased to 120 hours the inhibitory effect on sialyltransferase was lost. The fact that sialyltransferase inhibitory activity could eventually be destroyed by pronase treatment indicated that there must be at least some protein component which was significant for its function. Incubation with heparatinase, chondroitinase ABC, or hyaluronidase (from *Streptomyces*) did not affect sialyltransferase inhibition. Hyaluronidase from *Streptomyces hyalurolyticus* is highly specific for hyaluronic acid, hydrolyzing only the β-GlcNAc-[1-4] glycosidic bonds by elimination resulting in 4,5-unsaturated tetra- and hexasaccharides (Ohya & Kaneko, 1970). The enzyme isolated from bovine testes is much less specific, randomly cleaving β-N-acetylhexosamine-[1-4] glycosidic bonds, usually in hyaluronic acid, chondroitin and chondroitin sulfates. Incubation with testicular hyaluronidase resulted in loss of sialyltransferase inhibitory activity, which indicated that there may be a functionally significant carbohydrate component in the inhibitor. The nature of the functionally significant component destroyed by testicular hyaluronidase was however unknown owing to the lack of specificity of this particular enzyme. The results indicated that the stable inhibitor contained functionally significant protein and carbohydrate components.

Table 7: Stability of the Inhibitor

Various purified inhibitor sources were used in the tests summarized here. For studies on the effect of freeze/thaw and of boiling treatments inhibitor purified from each stage of purification was monitored. For lyophilization/rehydration, inhibitor purified by Sephadex G100 was used. Details on the inhibitor sources and assay conditions for the various hydrolytic treatments are in the Methods, section VI. Effect of Hydrolysis of the Inhibitor on its Activity.

Treatment Type:	Effect on the Inhibitor
Freeze/Thaw Cycles	None
Boiling	None
Lyophilization/Rehydration	None
Acid hydrolysis	None
Trypsin	None
Pronase (24 hours at 37°C)	None
Pronase (120 hours at 37°C)	Abolished Activity
Heparatinase	None
Chondroitinase ABC	None
Hyaluronidase (*Streptomyces*)	None
Hyaluronidase (Bovine testes)	Abolished Activity

In an attempt to determine the molecular nature of the inhibitor, direct analysis of carbohydrate and protein were performed. Inhibitor from the Sephadex DEAE A25 and Sepharose CL 4B purifications were used for these determinations. The proportions of protein and carbohydrate were determined relative to the total weight. As indicated in Table 8, the inhibitor is primarily protein in nature, with a minor carbohydrate component.

Figure 7 shows the UV-VIS spectrum for a dilute solution of the inhibitor from the Sepharose CL 4B purification. A major peak is seen at 220 nm, and a minor peak at 280 nm. The peak at 220 nm is indicative of peptide bonds, and that at 280 nm indicates the presence of tyrosine and/or tryptophan. Also noteworthy is the absence of any absorbance at 254 nm, excluding the possibility that the α2,6 sialyltransferase inhibitor preparation contained any major nucleotide component.

Table 8: Inhibitor Protein and Carbohydrate Proportions

Protein was determined by the Bio-Rad method (Bradford, 1976) using BSA to construct the standard calibration curve. Carbohydrate was analyzed by the resorcinol method (Svennerholm, 1957) using mannose for the standard calibration curve. The weight of protein or carbohydrate of the test samples is determined from the standard curves. The sum of the weight of protein and the weight of carbohydrate is taken as the total weight. The percentage protein is calculated from the ratio of protein weight to total weight. The percentage carbohydrate is calculated from the ratio of carbohydrate weight to total weight. The results are therefore expressed as weight per weight percentages.

Inhibitor Preparation:	% Protein	% Carbohydrate
Sephadex DEAE A25, Peak IV	89	11
Sepharose CL 4B	85	15

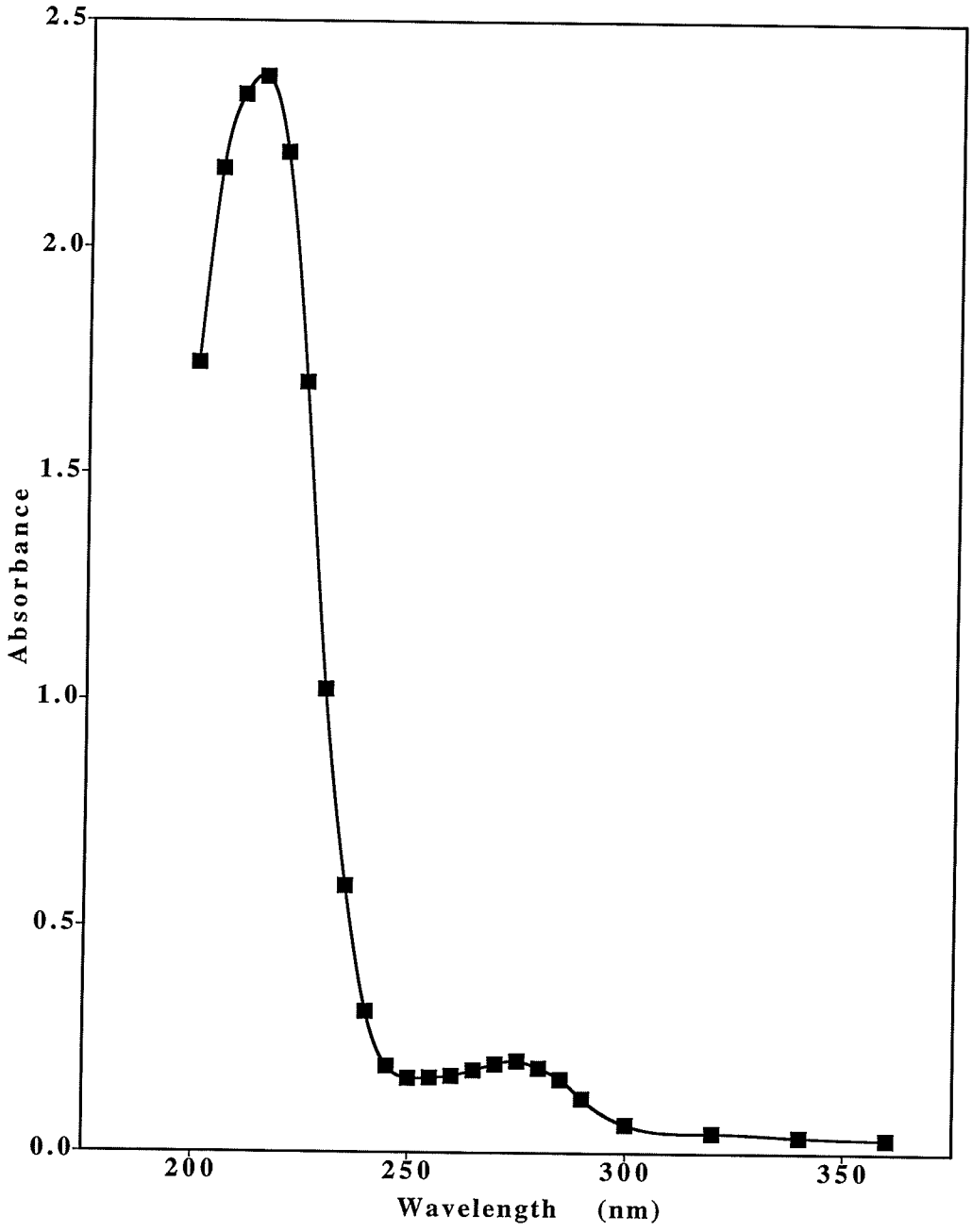


Figure 7: UV-VIS Spectrum of Inhibitor Purified by Sepharose CL 4B Fractionation

The absorbance of a dilute solution of Sepharose CL 4B purified inhibitor was scanned over a wavelength range of 200 to 1000 nm, relative to a 20 mM Tris, pH 7 buffer blank. The absorbance (■), over the range of 200 to 400 nm is shown. At wavelengths above 400 nm, absorbance values above the blank were not detected.

The inhibitor had been established to be a stable compound with protein and carbohydrate components. In addition to the general molecular nature of the inhibitor, it was important to investigate its mechanism of action on sialyltransferase. It was particularly critical to determine if the inhibitor was interfering with the sialyltransferase detection method, rather than acting as a true α2,6 sialyltransferase inhibitor. It was possible that the inhibitor was hydrolyzing the nucleotide sugar donor, or destroying the macromolecular acceptor, asialo α_1 AGP. If either of these situations were occurring, the lack of sialyltransferase activity would not be caused by its inhibition, but by the destruction of substrate(s) necessary for its detection. It was also possible that the inhibitor itself was acting as an acceptor. In this case if the inhibitor was used as a preferential substrate over the usual macromolecular acceptor, asialo α_1 AGP, the result may be a lack of precipitation of the labeled inhibitor, and therefore a lack of detection in activity. These possibilities, that the inhibitor was interfering with the detection of sialyltransferase activity, were investigated.

To test whether the inhibitor was hydrolyzing the nucleotide sugar donor, various mixtures were incubated for two hours at 37°C, chromatographed and the radioactivity profiles were determined. Figure 8 indicates the position of free [^{14}C]NeuAc, of intact CMP-[^{14}C]NeuAc, and of inhibitor-treated CMP-[^{14}C]NeuAc. Treatment of the ^{14}C-labeled nucleotide donor with inhibitor followed by chromatographic separation indicates a minor amount of the donor is partially hydrolyzed after the two hour incubation. The control CMP-[^{14}C]NeuAc gave one discrete peak of radioactivity with none of the

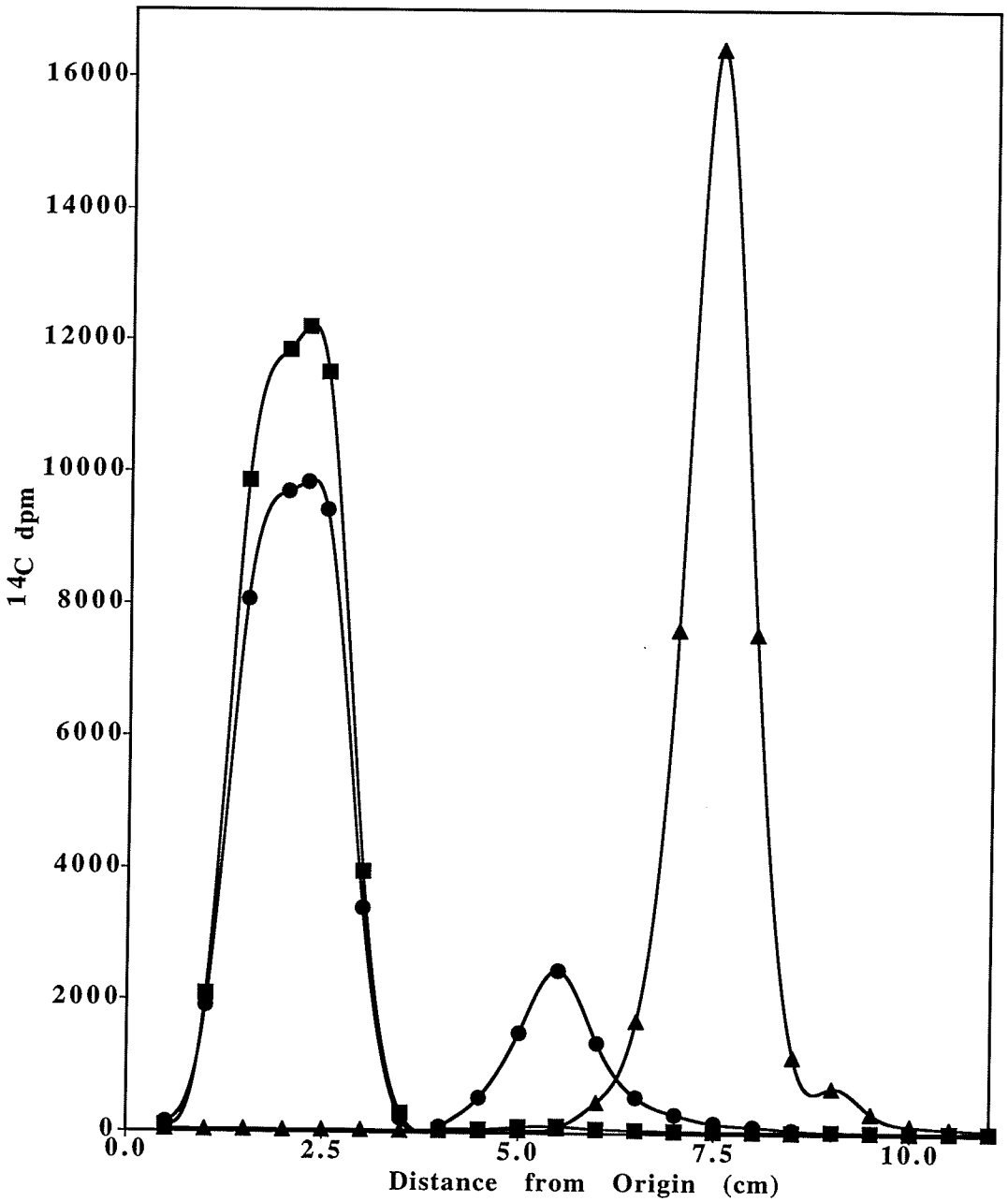


Figure 8: Chromatographic Profiles of CMP-[^{14}C]NeuAc, [^{14}C]NeuAc, and CMP-[^{14}C]NeuAc Treated with Inhibitor
The inhibitor treated CMP-[^{14}C]NeuAc mixture, and the controls were separated by paper chromatography, with the radioactivity monitored as a function of distance traveled from the origin of sample application. The chromatographic profile of inhibitor-treated CMP-[^{14}C]NeuAc (●) shown utilized inhibitor from the Sephadex G100 column incubated with 20 nCi (5 nmoles) CMP-[^{14}C]NeuAc at 37°C for 2 hours. The ratio of inhibitor to donor were as they would be in a routine assay in which sialyltransferase would be inhibited by greater than 90%. Controls included 20 nCi CMP-[^{14}C]NeuAc, (■), and [^{14}C]NeuAc prepared by acid hydrolysis of 20 nCi CMP-[^{14}C]NeuAc at 80°C for 1 hour, (▲).

partial hydrolysis seen when the donor was treated with inhibitor. The difference between these two samples was that the inhibitor-treated donor was incubated for two hours at 37°C, while the control was not. The 2 hour incubation at 37°C of the inhibitor-treated CMP-NeuAc probably caused a slow, and partial hydrolysis of the donor by water. The major peak of radioactivity for all samples were in positions identical to that of the CMP-[^{14}C]NeuAc control, and were well separated from the free [^{14}C]NeuAc that migrated much further down the chromatogram. These results indicate that the inhibitor does not interfere with the detection of sialyltransferase activity by hydrolysis of the donor, CMP-[^{14}C]NeuAc.

Tests were also performed to determine if the inhibitor was interfering with the detection of sialyltransferase activity by destroying the macromolecular acceptor, asialo α_1 AGP. Here inhibitor-treated asialo α_1 AGP, and untreated asialo α_1 AGP were passed through a Sephadex G100 sizing column, and the absorbance profiles at 280 nm were monitored. The results of these separations are shown in Figure 9. Inhibitor-treated asialo α_1 AGP, and the control asialo α_1 AGP eluted in exactly the same position, indicating that incubation of the acceptor with the inhibitor did not alter the macromolecular acceptor size. After separation and recovery of the acceptor, inhibitor-treated acceptor, and mock-treated acceptor, and substitution of these acceptors in the standard assay, no detectable difference in sialyltransferase activity was found (data not shown). These results indicate that the inhibitor is not interfering with sialyltransferase activity or detection, by altering the acceptor size or its function.

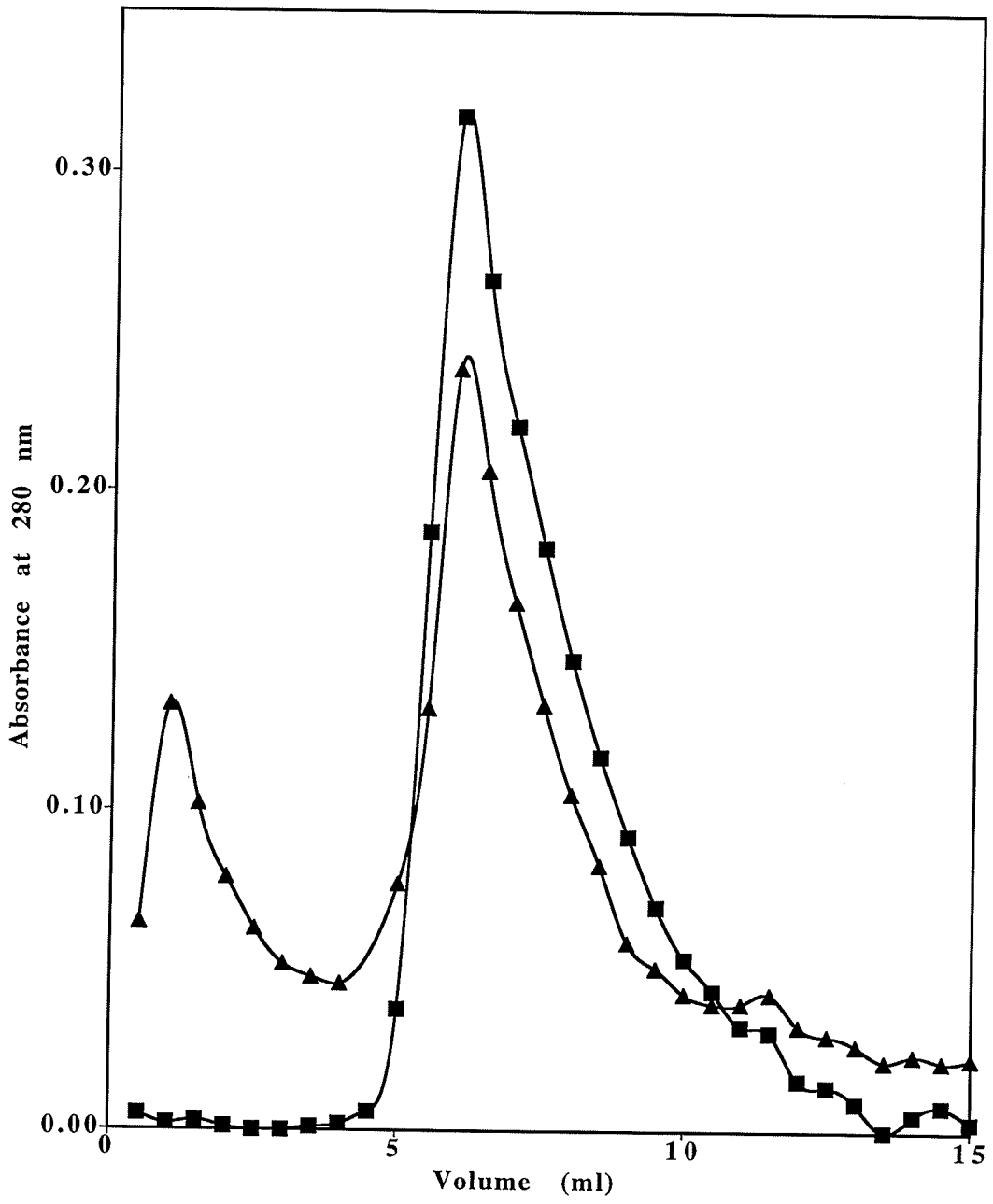


Figure 9: Chromatographic Profiles of asialo α_1 AGP, and asialo α_1 AGP Treated with Inhibitor

Asialo α_1 AGP (■), or asialo α_1 AGP mixed with inhibitor (▲), were incubated for 18 hours at 37°C prior to fractionation on a Sephadex G100 column (22cm x 0.8cm). Inhibitor was the Peak IV material from the Sephadex DEAE A25 fractionation. The samples were eluted with 20 mM Tris, pH 7 buffer, and 10 drops per fraction were collected and monitored for absorbance at 280 nm, relative to buffer as a blank.

Investigations were also performed to determine if the inhibitor itself was acting as an acceptor in the standard sialyltransferase assay. If the inhibitor were acting as an acceptor, and if it did not precipitate on the filter paper discs in the manner that the standard macromolecular acceptor does, the results would mimic α2,6 sialyltransferase inhibition, by the lack of detection of any precipitated radioactivity. Figure 10 shows the radioactive profiles of various reaction mixtures after separation on a Sephadex G100 column. Incorporation of radioactivity from the sugar nucleotide donor could only be detected when the acceptor was asialo α_1 AGP. No incorporation of radioactivity into the inhibitor or asialo inhibitor (prepared by acid hydrolysis) was observed. All of the radioactivity was recovered in peaks eluting at the same position as the free CMP-[^{14}C]NeuAc. The inhibitor itself was therefore not acting as an acceptor.

Since the inhibitor was apparently not altering the donor or acceptor, or acting itself as an acceptor, all indications were that it must therefore be interacting directly with sialyltransferase. Although the exact nature of the mechanism of inhibition was not known, the possibility that it was simply an interference in the detection method had been ruled out, suggesting that a true sialyltransferase inhibitor had been found. Further investigations into the mechanism of inhibition were therefore warranted, and undertaken.

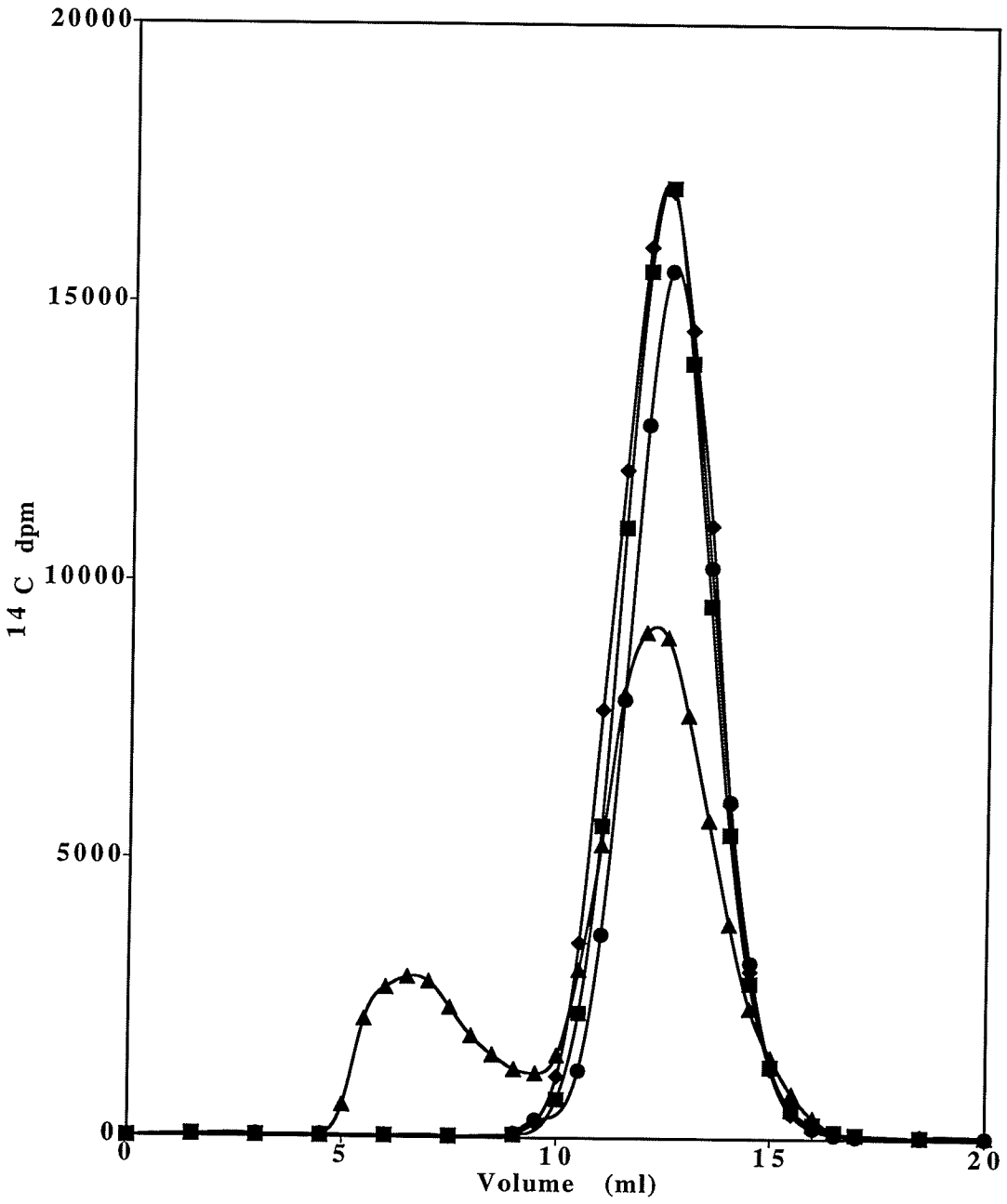


Figure 10: Chromatographic Profiles of Radioactivity used to Detect if Sialyltransferase Incorporated [^{14}C]NeuAc into Inhibitor, or asialo Inhibitor

Sialyltransferase was incubated with CMP-[^{14}C]NeuAc donor, and the usual asialo α_1 AGP (▲) as acceptor, or inhibitor (●), or asialo inhibitor (●), as potential acceptors, for 2 hours at 37°C, followed by fractionation on a Sephadex G100 column (25cm x 0.8cm). CMP-[^{14}C]NeuAc, (■), was also fractionated to determine the position of unincorporated radioactivity. Inhibitor was the Peak IV material from the Sephadex DEAE A25 fractionation. The column was eluted with 20 mM Tris, pH 7, with 10 drops per fraction collected, added to 10 ml of ACS cocktail, and radioactivity was monitored. Results are shown as dpm versus elution volume.

The possibility that sialyltransferase inhibition was by a proteolytic mechanism also had to be addressed. This possibility did however, seem unlikely since the standard assay included excess exogenous protein (0.63 mg/ml of BSA, and 3.13 mg/ml of the macromolecular protein acceptor) which would be expected to protect the relatively small quantity of sialyltransferase protein (at most 0.00031 mg/ml for 0.2 mU and a specific activity of 8 U/mg). Despite this, experiments were performed to investigate the possibility that the mechanism of inhibition was by proteolysis.

Initially, protease inhibitors were used as a tool to determine if sialyltransferase inhibition could be blocked. In these experiments, various protease inhibitors were mixed and incubated with the sialyltransferase inhibitor purified by Sephadex G100 fractionation, followed by the standard inhibition assay. Protease inhibitors against every major class of protease (EC 3.4.21-24), i.e., serine, thiol, acid, and metallo-proteases (Nomenclature Committee, IUBMB, 1992) were monitored for their potential to block the inhibition of sialyltransferase. PMSF, pepstatin A, leupeptin, and EDTA were tested, and as shown in Table 9, none of these protease inhibitors significantly altered sialyltransferase inhibition. This provided indirect evidence that the inhibition of sialyltransferase was not through a proteolytic mechanism.

Table 9: Effect of Protease Inhibitors on α2,6 Sialyltransferase Inhibition

Inhibitor purified by Sephadex G100 fractionation was mixed with either PMSF, pepstatin A, leupeptin, or EDTA, so that the final concentration of each protease inhibitor was 1 mM. The mixtures were incubated at 37°C for 2 hours followed by the standard inhibition assay using 0.1 mU sialyltransferase. The controls used for the calculation of percent inhibition contained no sialyltransferase inhibitor, but were otherwise identical.

Protease Inhibitor:	% Inhibition of Sialyltransferase
PMSF	81.0
Pepstatin A	90.8
Leupeptin	96.0
EDTA	94.2

Proteolytic activity was also monitored directly, using the very sensitive FITC-casein hydrolysis method in an attempt to determine if any trace proteolytic activity was present in the sialyltransferase inhibitor preparations. The inhibitor preparations monitored were from the Sepharose CL 4B and the isoelectric focusing purifications. As detailed in the Methods, this technique is easily capable of detecting 10 ng of tryptic activity. Various concentrations of inhibitor, containing up to 10 μg of protein, were tested. Also, incubation times at 37°C were varied, for up to 24 hours, in order to give any trace contaminating protease ample opportunity to hydrolyze the fluorescently labeled substrate. In all cases the relative fluorescence of assay mixtures containing inhibitor were equivalent to the blank values, and therefore the data are not shown. According to the lack of release of fluorescence the inhibitor preparations contained no proteolytic activity.

Since excess by-stander protein or the presence of protease inhibitors did not alter sialyltransferase inhibition, and since no proteolytic activity was directly detectable, it was concluded that sialyltransferase activity was not simply being destroyed by proteolysis. These experiments showing that the inhibition was not the result of proteolysis, and the earlier studies showing that it was not due to a simple interference in the detection of sialyltransferase activity provided strong evidence that a true sialyltransferase inhibitor had been discovered. The precise mechanism of sialyltransferase inhibition was still, however, unknown, and was therefore further investigated.

To gain further insight into how the inhibitor was affecting α2,6 sialyltransferase activity, kinetic analyses were performed. Sialyltransferase kinetics were performed with varied substrate concentrations as described in the Methods, in the absence and presence of varying quantities of inhibitor. The inhibitor was found to affect both the rate of the enzyme reaction (k_{cat}), and the apparent K_m, with respect to both the asialo α_1 AGP acceptor, and the CMP-NeuAc donor. The reaction rates in the presence of varying concentrations of asialo α_1 AGP substrate (Figure 11), or CMP-NeuAc substrate (Figure 13) indicates Michaelis-Menten type of kinetics. Lineweaver-Burk (double reciprocal) plots were constructed from the data used for the Michaelis-Menten plots.

The Lineweaver-Burk plots from Figure 12, in the presence of varied acceptor, were used to calculate the apparent K_m and V_{max} values in the absence and presence of varied amounts of inhibitor, and the resulting values are summarized in Table 10. As increasing amounts of inhibitor are added V_{max} clearly decreases, as seen in Figure 11, in Figure 12 by the changing y-intercepts, and as summarized in Table 10. In the absence of any inhibitor the apparent V_{max} has a value of 4.11 pmole/min, and decreases to 1.57 pmole/min in the presence of 10 µl of inhibitor preparation purified from the Sepharose CL 4B column. The apparent K_m values for acceptor increase with increasing inhibitor quantity, as evidenced by the changing x-intercepts in Figure 12. The values are elevated from 0.125 mM Gal in the absence of inhibitor to 0.198 mM Gal in the presence of 10 µl of inhibitor, as summarized in Table 10.

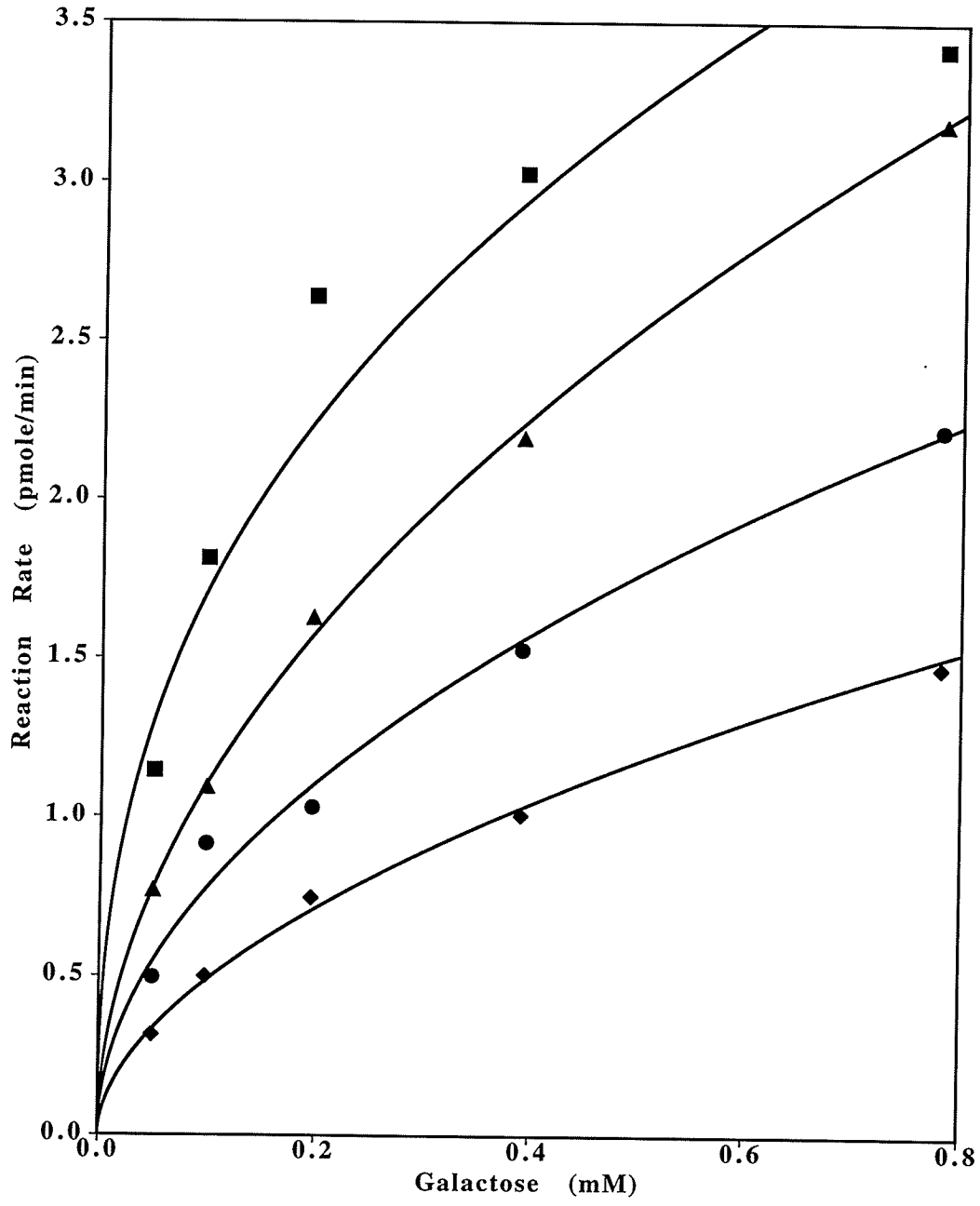


Figure 11: Michaelis-Menten Plots in the Presence of Varied Quantities of asialo α_1 AGP and Inhibitor

With rat asialo α_1 AGP as the varied acceptor in the assay described in the Methods, the rate of reaction was determined in the absence of inhibitor (■), and with 2.5 µl (▲), 5.0 µl (●), and 10 µl (◆) of inhibitor purified by the Sepharose CL 4B fractionation. The protein concentration of the inhibitor preparation was 0.3 mg/ml. The concentration of asialo α_1 AGP is expressed as the number of moles of galactose, given that each mole of protein contains 10 moles of galactose sites.

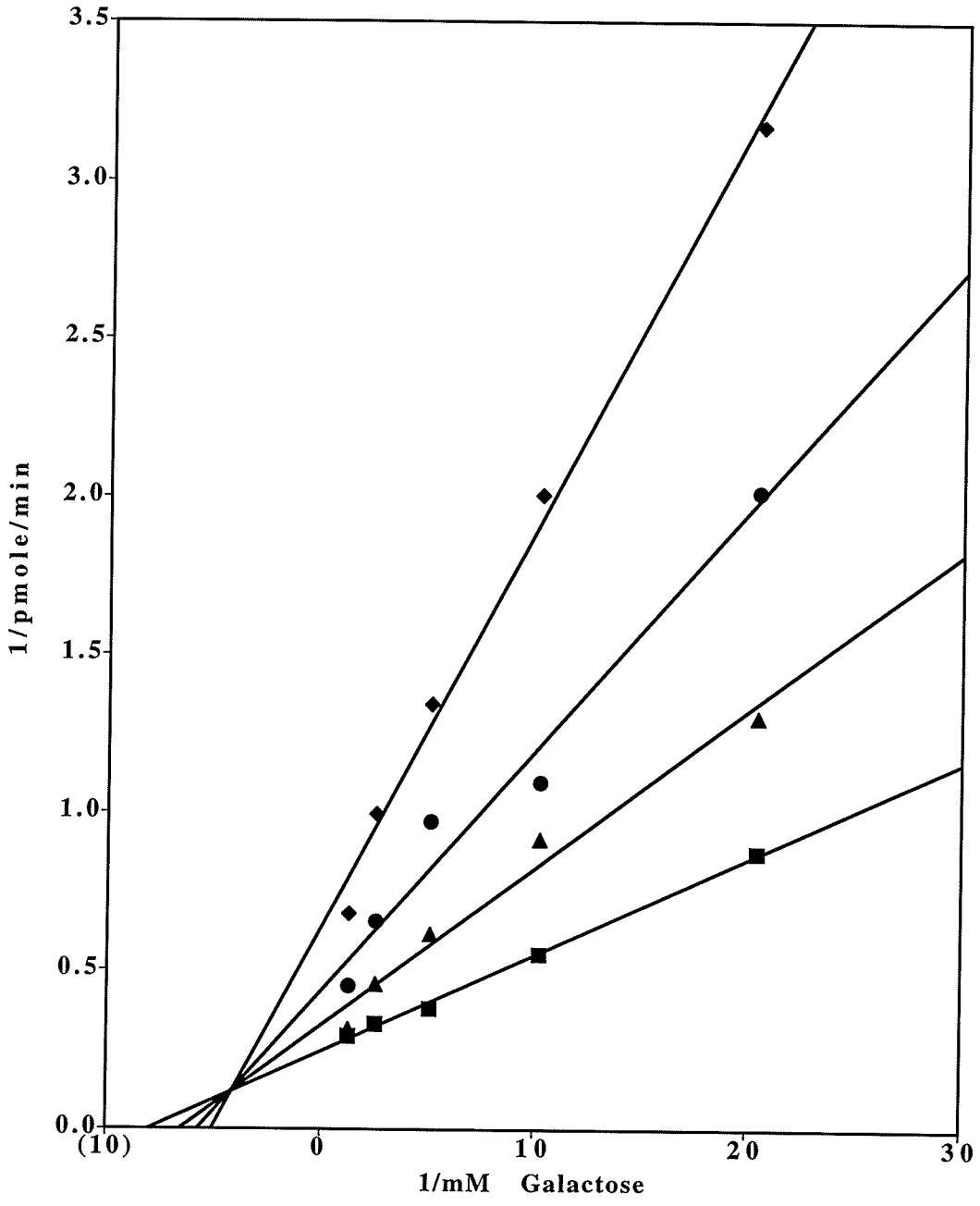


Figure 12: Lineweaver-Burk Plots in the Presence of Varied Quantities of asialo α_1 AGP and Inhibitor

With rat asialo α_1 AGP as the varied acceptor in the assay described in the Methods, the rate of reaction was determined in the absence of inhibitor (■), and with 2.5 µl (▲), 5.0 µl (●), and 10 µl (◆) of inhibitor purified by the Sepharose CL 4B fractionation. The protein concentration of the inhibitor preparation was 0.3 mg/ml. The concentration of asialo α_1 AGP is expressed as the number of moles of galactose, given that each mole of protein contains 10 moles of galactose sites. Inversion of the reaction rates and galactose concentrations, as seen in Figure 11, were used to construct these double reciprocal plots.

Table 10: Apparent K_m and V_{max} Values with Respect to the Acceptor, asialo α_1 AGP, for Sialyltransferase in the Absence and Presence of Inhibitor

Summary of analysis of Lineweaver-Burk Plot in Figure 12, in the presence of varied quantities of asialo α_1 AGP and inhibitor. Inhibitor purified by the Sepharose CL 4B fractionation with a protein concentration of 0.3 mg/ml was used in the kinetic analysis over a variable range of asialo α_1 AGP concentrations. The donor, CMP-NeuAc quantity was fixed, with each assay mixture containing 0.10 mM. The equations were computer generated using linear analysis of the graphs as described in the Methods. K_m and V_{max} values were determined from the generated equations.

Inhibitor Volume (µl)	Equations of Straight Lines from the Lineweaver-Burk Plots in Figure 12	Apparent K_m (mM Gal)	Apparent V_{max} (pmole/min)
0	y = 0.030501x + 0.243463	0.125	4.11
2.5	y = 0.049850x + 0.324684	0.154	3.08
5.0	y = 0.076041x + 0.434016	0.175	2.30
10.0	y = 0.126102x + 0.637348	0.198	1.57

The Lineweaver-Burk plots from Figure 14, in the presence of varied donor concentrations, were used to calculate the apparent K_m and V_{max} values in the absence and presence of varied amounts of inhibitor, and the resulting values are summarized in Table 11. As increasing amounts of inhibitor are added V_{max} clearly decreases, as seen in Figure 13, in Figure 14 by the changing y-intercepts, and as summarized in Table 11. In the absence of any inhibitor the apparent V_{max} has a value of 5.84 pmole/min and decreases to 2.77 pmole/min in the presence of 10 µl of inhibitor preparation purified from the Sepharose CL 4B column. The apparent K_m values for the donor increase with increasing inhibitor quantity, as noted by the changing x-intercept values in Figure 14. They are elevated from 0.0514 mM CMP-NeuAc in the absence of inhibitor to 0.0742 mM CMP-NeuAc in the presence of 10 µl of inhibitor, as summarized in Table 11.

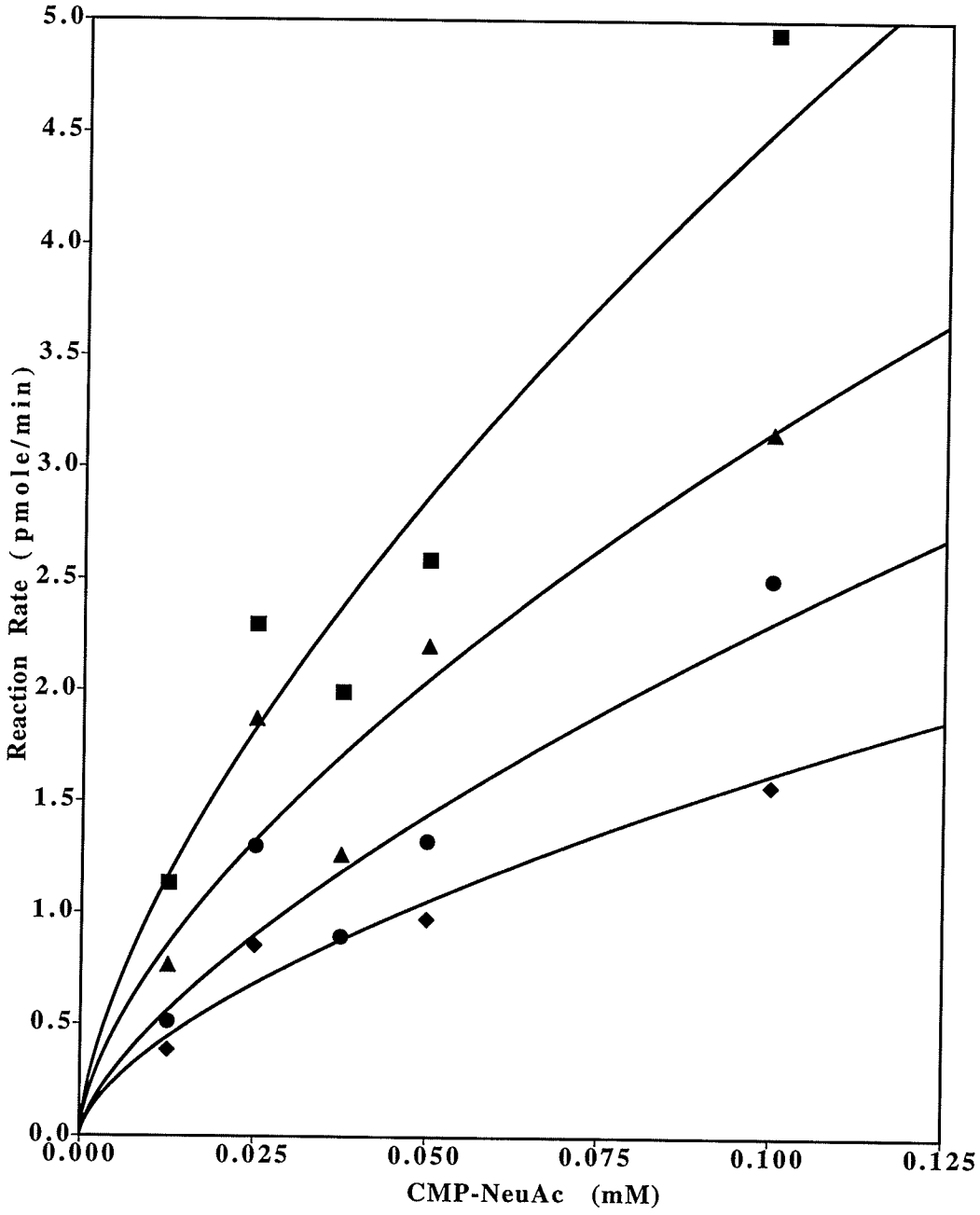


Figure 13: Michaelis-Menten Plots in the Presence of Varied Quantities of CMP-[^{14}C]NeuAc and Inhibitor

With the donor, CMP-[^{14}C]NeuAc as the varied substrate in the assay described in the Methods, the rate of reaction was determined in the absence of inhibitor (■), and with 2.5 µl (▲), 5.0 µl (●), and 10 µl (◆) of inhibitor purified by the Sepharose CL 4B fractionation. The protein concentration of the inhibitor preparation was 0.3 mg/ml. The rat asialo α_1 AGP acceptor quantity was fixed, with each assay mixture containing 0.78 mM Gal acceptor sites.

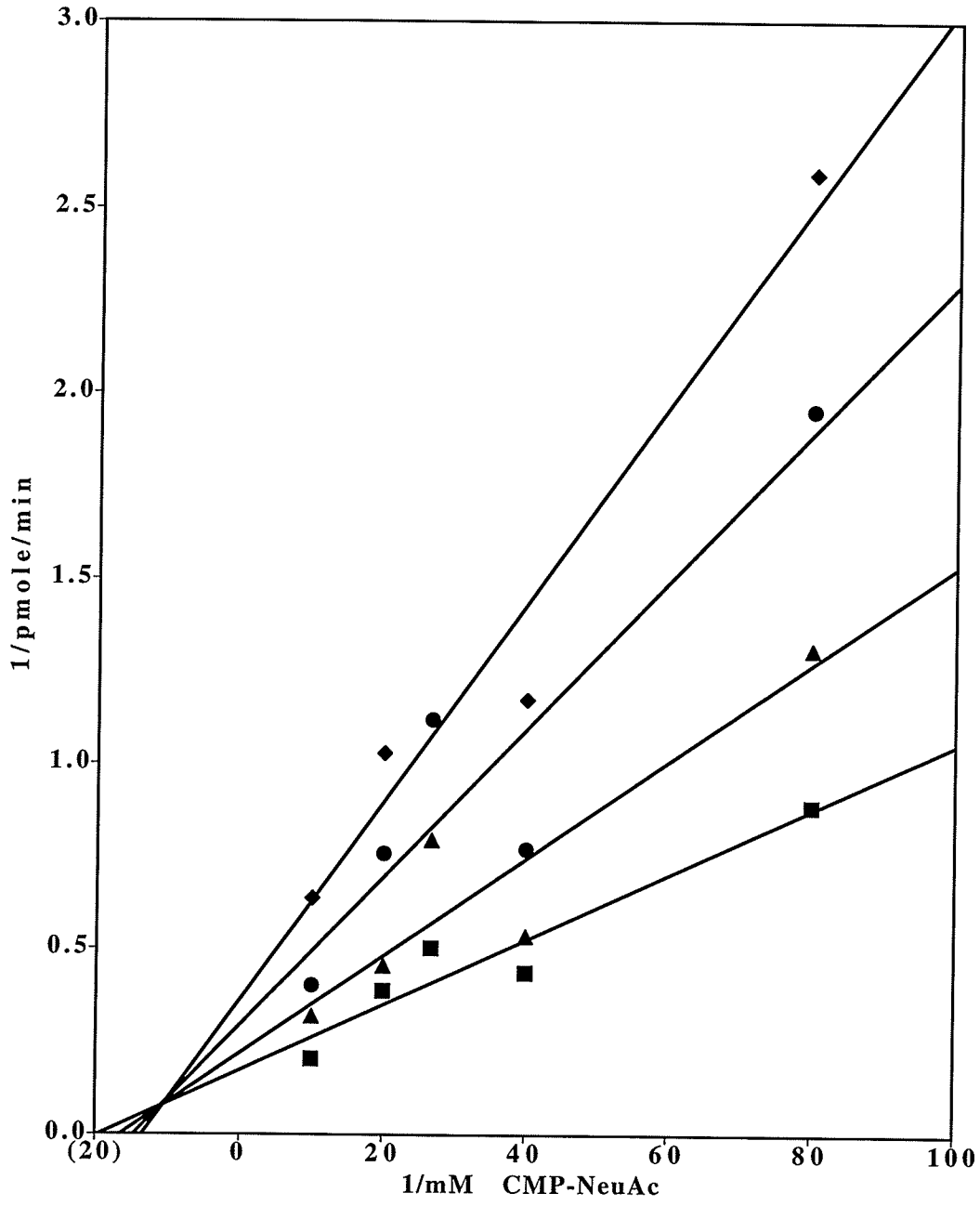


Figure 14: Lineweaver-Burk Plots in the Presence of Varied Quantities of CMP-[^{14}C]NeuAc and Inhibitor

With the donor CMP-[^{14}C]NeuAc as the varied substrate in the assay described in the Methods, the rate of reaction was determined in the absence of inhibitor (■), and with 2.5 µl (▲), 5.0 µl (●), and 10 µl (◆) of inhibitor purified by the Sepharose CL 4B fractionation. The protein concentration of the inhibitor preparation was 0.3 mg/ml. The rat asialo α_1 AGP acceptor quantity was fixed, with each assay mixture containing 0.78 mM Gal acceptor sites. Inversion of the reaction rates and CMP-[^{14}C]NeuAc concentrations, as seen in Figure 13, were used to construct these double reciprocal plots.

Table 11: Apparent K_m and V_{max} Values with Respect to the Donor, CMP-NeuAc, for Sialyltransferase in the Absence and Presence of Inhibitor

Summary of analysis of Lineweaver-Burk Plot in Figure 14, in the presence of varied quantities of CMP-[^{14}C]NeuAc and inhibitor. Inhibitor purified by the Sepharose CL 4B fractionation with a protein concentration of 0.3 mg/ml was used in the kinetic analysis over a variable range of CMP-NeuAc concentrations. The rat asialo α_1 AGP acceptor quantity was fixed, with each assay mixture containing 0.78 mM Gal acceptor sites. The equations were computer generated using linear analysis of the graphs as described in the Methods. K_m and V_{max} values were determined from the generated equations.

Inhibitor Volume (µl)	Equations of Straight Lines from the Lineweaver-Burk Plots in Figure 14	Apparent K_m (mM CMP-NeuAc)	Apparent V_{max} (pmole/min)
0	y = 0.008801x + 0.171232	0.0514	5.84
2.5	y = 0.013154x + 0.216639	0.0607	4.62
5.0	y = 0.020012x + 0.292628	0.0684	3.42
10.0	y = 0.026826x + 0.361291	0.0742	2.77

Figures 12 and 14 each show that with varied inhibitor quantities, the inhibition is of a mixed type, as evidenced by the intersections which occur in the second quadrant. According to the effect of the inhibitor on sialyltransferase kinetics seen in the Michaelis-Menten plots, and the double reciprocal plots, the inhibitor does not compete for binding at the substrate site(s). The inhibition of sialyltransferase is not competitive with the asialo α_1 AGP substrate, or the CMP-NeuAc substrate. The inhibitor does however affect sialyltransferase kinetics by interacting with sites or forms of the enzyme that subsequently affect substrate binding. The apparent K_m values indicate that as increasing quantities of inhibitor are added, sialyltransferase has a decreasing affinity for asialo α_1 AGP, as well as for CMP-NeuAc. The kinetic evaluations indicated that the mechanism was not simply through competition for substrate binding, but was more complex, and very intriguing.

Understanding the mechanism of inhibition would require more information. A variety of approaches could have been taken to access this information. Since identification of the inhibitor was the major goal, and since successful identification would also provide insight into the mechanism of inhibition, it was the approach of choice. Direct identification of the inhibitor was therefore undertaken.

VI. Identification of the Inhibitor by Electrophoretic Analysis and Sequencing

The inhibitor pool purified by isoelectric focusing was analyzed by acrylamide gel electrophoresis, under non-reducing and reducing conditions. Typical Coomassie blue stained gels of the inhibitor are shown in Figure 15. Under non-reducing conditions (Figure 15, I) two protein bands were observed, labeled bands A and B. Under reducing conditions (Figure 15, II) this same inhibitor preparation separated into four major protein bands, labeled C, D, E, and F.

For N-terminal amino acid sequence analysis, lanes identical to those shown in Figure 15 were run in parallel, but were removed prior to staining, and electroblotted from the acrylamide to PVDF membranes. The PVDF membranes were Coomassie blue stained for detection of the proteins. Bands A through F were identified, excised, and analyzed for their N-terminal amino acid sequences. Sequencing of band A resulted in equimolar amounts of two distinguishable amino acids in each cycle, as detailed in Table 12. Band B gave a clean, definitive sequence, H-E-D-M-S-K-Q-A-F-V-F-P-G-V-S-A-T-A-Y-V-S-L-E-A-E. In a search of the NBRF PIR database, using the BLAST program, a match to this sequence was found, having accession # A42579, and corresponding to the N-terminal sequence of rat CRP (Rassouli *et al*, 1992). No other proteins were detected under non-reducing conditions. Under reducing conditions, band F was found to have the same N-terminal sequence as rat CRP. Band B (non-reduced) corresponds to the CRP homodimer Mr 56,000, and band F (reduced) corresponds to the CRP monomer, Mr 30,000. The

inhibitor purified by isoelectric focusing appeared to contain two protein components, with one now identified as rat CRP.

Under non-reducing conditions, in addition to rat CRP (band B), there was one other component, band A, which appeared to be a heterodimer since N-terminal amino acid sequence analysis resulted in two equimolar sequences (see Table 12). After reduction, band A appeared to dissociate into three polypeptides, bands C, D, and E. Bands C and D from the reduced gel each had the same N-terminal amino acid sequence as shown in Table 12. The sequences determined for bands C and D were used in the search for matching proteins using the BLAST program as detailed in the Methods. A match was found as accession # A42210 in the NBRF PIR database, and as accession # M77183 in the GenPept database (a derivative of the GenBank database), and was identified as the published sequence for the heavy, or α chain of rat α_1 macroglobulin (Eggertsen *et al*, 1991; Wärmegård *et al*, 1992). Band E was blocked and could not be sequenced. Subtraction of the N-terminal sequence of the heavy chain of α_1 macroglobulin from the double sequence in band A gave the single sequence shown in Table 12. A comparison of this sequence with the N-terminal sequence of the light, or β chain of α_1 macroglobulin also shown in Table 12, indicates that in the first ten amino acids, where a comparison could be made, all of the amino acids match, with the exception of cycle four where a glutamic acid is deduced, but a serine is the published amino acid for this position. The double sequence observed under non-reducing conditions is therefore, the disulfide linked (α,β) subunit of rat α_1 macroglobulin.

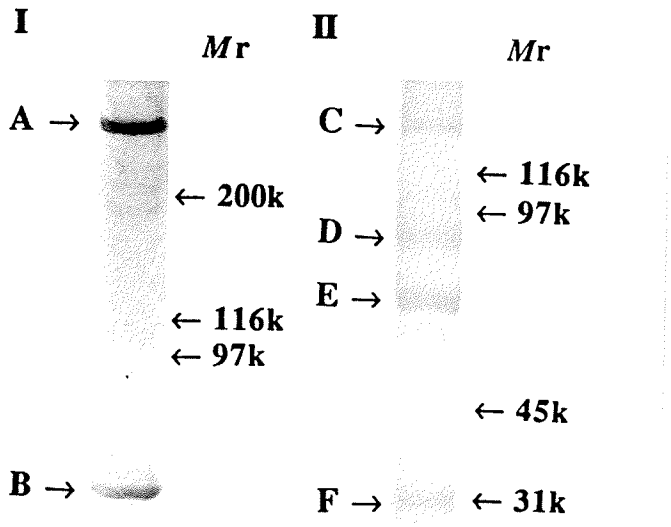


Figure 15: SDS-Polyacrylamide Gel Electrophoresis of the Sialyltransferase Inhibitor Isolated By Isoelectric Focusing
Fractions purified by isoelectric focusing, as indicated by the bar in Figure 5, were pooled, concentrated, and subsequently separated by acrylamide gel electrophoresis, under non-reducing (**I**), and reducing conditions (**II**), followed by protein detection using coomassie blue staining. **I** is a 4.8% acrylamide gel, and **II** is a 7.5% acrylamide gel. Under non-reducing conditions (**I**) two protein bands were visualized by detection with coomassie blue, labeled **A** and **B**. Under reducing conditions (**II**), the same inhibitor preparation separated into four protein bands, labeled **C**, **D**, **E**, and **F**. The migration of the standard *M*r markers are indicated.

Table 12: N-Terminal Amino Acid Sequences of Proteins Eluted from Acrylamide Gels Compared with Published Sequences

After separation by gel electrophoresis (see Figure 15), and transfer to PVDF membranes, the isolated proteins were analyzed for N-terminal amino acid sequences. Bands C and D were identical to the published α chain sequence of α_1 macroglobulin. After subtraction of the sequence of band C or D from the double sequence for band A, the single sequence deduced corresponds to the published β chain sequence of α_1 macroglobulin (Eggertsen *et al*, 1991; Wärmegård *et al*, 1992). Standard single-letter abbreviations are used for the amino acids, with X referring to those not determined.

Cycle	Band A	Band C or Band D	Published α Chain Sequence	Band A - Band C or Band D	Published β Chain Sequence
1	D, A	A	A	D	D
2	L, T	T	T	L	L
3	S, G	G	G	S	S
4	E, K	K	K	E	S
5	S, P	P	P	S	S
6	D, R	R	R	D	D
7	L, Y	Y	Y	L	L
8	T, V	V	V	T	T
9	T, V	V	V	T	T
10	A, L	L	L	A	A
11	S, V	V	V	S	S
12	X, P	P	P	X	K
13	I, S	S	S	I	I
14	X,E	E	E	X	V
15	X,L	L	L	X	K
16	X, Y	Y	Y	X	W
17	X, A	X	A	X	I
18	X,G	X	G	X	S
19	X, V	X	V	X	K
20	X, P	X	P	X	Q

The average *M*r for band C was found to be 164,000, which is comparable to previously reported values for the α chain of α_1 macroglobulin as determined by gel electrophoresis (Eggertsen *et al*, 1991; Geiger *et al*, 1987). The average *M*r for band D was found to be 92,000, which is similar to other values reported for the N-terminal fragment of the α chain of α_1 macroglobulin which results from autolytic cleavage (Eggertsen *et al*, 1991; Nelles & Schnebli, 1982; Schaeufele & Koo, 1982). An average *M*r of 71,700 was determined for the N-terminally blocked band E, and this corresponds to values reported for the carboxyl fragment of the α chain, that is the other product created by autolytic cleavage (Eggertsen *et al*, 1991). The carboxyl fragments of the α chain, from pure α_1 macroglobulin, like band E, were also found to be N-terminally blocked (Eggertsen *et al*, 1991; Nelles & Schnebli, 1982; Schaeufele & Koo, 1982). Although band E could not be sequenced, it is believed, by its nearly identical size and its similarly blocked N-termini, to the fragments found in electrophoresis of pure α_1 macroglobulin, that it is part of the α_1 macroglobulin already determined to be present in the inhibitor preparation.

As indicated earlier, the two protein peaks identified in the fractionation of the isoelectric focused inhibitor (see Figure 5), were examined separately by electrophoresis, and found to contain different proportions of the same two proteins, according to their migration distances. Under non-reducing conditions the peak at pH 4.20, contained greater amounts of the lower *M*r band B (Figure 15, I), relative to the higher *M*r band A (Figure 15, I). The second protein peak at pH 4.45, contained greater amounts of band A than

band B. Since the identity of these proteins has been determined, band A as rat α_1 macroglobulin, and band B as rat CRP this further corroborates that the mixture obtained by isoelectric focusing contains only two proteins. Furthermore, comparison of the pI values previously estimated for the two proteins from the isoelectric focusing purification can now be made to the literature values for the known proteins. The pI value for rat CRP has a reported pI of 3.8 - 4.0 (De Beer *et al*, 1982) which is close to the pH of 4.2 for the first protein peak in the isoelectric focusing purification and which is now known to contain more CRP (Figure 15, I, band B) than α_1 macroglobulin (Figure 15, I, band A). The reported pI for rat α_1 macroglobulin is 4.4 (Gordon, 1976), which closely agrees with the pH 4.45 found for the second protein peak separated by isoelectric focusing which is now known to contain a greater amount of α_1 macroglobulin (Figure 15, I, band A) than CRP (Figure 15, I, band B). The two proteins in the isoelectric focusing pool have been identified by N-terminal amino acid sequencing, and the published pI values for the proteins are in agreement with the pH values for the separate protein peaks identified in the isoelectric focusing purification.

Since bands A, B, C, D, and F could be identified by N-terminal sequence analysis, and band E is believed to be a blocked fragment from the α_1 macroglobulin, and since no other protein bands could be identified, it is concluded that the inhibitor purified by isoelectric focusing is a mixture of two proteins, rat CRP and rat α_1 macroglobulin.

To test whether CRP could act as an independent inhibitor, pure CRP, from *Limulus polyphemus*, human, and rat species were

monitored for their effect on sialyltransferase activity. Human CRP is approximately 70% similar in amino acid sequence to the rat CRP protein, but unlike the rat protein, it is not glycosylated. *Limulus* CRP is much less similar in amino acid sequence, approximately 50% similar to the rat protein, but is structurally similar, and is glycosylated. The standard inhibition assay was conducted using 0.2 mU α2,6 sialyltransferase and with pure CRP substituted for the inhibitor. When *Limulus polyphemus* or human CRP were monitored for α2,6 sialyltransferase inhibition, over a wide range of concentrations no inhibition of sialyltransferase was detected. Although this evidence provided a good indication that CRP was not the inhibitory component, the fact that the rat protein is structurally different than the *Limulus polyphemus* and human proteins meant it could not be strictly ruled out as a potential sialyltransferase inhibitor. Pure native rat CRP was therefore tested for α2,6 sialyltransferase inhibition and as summarized in Table 13, it also did not inhibit sialyltransferase. The addition of rat CRP to α2,6 sialyltransferase was noted (data not shown) to have a stabilizing effect on the enzyme, with its addition causing the enzyme activity to be 10 - 30% greater than the controls which contained no CRP. Since pure rat CRP did not inhibit sialyltransferase, and since the only other known protein present in the inhibitor preparation was rat α_1 macroglobulin, by default it seemed that the sialyltransferase inhibitor must be rat α_1 macroglobulin.

Table 13: Effect of Rat C Reactive Protein on Galβ1-4GlcNAc α2-6 Sialyltransferase Activity

Varying quantities of rat CRP were mixed with 0.2 mU α 2,6 sialyltransferase, incubated for 2 hours at 37°C, and then assayed for sialyltransferase activity. Controls contained no CRP but were otherwise treated identically to the test samples, and were used to determine the % inhibition of sialyltransferase.

Rat CRP (μg):	% Inhibition of α2,6 Sialyltransferase
0	0
0.3	0
0.9	0
1.5	0
1.8	0
2.7	0
3.0	0
6.0	0

To determine if rat α_1 macroglobulin was responsible for inhibition of α2,6 sialyltransferase, the most obvious option would have been to test pure rat α_1 macroglobulin. Pure native rat α_1 macroglobulin was however, unavailable. Since pure rat CRP did not inhibit sialyltransferase and α_1 macroglobulin was the only other identified protein in the mixture, and since the macroglobulins are very well-known for their scavenger nature, capable of binding tremendously diverse molecules, it was concluded that the identity of the inhibitor was rat α_1 macroglobulin.

To provide supporting evidence that α_1 macroglobulin was the sialyltransferase inhibitor, monospecific antibodies against the heavy and light chains of rat α_1 macroglobulin were tested for their effect on the inhibition of sialyltransferase by the inhibitor purified by isoelectric focusing. The aim was to determine if antibodies to rat α_1 macroglobulin could block the action of the inhibitor, thereby confirming the inhibitor identity. Incubation of the inhibitor purified by isoelectric focusing, with antibodies against the heavy, or α chain of α_1 macroglobulin indicates a dose dependent response, as shown in Figure 16. In the presence of sufficient antibody all the sialyltransferase inhibition was essentially eliminated. Also shown in Figure 16 is the dose response of sialyltransferase inhibition by antibodies against the light, or β chain of α_1 macroglobulin. Antibodies against the light chain had no effect on sialyltransferase inhibition, except at high doses (7.5 μl) when a slight loss, from 90% to 75% inhibition is observed. Controls containing antibodies to other rat serum proteins were also found to have no effect on sialyltransferase inhibition. The ability of antibodies monospecific

for the α chain of rat α_1 macroglobulin to block sialyltransferase inhibition, verifies that α_1 macroglobulin is the inhibitor.

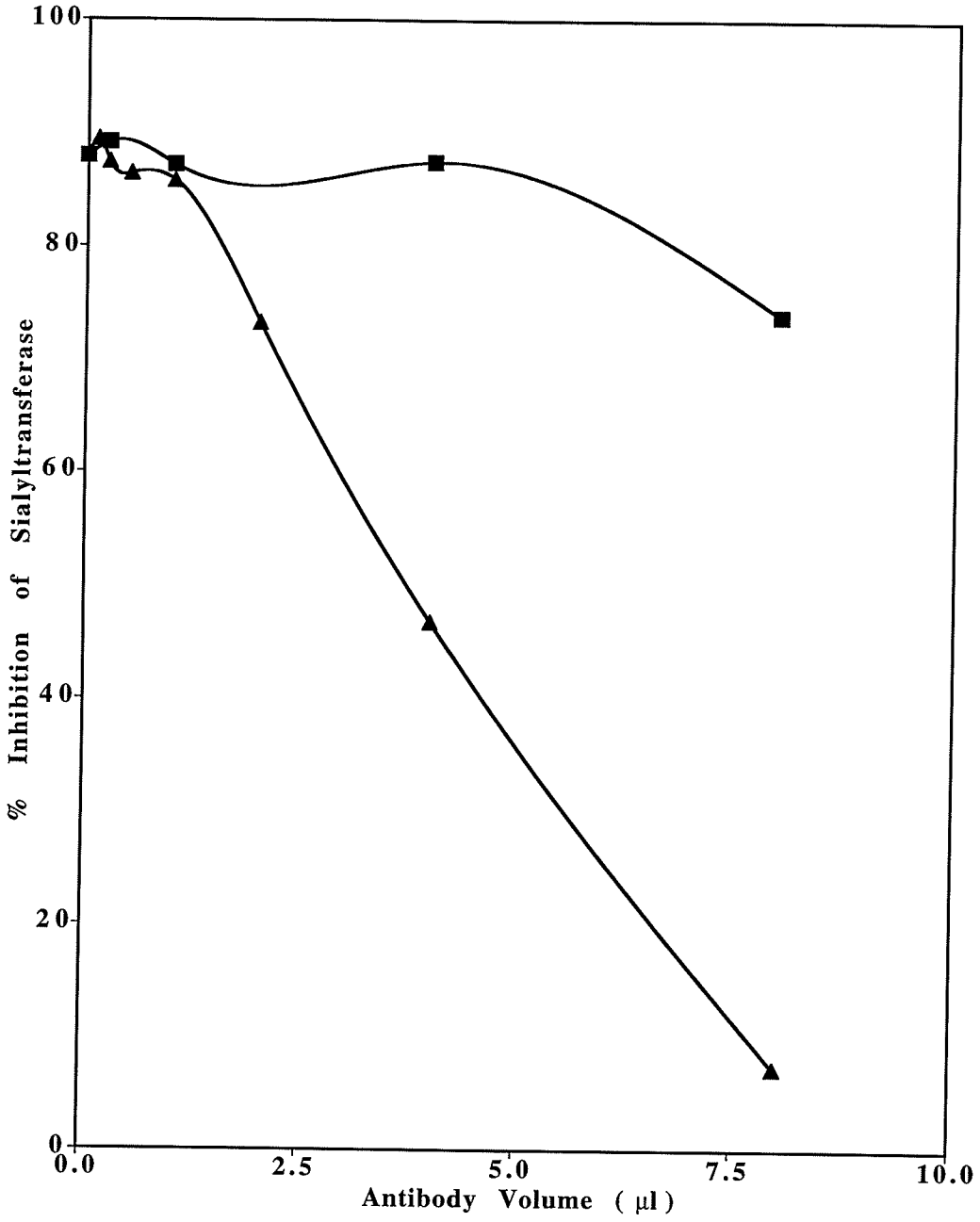


Figure 16: Dose Response of α2,6 Sialyltransferase Inhibition by Monospecific Antibodies to Rat α_1 Macroglobulin

Inhibitor, isolated by isoelectric focusing as shown in Figure 5, was adjusted to contain approximately 0.5 µg protein in 10 µl of 20 mM Tris, pH 7, and mixed with increasing amounts of antibodies. The mixtures were incubated at 37°C for 2 hr, and the standard inhibition assay was performed using 0.2 mU pure sialyltransferase. Antibodies (■) monospecific for the light or β chain, and antibodies (▲) monospecific for the heavy or α chain of rat α_1 macroglobulin are tested over a range of quantities, and the resulting percentage inhibition of sialyltransferase is calculated relative to controls containing no inhibitor.

VII. Mechanism of Inhibition

It is postulated that rat α_1 macroglobulin acts to inhibit sialyltransferase by functioning as a molecular trap, encaging the enzyme, thereby preventing it from being catalytically active. What might be required to activate the α_1 macroglobulin into the form which is capable of inhibiting sialyltransferase is unknown. In its native form, which is traditionally referred to as the 'slow' form of α_1 macroglobulin, it is a tetramer of the α,β disulfide linked subunit, and sometimes exists in multimeric forms of the tetramer. In its native state, the molecule is loose and has a large hydrodynamic volume, and functions exceptionally well to trap proteases, as well as other proteins. Once a protease has been trapped, the α_1 macroglobulin contracts, becoming much more compact having a smaller hydrodynamic volume. This form of the molecule is traditionally referred to as the 'fast' form. Conversion into the 'fast' form can be accomplished by treatment with methylamine. In its methylamine form the macroglobulin can no longer function to trap proteases, but has been shown to be capable of trapping other proteins. Since it was possible that sialyltransferase could be inhibited by an already activated α_1 macroglobulin, the methylamine α_1 macroglobulin form was tested. The results of experiments which tested the effect of rat methylamine α_1 macroglobulin on $\alpha 2,6$ sialyltransferase are summarized in Table 14. As indicated by the data in Table 14, the methylamine form of α_1 macroglobulin over the range of quantities utilized shows marginal inhibitory capability, with 10 µg causing approximately 25% inhibition of sialyltransferase. This is a very high ratio of α_1 macroglobulin to sialyltransferase,

when compared to the potency that is obtained from the inhibitor purified by isoelectric focusing. Using inhibitor purified by isoelectric focusing, as little as 0.25 μg of protein (CRP and α_1 macroglobulin mixture) could cause 95% inhibition of the same quantity of sialyltransferase, i.e., 0.2 mU or 0.025 μg protein. It is possible that the methylamine α_1 macroglobulin does have the ability to inhibit sialyltransferase, albeit with less potency than the α_1 macroglobulin isolated here. However, it could also be possible that the less potent inhibition by the methylamine α_1 macroglobulin is caused by residual α_1 macroglobulin not converted into the methylamine form. Although these studies did not determine the definitive form of α_1 macroglobulin required for sialyltransferase inhibition, the fact that the methylamine preparation was inhibitory further corroborates the identity of the inhibitor as rat α_1 macroglobulin.

Table 14: Effect of Rat Methylamine α_1 Macroglobulin on Galβ1-4GlcNAcα2-6 Sialyltransferase Activity

Varying quantities of rat methylamine α_1 macroglobulin were mixed with 0.2 mU α2,6 sialyltransferase, incubated for 2 hours at 37°C and then assayed for sialyltransferase activity. Controls contained no methylamine α_1 macroglobulin, but were otherwise treated identically to the test samples and were used to determine the % inhibition of sialyltransferase.

Methylamine α_1 Macroglobulin (μg)	% Inhibition of Sialyltransferase
0	0
0.1	12
0.5	14
2.5	13
5.0	13
10.0	24

VIII. Specificity of the Macroglobulin Inhibitor

The macroglobulin family of proteins are often referred to as general traps, since they act rather non-specifically, trapping a wide variety of molecules, but they are most well-known for their ability to trap and inhibit proteases. Of the numerous non-protease proteins the macroglobulins have been shown to bind, none have been glycosyltransferases. Like other members of its family, α_1 macroglobulin also functions to trap various molecules. The greatest proportion of rat α_1 macroglobulin *in vivo* has been shown to be complexed with proteases (Tsuji *et al*, 1994). Therefore the inhibition of rat α2,6 sialyltransferase by rat α_1 macroglobulin, i.e., its trapping of the enzyme, is a unique finding. Furthermore, it may be unique among the glycosyltransferase group of proteins. In order to provide some insight into whether this was a possibility, the effect of the inhibitor on galactosyltransferase was monitored. Experiments that tested the effect of the inhibitor preparations on galactosyltransferase showed that this enzyme was completely unaffected. Table 15 shows the results from this experiment. As indicated sialyltransferase is 95% inhibited, while under the same conditions galactosyltransferase maintains full activity. These results indicate that the inhibitor, α_1 macroglobulin, may have glycosyltransferase specificity, and in particular may target the sialyltransferases.

Table 15: Specificity of the Inhibitor

The effect of inhibitor purified by isoelectric focusing on 0.1 mU sialyltransferase or 1.0 mU galactosyltransferase activity was monitored using the standard inhibition assay. Equivalent amounts of inhibitor were used for each enzyme. The controls were the enzyme activities determined in the absence of inhibitor, but otherwise treated identically.

	% Inhibition
α2,6 Sialyltransferase (EC 2.4.99.1)	97
Galactosyltransferase (EC 2.4.1.38)	0

DISCUSSION

During studies on the regulation of Galβ1-4GlcNAcα2-6 sialyltransferase as an acute phase reactant a sialyltransferase inhibitor was discovered and subsequently identified. In retrospect, the minimal potential for success in this pursuit was perhaps not fully appreciated. Previous reported cases of sialyltransferase inhibitors have to date only been preliminary findings, with no reported subsequent follow-up on the specific nature, the mechanism of inhibition, or the identity of these inhibitors (Albarracin *et al*, 1988; Duffard & Caputto, 1972; Van den Eijnden & Schiphorst, 1988). The lack of further reports on these putative inhibitors may be due to a variety of reasons and include the possibilities that they may have been found to be proteases, to be non-specific, or to be substrates for the enzyme, rather than true inhibitors. Despite the risk that a true sialyltransferase inhibitor had not been discovered, the potential of such a finding perpetuated the study. The investigations into the nature of the inhibition and the information obtained throughout the isolation procedure provided increasing assurance that a true inhibitor had been found, and resulted in the final successful identification of the inhibitor as rat α_1 macroglobulin.

The discovery was made when rat serum, presumed to be a species-compatible and complete physiologic source of the regulators of the acute phase response, was added to the FAZA cell culture system in an attempt to induce the acute phase response of sialyltransferase. Rather than the anticipated stimulation in sialyltransferase activity, the addition of the rat serum to the FAZA cells resulted in a complete loss of all intracellular and secreted

sialyltransferase activity. Despite the sudden loss of sialyltransferase activity no detectable change in the morphology or growth of the FAZA cells was noted, even when cells were cultured in the presence of the inhibitory rat serum for up to 5 days. Whether cell integrity could have been maintained over longer periods of time, i.e., for weeks, or months, in the presence of this rat serum, and hence the lack of detectable sialyltransferase activity, is not known. Sialyltransferase activity was inhibited under normal (basal) growth conditions, and also when the cells were challenged with dexamethasone or PMA. The mechanisms regulating sialyltransferase synthesis under basal conditions, or when stimulated with dexamethasone or with PMA, are different, and therefore the loss in enzyme activity could not be attributed to some common mechanism targeting its synthesis. The inhibitor in the rat serum causing the loss of sialyltransferase activity was apparently capable of crossing the cell membrane since intracellular sialyltransferase activity as well as the secreted activity was lost, did not affect the viability of the FAZA cells, and was apparently not acting at the level of transcription since it was effective even in the presence of dexamethasone or PMA. It appeared that the inhibitor in the rat serum was acting directly on the activity of the enzyme, and therefore investigations to determine if a true sialyltransferase inhibitor had been discovered were undertaken.

The rat serum found to inhibit sialyltransferase in the FAZA cell system was tested directly on pure sialyltransferase, by addition to the standard assay, using purified Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1). The enzyme activity in the presence

of the inhibitory rat serum was monitored relative to normal sialyltransferase activity in the absence of the rat serum. Pure sialyltransferase was also inhibited, eliminating the possibility that the FAZA cells themselves had some role in the loss of sialyltransferase activity. Upon determination that pure sialyltransferase could be inhibited by simply adding the rat serum to the standard assay, studies in the FAZA cells were discontinued. Although the sialyltransferase synthesized by the FAZA cells could have been used as the enzyme source, it was not pure, and the effort and difficulties associated with the maintenance of cell lines made the use of pure sialyltransferase more sensible.

The inhibitory assay was designed to use a minimal amount of pure enzyme while retaining reliable amounts of [^{14}C]NeuAc transferred to the acceptor, as evidenced by the resulting dpm which ranged from two to four thousand in the absence of any inhibitory rat serum. Inhibitory rat serum and sialyltransferase were pre-incubated prior to measurement of enzyme activity since at low levels of serum relative to sialyltransferase, the inhibition had been observed to be time-dependent. The two hour pre-incubation at 37°C was chosen since it allowed ample time for the inhibition to occur at the ratios of 0.1 mU - 0.2 mU sialyltransferase to the varying quantities of inhibitory rat serum, or any of the subsequently purified inhibitor fractions or pools. Once the standard inhibition assay was developed it was used in all routine investigations, and throughout the purification process to track and quantitate the inhibitor.

Isolation of the inhibitor was undertaken simultaneously with experiments designed to provide insight into the nature and mechanism of the sialyltransferase inhibition. This was done since, with the exception that it came from rat serum prepared by partial decapitation to sever the jugular vein, the inhibitory compound was an entirely unknown entity. It could have been an organic compound, such as a glycan (for example heparin), protein, lipid, or nucleotide (DNA, RNA, or various other nucleotide compounds). Alternatively, it could have been inorganic including various ionic compounds, for example Na^{+}, K^{+}, Mn^{2+}, Mg^{2+}, or Cl^{-}. The inhibition could also have resulted from any of the various possible combinations of serum components. The loss of enzymatic activity was not necessarily the result of a sialyltransferase inhibitor. It could have resulted from a lack of the appropriate conditions for the enzymes activity, or to attack by glycosidases, proteases, or various hydrolyzing enzymes. Furthermore, it could have simply resulted from interference with the method of detection of enzyme activity. Therefore, it was essential to begin studies which would provide insight into the mechanism of the inhibition. Isolation was undertaken concurrently, since this process itself could provide information on the properties of the inhibitor, and since this would inevitably be necessary for identification.

At the outset of the purification the only available knowledge of the sialyltransferase inhibitor was that it was found in rat serum. Although it was possible that the noted sialyltransferase inhibition could have been caused by numerous types of molecular components, the major serum components are water and protein.

Early investigations indicated that the inhibitory component was non-dialyzable, having a *M*r of at least 10,000. Therefore, the assumption that the inhibitor was protein in nature was made, and the isolation procedure used was based on previous methods for the purification of serum proteins. The initial Sephadex G100 and Sephadex DEAE A25 separations resulted in substantial removal of uninhibitory protein, and therefore substantial increased purification. The Sepharose CL 4B separation did not contribute as significantly to the separation of the inhibitor from the non-inhibitory proteins, but provided information on the *M*r of the inhibitor. Also, it was highly reproducible, providing assurance that the same compound was being isolated in the many individual fractionations that were performed. The isoelectric focusing was also highly reproducible, and was successful in the removal of a substantial amount of non-inhibitory protein in the protein peak near the anode, at the alkali end of the gradient. Overall, the isolation procedure resulted in a substantial purification, with only two proteins, rat CRP and rat α_1 macroglobulin, identified in the final inhibitor-containing pool obtained from the purification by isoelectric focusing.

The purification by isoelectric focusing revealed two protein peaks, each containing CRP and α_1 macroglobulin, incompletely resolved. In addition to its many binding properties, rat CRP has been shown to function as a galactose-specific lectin (Kolb-Bachofen, 1991), binding a variety of glycoproteins. A lectin-like affinity of CRP for the α_1 macroglobulin glycoprotein (Geiger *et al*, 1987; Gordon, 1976) may therefore exist. It is possible that the affinity of

CRP for α_1 macroglobulin may have caused the apparent pI of the major CRP peak at 4.2, to be higher than the reported 3.8 - 4.0 for rat CRP (De Beer *et al*, 1982). Additionally, the speculated lectin-like affinity between CRP and α_1 macroglobulin, coupled with their close isoelectric values, may have resulted in the co-purification in the early separations, and their final incomplete resolution by isoelectric focusing.

Calculation of the total protein which would have been recovered from the final isoelectric focusing step indicates that 12 mg of protein would have recovered from the initial 40 ml of rat serum applied to the Sephadex G100 column. The relative proportion of this protein to the total serum protein is 0.6%. Although this may appear low, it must be realized that the total serum protein contains many proteins. CRP and α_1 macroglobulin actually only account for a very small percentage of the total serum protein. Normal healthy rats contain from 0.3 - 0.6 mg CRP per ml of serum (De Beer *et al*, 1982), and from 1 - 2 mg α_1 macroglobulin per ml of serum (Gordon, 1976; Lonberg-Holm *et al*, 1987). Therefore, at the low end the total amount of CRP and α_1 macroglobulin would be 1.3 mg per ml of serum, and at the high end would be 2.6 mg per ml of serum. The total amount of CRP and α_1 macroglobulin in the 40 ml of serum is therefore calculated to range from 52 mg (1.3 mg/ml x 40 ml) to 104 mg (2.6 mg/ml x 40 ml). Based on the calculated recovery of 12 mg of the mixture of CRP and α_1 macroglobulin, and on the assumption that the proportions of each recovered were the same, the resulting recovery would range from 12 to 24%. This range of percentage yield of protein is reasonable, and similar to

most protein purifications. To further purify this mixture of CRP and α_1 macroglobulin, either pneumonococcal C polysaccharide (De Beer *et al*, 1982), or phosphorylcholine (Nagpurkar & Mookerjea, 1981) affinity columns could be used, which would strongly bind CRP removing it from the mixture. Based on the knowledge that the inhibitor is rat α_1 macroglobulin traditional methods could also now be used for its purification (Gordon, 1976; Lonberg-Holm *et al*, 1987). These methods also require multiple steps resulting in very similar percentage yields as obtained with the method here, but they have the advantage that the final product would be pure, containing no CRP. Alternatively, new α_1 macroglobulin purification strategies could be developed, using information from the traditional α_1 macroglobulin purification methods, coupled with the information obtained here to provide a simpler, more efficient overall method.

Quantitation of the inhibitor using the defined unit of sialyltransferase inhibitory activity was also used to monitor the efficiency of the purification procedure. From the units of inhibitory activity and the protein quantitations, calculation of the specific activities for each purified inhibitor pool was possible. The specific activity of rat serum was determined to be 0.3 U/μg, and the final product from the isoelectric focusing purification was determined to be 18.1 U/μg, with an approximate overall 60-fold increase in the purity of the inhibitor.

During the purification process, various properties of the inhibitor were investigated, and some of these were difficult to understand. Examples of these properties include the time-dependence of the inhibition, the apparent inability to hydrolyze the

inhibitor with proteolytic enzymes, yet the ease with which inhibition could be destroyed by treatment with hyaluronidase. The time-dependence indicated that the process may be enzymatic, for example by hydrolysis of sialyltransferase, the acceptor, or the donor used for the detection of its activity. The significant difficulty found using proteases in an attempt to hydrolyze the inhibitor, and the ability to destroy the inhibitor activity with hyaluronidase indicated that the inhibitor may not be primarily protein in nature, and that it was potentially a proteoglycan, with the functionally significant portion being the glycan component. Its large size and acidic nature further indicated that this component may be a proteoglycan. Other properties found, including the UV-VIS spectrum, the quantitation of protein and carbohydrate indicated that the inhibitor was primarily protein, with a small proportion of carbohydrate relative to what might be expected in a proteoglycan. So, at the time of the determination of these properties, they were difficult to understand, and even contradictory. However, after the inhibitor was identified as α_1 macroglobulin, many of these properties were in retrospect much more understandable.

During the initial experiments it was noted that the reaction between sialyltransferase and the inhibitor was a time-dependent phenomenon, and that the length of time required for the reaction to be complete was dependent on the ratio of inhibitor to sialyltransferase. The greater the quantity of inhibitor to sialyltransferase, the less time was required for complete inhibition to be achieved. This indicated that the mechanism could be a non-specific enzymatic process, and may not be the result of a specific

sialyltransferase inhibitor. Investigations into whether the macromolecular asialo α_1 AGP acceptor, or the CMP-[^{14}C]NeuAc donor were being hydrolyzed, eliminated these possibilities, but the potential that the sialyltransferase molecule itself was being hydrolyzed could not be eliminated.

Macroglobulins function as molecular traps (Barrett & Starkey, 1973), and do so in a time-dependent manner. For example, incubation at 37°C for up to an hour is required for completion of complex formation between trypsin and native α_1 or α_2 macroglobulin, depending on the relative proportions of protease to macroglobulin (Gordon, 1976). Another example is the complex formation between IL-1 and activated α_2 macroglobulin (the methylamine form), which requires up to 200 minutes for the reaction to be complete (Borth *et al*, 1990). Thus, the dependence of inhibition of sialyltransferase on the quantities of enzyme and α_1 macroglobulin, and on the interaction time, is consistent with the known behavior of the macroglobulins, and the identification of the inhibitor as rat α_1 macroglobulin.

The large size of the sialyltransferase inhibitor, with a Mr found to range from 370,000 to 728,000 by gel filtration on Sepharose CL 4B, was also retrospectively, consistent with the identification of the inhibitor. The final inhibitor preparation from the isoelectric focusing purification was found to contain CRP and α_1 macroglobulin. The inhibitor pool obtained from the Sepharose CL 4B purification must therefore contain both of these, and possibly other components. The more potent inhibition occurring at the higher 728,000 Mr is probably indicative of the native tetramer of α_1

macroglobulin that has a reported *Mr* in the range of 730,000 to 746,000 (Gordon, 1976; Lonberg-Holm *et al*, 1987). The lower 370,000 *Mr* component in the inhibitor pool could potentially correspond to the reported 300,000 to 360,000 *Mr* component of α_1 macroglobulin believed to be a dimeric form (Lonberg-Holm *et al*, 1987). The lower 370,000 *Mr* component could however, also contain oligomeric forms of CRP (De Beer *et al*, 1982). Additionally, the Sepharose CL 4B inhibitor pool could also include various associated complexes between α_1 macroglobulin subunits and CRP monomers. Although the components recovered in the Sepharose CL 4B inhibitor pool would contain CRP components, and potentially other proteins, the largest and most potent component, with a *Mr* of 728,000, is consistent with the identification of α_1 macroglobulin as the inhibitor.

The acidic nature of the inhibitor, as observed by its elution at pH 5 from the Sephadex DEAE A25 column, and its pH range of 4.2 - 4.5 observed on isoelectric focusing, is consistent with the presence of CRP in the final purified product, and the known identity of the inhibitor as α_1 macroglobulin. The separation by isoelectric focusing was observed to contain three protein peaks, two of which were inhibitory. The peak at pH 4.2 was noted to contain relatively higher amounts of the protein band corresponding to CRP, and less of that corresponding to α_1 macroglobulin. Rat CRP has been reported to have a pI of 3.8 - 4.0 (De Beer *et al*, 1982). The peak at pH 4.45 contained relatively higher amounts of α_1 macroglobulin than of CRP. This is consistent with the reported isoelectric point for rat α_1 macroglobulin of 4.4 (Gordon, 1976). The inhibitory activity was also

found to be more potent in the second protein peak, at pH 4.45 which further confirms the identification of α_1 macroglobulin as the sialyltransferase inhibitor.

The significance of the difficulty found in the attempts to hydrolyze the inhibitor with proteolytic enzymes could also now be understood, since macroglobulins are most well known for their function as protease inhibitors (Barrett & Starkey, 1973). During those experiments which utilized trypsin or pronase in an attempt to hydrolyze the inhibitor, the lack of destruction of the inhibitor was not understood. At the time, the concern was that the inhibitor was not primarily protein in nature, but in retrospect it is evident that the sialyltransferase inhibitor was also acting as a protease inhibitor, and that this is consistent with the identification of the inhibitor as rat α_1 macroglobulin.

The ability of hyaluronidase, from bovine testes, to destroy the inhibitory activity, coupled with the difficulties in the attempts to hydrolyze the inhibitor with proteases, may have indicated that the inhibitor was a proteoglycan. But this seemed contradictory to the protein and carbohydrate analysis, which indicated that the inhibitor was approximately 85% protein and 15% carbohydrate. The ability of hyaluronidase from bovine testes to hydrolyze the inhibitor is presumed to be the result of its lack of specificity. The specific glycosaminoglycan hydrolyzing enzymes, hyaluronidase from *Streptomyces hyalurolyticus*, chondroitinase ABC, and heparatinase each had no effect on the inhibitor, further indicating that the glycan portion of the inhibitor was not of the glycosaminoglycan-type. Rat CRP has been reported to be 18% carbohydrate by weight (Nagpurkar

& Mookerjea, 1981), and rat α_1 macroglobulin is N-linked glycosylated containing approximately 10% carbohydrate (Geiger *et al*, 1987; Gordon, 1976). Thus, the 15% carbohydrate determined here is consistent with the identification of the two components, CRP and α_1 macroglobulin, in the final inhibitor-containing pool purified by isoelectric focusing.

Since macroglobulins are most well known for their ability to trap proteases (Barrett & Starkey, 1973), the importance of eliminating the possibility that sialyltransferase activity was being destroyed by any trace proteolysis, was in retrospect perhaps even more critical. As previously described, addition of protease inhibitors, prior to and during the reaction between the inhibitor and sialyltransferase, had no effect on the inhibitory activity, and no protease activity was detected directly using the FITC-casein method. Additionally, galactosyltransferase activity was unaffected by addition of the inhibitor preparations. These findings provide strong evidence for the absence of any accessible contaminating proteolytic component in the inhibitor preparations, which could cause proteolysis of either the macromolecular acceptor, α_1 AGP, or the sialyltransferase molecule itself.

Identification of rat α_1 macroglobulin and CRP in the complex was accomplished by electrophoretic separation followed by N-terminal sequencing of the isolated proteins. In the case of CRP the sequence was easily determined, since this protein consists of five identical subunits of single polypeptide chains. Native α_1 macroglobulin is a tetramer of subunits, each consisting of a disulfide linked α,β polypeptide. Under non-reducing conditions the α,β

subunit was isolated which gave a double sequence (band A, Figure 15, I). Under reducing conditions the α chain (band C, Figure 15, II), and an N-terminal fragment of the α chain (band D, Figure 15, II), were separated and sequenced, and were identical to the α chain sequence of rat α_1 macroglobulin (Eggertsen *et al*, 1991; Wärmegård *et al*, 1992). An intact β chain was not detected on separation by electrophoresis, probably due to fragmentation occurring during sample preparation. Identification was inferred by subtraction of the α chain sequence from the double sequence, and this sequence matched the published β chain sequence of α_1 macroglobulin (Eggertsen *et al*, 1991; Wärmegård *et al*, 1992). One sequence discrepancy was observed in the fourth cycle where a glutamic acid was deduced rather than the published serine. This discrepancy may be due to the differences in the strain of rat used, or to microheterogeneity in amino acid sequence for this protein. Microheterogeneity has been observed in this family of proteins, for example in $\alpha_1 I_3$ cDNA sequences and genes (Braciak *et al*, 1988; Northemann et al, 1988), and at the protein level (Sottrup-Jensen, 1987; Sottrup-Jensen *et al*, 1989). For α_1 macroglobulin specifically six cDNA clones have been isolated, three of which show distinctly different restriction enzyme patterns (Eggertsen *et al*, 1991), indicating microheterogeneity in the cDNA sequence. Additionally, on gel electrophoresis a doublet of *M*r 36,000 and 38,000 has been shown for the β chain (Geiger *et al*, 1987), indicating a potential for microheterogeneity at the protein level.

The average *M*r for the α chain determined by electrophoresis was 164,000, which is comparable to previously reported values of

160,000 (Geiger *et al*, 1987) and 165,000 (Eggertsen *et al*, 1991), although lower values of 140,000 (Gordon, 1976) and 143,000 (Lonberg-Holm *et al*, 1987) have also been reported. Bands D and E are fragments of the heavy chain, probably resulting from autolytic cleavage at the thiol ester site (amino acids 962 - 965). The fragment of average *M*r 92,000 (band D, Figure 15, II) gave an identical N-terminal sequence to the intact α chain, and probably corresponds to previously identified fragments of *M*r 82,500 (Schaeufele & Koo, 1982), 85,000 (Eggertsen *et al*, 1991), and 90,000 (Nelles & Schnebli, 1982). The smaller band E (Figure 15, II) with a *M*r of 71,700 is probably the carboxyl fragment of the α chain generated by the autolytic cleavage. Addition of the *M*r for the fragments, band D (92,000) and E (72,000) gives a total *M*r of 164,000, which corresponds to the *M*r of the intact heavy chain of α_1 macroglobulin. Sequencing of band E, presumably the carboxyl fragment of the heavy chain with the N-terminal being at the thiol ester site, was blocked. Similar N-terminally blocked *M*r fragments have been observed by other researchers in electrophoretic profiles of α_1 macroglobulin under reducing conditions (Eggertsen *et al*, 1991). So, the electrophoretic profiles, both under non-reducing and reducing conditions, and N-terminal sequence analysis of the components indicated that the only two proteins in the isoelectric focusing purified product were rat CRP and α_1 macroglobulin.

After identification of the rat CRP and α_1 macroglobulin in the final purified product, the inhibitor was identified by the combination of independent tests with pure rat CRP, and verified by the use of antibodies monospecific for α_1 macroglobulin. Pure CRP

did not inhibit sialyltransferase, when it was substituted for the inhibitor, in the standard inhibition assay. Therefore, pure CRP does not, independently inhibit sialyltransferase. Pure native α_1 macroglobulin was unavailable, and therefore could not be tested for its potential as an independent inhibitor. To verify that α_1 macroglobulin was the major inhibitory component, antibodies monospecific for the heavy and light chains of α_1 macroglobulin were used, and those for the heavy chain abolished sialyltransferase inhibition in a dose-dependent manner. The antibodies to the light chain had only a marginal effect, probably since these antibodies only react with the β chain when it is free from the α,β disulfide linked subunits, and do not react with the intact native molecule (Geiger *et al*, 1987). The observed loss of activity in the presence of antibodies to the α, or heavy chain does however, verify the inhibitor identity as rat α_1 macroglobulin.

The macroglobulin family of proteins are conserved throughout evolution and include a variety of proteins, for example α_1 macroglobulin, α_2 macroglobulin, α_1I_3, pregnancy zone protein, and various complement proteins, for example C3 and C4 (for reviews see Sottrup-Jensen, 1987; Pizzo & Gonias, 1984; Salvesen & Enghild, 1993; Sottrup-Jensen, 1989; Travis & Salvesen, 1983). The distinguishing feature of this family of proteins is the presence of an internal β-cysteinyl-γ-glutamyl thiolester (Sottrup-Jensen, 1987; Sottrup-Jensen, 1989; Sottrup-Jensen *et al*, 1990). The macroglobulins (α_1 and α_2) are among these proteins, and are known for a variety of functions, in a variety of contexts.

As protease inhibitors the macroglobulins are unique since unlike other protease inhibitors, the mechanism is not by inactivation of the protease. The mechanism of protease inhibition is traditionally described as a molecular trapping mechanism (Barrett & Starkey, 1973). Trapping of a protease is usually achieved when a protease encounters a macroglobulin through recognition of the exposed bait region. The bait region is a unique stretch of amino acids located approximately midway in the polypeptide chain of macroglobulin subunits, that functions to lure proteases with specificity (Sottrup-Jensen *et al*, 1989). On contact with a protease, the bait region undergoes limited proteolysis, with exposure of the previously buried labile thiolester that is subsequently available for interaction with the protease. Often a new covalent linkage is formed between nucleophilic groups in the protease (for example the ε-amino group of lysine residues) and the γ-glutamyl residue in the thiolester of the macroglobulin. In this manner macroglobulins act to trap the protease, surround it, and hence prevent it from acting on large protein substrates by steric hindrance. The trapped protease is still capable of hydrolysis, but the substrates must be small enough to access the protease embedded in the large macroglobulin molecule. Most proteins are of sufficient size and are therefore protected from proteolysis by the macroglobulin-trapped protease, since they cannot penetrate the macroglobulin shield.

In native form macroglobulins have large hydrodynamic volumes, and travel slowly when subjected to sedimentation analysis, and hence are often referred to as the 'slow', or S form. Once interaction with a protease has occurred, the conformation of

the macroglobulin changes drastically, causing the molecule to become much more compact. In this form, it travels relatively fast by sedimentation analysis, and hence is often referred to as the 'fast' or F form. The fast form of macroglobulins can also be created, for example by treatment with small nucleophiles such as methylamine. This treatment does not result in bait region cleavage, but attacks the thiolester directly, resulting in a bond formation between the amine group of the methylamine and the γ-glutamyl group in the thiolester. These forms of macroglobulin are usually referred to as the activated forms, which are compact and sediment identically to their respective fast forms.

Macroglobulin activation, or conversion into the fast form by interaction with various ligands, results in drastic conformational changes. The entire shape of the macroglobulin changes, with previously buried portions of the structure becoming presented on the surface. With the conformational change, previously buried receptor recognition sites become exposed. These receptor recognition sites can recognize receptors on a variety of cell types, including hepatocytes (Davidsen *et al*, 1985), fibroblasts (Van Leuven *et al*, 1979), and macrophages (Debanne *et al*, 1975). Interaction of the receptor recognition sites and the various receptors, leads to internalization of the macroglobulin, whether it be the activated form, or complexed to particular ligands. The fate of the complex is dependent on the ligand, and the cell type which has internalized the complex. When the capture of the ligand is a protective mechanism, for example if the ligands are undesirably destructive proteases, the

complexes are rapidly cleared and degraded in the lysosomes (Sottrup-Jensen, 1987; Pizzo & Gonias, 1984).

Although the best studied function of the macroglobulins is their ability to bind proteases, they also bind a variety of other ligands. They have been shown to bind other non-protease enzymes, such as transglutaminase (Hall & Söderhäll, 1994), lipoprotein lipase (Vilella *et al*, 1994), and choline acetyltransferase (Liebl & Koo, 1994). Many other molecules have also been shown to be ligands for these scavengers. Macroglobulins can interact with, capture, or bind these ligands. Examples of some of these ligands include zinc, lectins, liposomes, histones, insulin, cytokines and growth factors, as well as toxins, bacteria, and viruses (Bonner *et al*, 1989; Borth *et al*, 1990; Crookston & Gonias, 1994; Dennis *et al*, 1989; Huang, 1989; O'Connor-McCourt & Wakefield, 1987; Philip *et al*, 1994; Soker *et al*, 1993; Wolf & Gonias, 1994). The mechanisms by which the ligands interact with the macroglobulins vary considerably. Examples of some of the mechanisms involved in ligand interaction include non-covalent mechanisms such as ionic, hydrophobic, and lectin-like interactions. For example, lectin-like interactions include recognition and binding to terminal sialic acids on viruses. Mechanisms can also be covalent in nature, with bonds being formed between the ligand and the γ-glutamyl group in the thiolester, or by disulfide linkage between the ligand and the β-cysteinyl group of the thiolester after disruption of the ester bond has occurred. The mechanisms vary with the nature of the ligand, and in some cases with the availability of the components potentially required for pre-requisite disruption of the thiolester. The mechanisms are often sequential with appropriate

timing being required to facilitate the formation of many of the various ligand-macroglobulin complexes. Since the mechanisms involved in the interaction between the many different types of ligands and the macroglobulins, are dependent on the ligand, the environment, and the timing, any specific interaction must be considered uniquely and investigated accordingly.

Although the precise mechanism of sialyltransferase inhibition by α_1 macroglobulin is not known, the experimental information determined thus far, provides significant insight. Apparently α_1 macroglobulin does not interfere with the method of detection of sialyltransferase activity by altering or disabling the donor or acceptor substrates. It appears that the inhibitor reacts directly with the enzyme, disabling its catalytic activity. When sufficient inhibitor is used, sialyltransferase activity approaches complete inhibition. Furthermore, the inhibition is not competitive with either the CMP-NeuAc donor, or the asialo α_1 AGP acceptor. The kinetic analyses indicate that a mixed type of inhibition is occurring. The inhibitor apparently interacts with parts or forms of sialyltransferase that subsequently affect its interaction with the donor and the acceptor. The antibody experiments indicate that the functionally significant portion of the macroglobulin may be the α chain, known to contain the functionally significant bait region and the thiolester group that are primarily responsible for the capture and trapping of ligands. Based on the experimental evidence, and the awareness of how the macroglobulins function to trap other molecules, it is believed that the α_1 macroglobulin isolated here traps Galβ1-4GlcNAcα2-6 sialyltransferase thereby preventing its catalytic activity.

The mechanism of sialyltransferase entrapment by α_1 macroglobulin remains to be determined. Based on other reports of the mechanisms of trapping by macroglobulins, there may be a pre-activation requirement. Although it is possible that trapping of sialyltransferase could occur with native α_1 macroglobulin, it may also require that α_1 macroglobulin be pre-activated prior to contact with sialyltransferase. This pre-activation may involve the release of complexed α_1 macroglobulin, by an unknown mechanism, creating a free macroglobulin in a conformationally appropriate form that could subsequently function to trap sialyltransferase. It is also possible that limited, and potentially specific, proteolysis of α_1 macroglobulin may be a pre-requisite for the trapping of sialyltransferase. Further investigations will be required to determine if pre-activation is required, and if so what this would involve.

Trapping of ligands by macroglobulins can occur through non-covalent interactions, or through covalent linkage between the two species. It is unclear if the trapping of sialyltransferase by α_1 macroglobulin involves covalent linkage. Preliminary experiments, not included here, do however indicate that once complex formation between sialyltransferase and α_1 macroglobulin has occurred, it cannot be reversed by treatments which should dissociate any non-covalent forces, but that on reduction by β-mercaptoethanol sialyltransferase can be separated from the α_1 macroglobulin molecule. This provides an indication that the interaction may be covalent potentially through reactive sulfhydryl groups, and in particular may involve the cysteinyl residue in the labile thiolester.

The methylamine form of α_1 macroglobulin, which does not contain the intact thiolester and does not function as a protease inhibitor, only caused a marginal inhibition of sialyltransferase. This inhibition may be due to residual native α_1 macroglobulin in the preparation, or to relatively small quantities of the methylamine form still having a reactive sulfhydryl group available at the cysteinyl site. Whether or not the cysteinyl site in the thiolester is functionally significant in the binding and subsequent inhibition of sialyltransferase is unknown. Sialyltransferase also contains functionally significant sulfhydryl groups, which are required for binding the CMP-NeuAc donor (Baubichon-Cortay, H. *et al*, 1989; Datta & Paulson, 1995), and it may be these that are functionally significant in its interaction with α_1 macroglobulin. Clearly, further investigations are required to determine if the interaction involves a covalent linkage between the two molecules, and if so, whether this occurs through the cysteinyl residue in the thiolester of α_1 macroglobulin and the functionally significant sulfhydryl groups in sialyltransferase, or at other potential sites.

The determination of the mechanisms required for the trapping of sialyltransferase by α_1 macroglobulin will also provide information that will aid in the understanding of how sialyltransferase activity can be detected in rat serum, known to contain relatively high amounts of α_1 macroglobulin. The rat serum collected for studies here did not contain any detectable sialyltransferase activity. The isolation process used here may have caused a release of *in vivo* ligand-complexed forms of α_1 macroglobulin, freeing the macroglobulin for availability to trap

sialyltransferase. Alternatively, the isolation process may have caused an activation of any existing free α_1 macroglobulin creating a form capable of complexing sialyltransferase. Whatever the cause of creating α_1 macroglobulin into a form capable of inhibiting sialyltransferase, it is clear that in the serum isolated here, the quantity of available α_1 macroglobulin is sufficient to completely inhibit all sialyltransferase activity. The requirements for creating α_1 macroglobulin into a functioning sialyltransferase inhibitor are unknown, and elucidation of this will be most valuable.

The fact that sialyltransferase activity has been reported in rat serum, despite the known fact that the serum contains α_1 macroglobulin, shown here to be a potent inhibitor of the enzyme, does however raise a key concern. This concern is whether previous reported values of serum sialyltransferase activity are indicative of the true levels of circulating sialyltransferase. The detected sialyltransferase activity in rat serum may only be that portion of sialyltransferase which is free from α_1 macroglobulin, and may depend on the relative proportion α_1 macroglobulin which exists in the form that is capable of inhibiting sialyltransferase. This is also true for measurements of sialyltransferase activity in liver, since α_1 macroglobulin in constitutively expressed in a variety of tissues (Eggertsen *et al*, 1991). Furthermore, dependent on the determination of the specificity of the reaction found here, and the potential that other macroglobulins may be capable of similar functions, this consideration may have to be extended to activity measurements of other sialyltransferases in a variety of tissues.

Whether any interaction between Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) isolated from rat liver and rat α_1 macroglobulin occurs *in vivo*, is unknown. Sialyltransferase activity in serum has been reported to be elevated about five-fold during the acute phase response, rising from about 5 μU/ml, to 25 μU/ml by 48 hours after turpentine administration (Kaplan *et al*, 1983). Quantitation of sialyltransferase is based on its activity, which monitors any enzyme that is accessible to the substrates, but not any that may be sequestered in complexes with α_1 macroglobulin or other molecular species. Rat α_1 macroglobulin is constitutively expressed (Eggertsen *et al*, 1991), but also increases in rat serum during the acute phase response from approximately 1-2 mg/ml to approximately 3-4 mg/ml following adjuvant arthritis induction (Lonberg-Holm *et al*, 1987). Quantitation of α_1 macroglobulin here is by rocket immunoelectrophoresis. Whether this immunodetection method, or those used by others (Gordon, 1976) detects free or complexed α_1 macroglobulin, or both, is not known. The measurements are therefore relative to the specificity of the reactivity with the antibodies, and are also to the purity of the α_1 macroglobulin standards. Since the quantitation of both sialyltransferase and α_1 macroglobulin are relative to the methods and their potential limitations, it is not possible to make any conclusive statements about the true levels of these proteins *in vivo*. Investigations to determine absolute quantities of each protein, and the quantities that exist in complexed forms, would provide significant insight into the potential for the physiologic association

between sialyltransferase and α_1 macroglobulin, and the circumstances under which this may occur.

It is, however, clear that whatever the mechanism involved in the inhibition of sialyltransferase by α_1 macroglobulin, and whether this interaction occurs *in vivo*, or not, under the conditions used here galactosyltransferase (EC 2.4.1.38) is unaffected. Therefore sialyltransferase must contain functional groups capable of interaction with α_1 macroglobulin, which are either absent or inaccessible in galactosyltransferase. The fact that sialyltransferase and not galactosyltransferase is inhibited by α_1 macroglobulin also indicates that this reaction may have specificity amongst the glycosyltransferases. While α_1 macroglobulin has been found to inhibit Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) from rat liver, further investigation using other sialyltransferases may reveal that it could inhibit the family of sialyltransferases, or that it may have specificity even within this family. This is an intriguing possibility since Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) from rat liver is an acute phase reactant intimately involved in the host defense response. No other glycosyltransferases have been found to be acute phase reactants, including the galactosyltransferase tested here. Furthermore, Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) from rat liver is the only known member of the sialyltransferase family of enzymes which functions as an acute phase reactant. It may therefore be possible that a specific reaction between Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) from rat liver and rat α_1 macroglobulin may occur, and may be functionally significant in the host defense response.

Subsequent to the determination of the quantities of α_1 macroglobulin and sialyltransferase, both the free and complexed forms, and the determination of the mechanism(s) required for the activation of α_1 macroglobulin into its sialyltransferase inhibitory capacity, it should be possible to determine the potential physiologic, and pathophysiologic role of any *in vivo* interaction between these two molecular species. Although it has been reported that α_1 macroglobulin functions *in vivo* mainly to trap proteases (Tsuji *et al*, 1994), it may also have the ability to trap sialyltransferase, as it does other non-protease molecules, with the subsequent potential for delivery to various cell types, either for degradation or for utilization in these cells. Some evidence indicates that macroglobulin complexes can be dissociated (Borth *et al*, 1990), with ligand recovery occurring on interaction with activated target immunocompetent cells (Deby-Dupont *et al*, 1994; Reddy *et al*, 1989; Reddy *et al*, 1994). Thus, α_1 macroglobulin could act as a sialyltransferase trap to deliver the enzyme to specific cell types, with important implications for the sialylation of glycoproteins, particularly if the target cells were involved in the host defense response.

α_1 macroglobulin is a member of a superfamily of proteins, many of which are acute phase reactants, for example α_2 macroglobulin and the complement components. The family of macroglobulins are intimately involved in the mammalian defense system (James, 1980), for example binding soluble antigens, immune complexes, viruses (Pritchett & Paulson, 1989) and bacteria (Jonsson & Muller, 1994), as well as cytokines (James, 1990; LaMarre et al, 1991). They function to protect, to scavenge, to deliver, to regulate

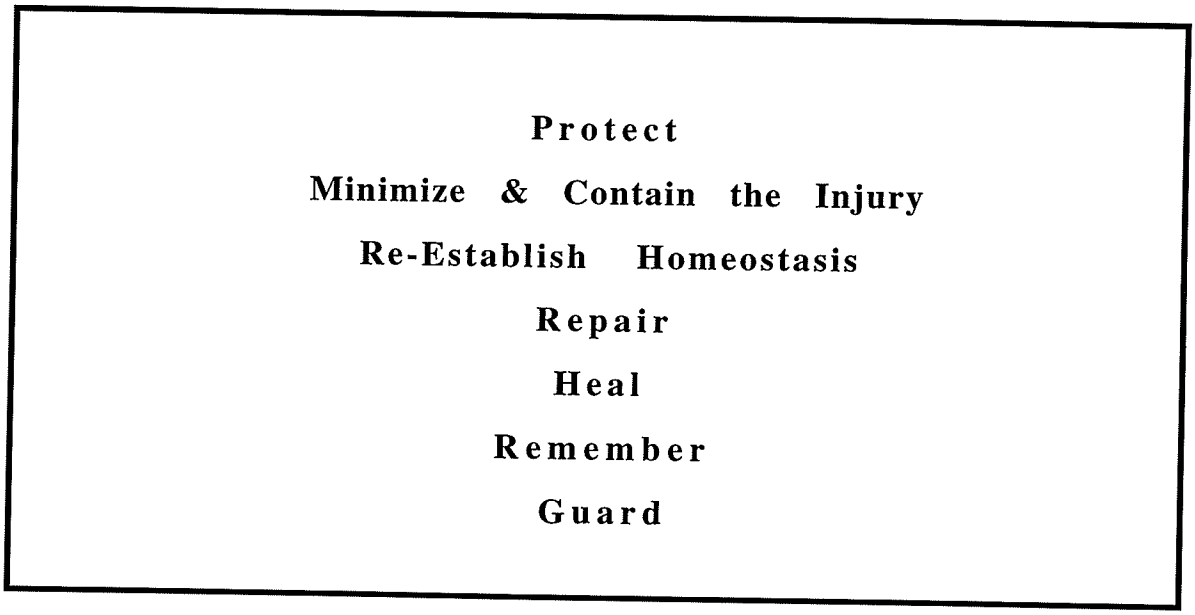
and modulate immune functions through their interactions with molecules, cells, tissues, and organisms. Based on the findings here that α_1 macroglobulin can function to inhibit Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1), it is clear that future studies on sialyltransferase as an acute phase reactant should not be limited to the studies on its regulation of synthesis, but should reach further to understand the interactions between the enzyme and other plasma proteins, and hence to potentially determine the purpose of Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1) from rat liver as an acute phase reactant. In general, all future investigations aimed at understanding the mechanisms involved in the acute phase response, should not be limited to studies of the mechanisms required for the regulation of the synthesis of these proteins, but must look beyond this to include the interactions between the reactants, and the subsequent impact on the entire process.

Whether the inhibition of sialyltransferase by α_1 macroglobulin is physiologically significant, the discovery and identification of this sialyltransferase inhibitor has other significant implications. α_1 macroglobulin could be used as a tool in studies on the biosynthesis of glycoproteins, specifically on the role of sialylation in the fate and function of glycoproteins. Elucidation of the mechanism and specificity of α_1 macroglobulin could potentially reveal that this reaction is specific for Galβ1-4GlcNAcα2-6 sialyltransferase (EC 2.4.99.1). Subsequently, sialyltransferase inhibitors with varying specificities could be designed using this particular interaction as the model foundation for the creation of a family of designed inhibitors. These inhibitors could be created from the functionally significant

portions of the α_1 macroglobulin, by excision or synthesis. Further modeling of inhibitors for specificity, potency, and half-life, could lead to the development of novel inhibitors, tailored specifically to meet the needs of particular studies. These would be invaluable as tools for investigations into the significance of sialylation at varying levels of complexity, for example at molecular, intracellular or intercellular levels, in extracellular interactions, in the directed and concerted development of various organisms, and in the interaction between organisms such as pathogens and hosts. Sialyltransferase inhibitors would be valuable tools for studies of the role of sialylation in normal physiology, and in various pathophysiologic conditions marked by altered states of sialylation, including numerous inflammatory conditions or imbalances, malignancy and metastasis. Ultimately, sialyltransferase inhibitors, such as the α_1 macroglobulin sialyltransferase inhibitor discovered here, or those thereby subsequently designed, could lead to the development of therapeutic agents for the use in any pathophysiologic conditions involving altered states of sialylation.

APPENDIX A

THE AIM OF THE HOST DEFENSE RESPONSE



THE HOST DEFENSE RESPONSE

TISSUE INJURY

Infection, Trauma, Neoplasia, Immunological Disorder...

↓

LOCAL REACTION

Release of Lysosomal Enzymes, Vasoactive Amines,
Arachidonate Acid Metabolites
Altered Vascular Permeability
Exudation of Plasma Fluids & Solutes
Leukocyte Migration & Phagocytosis
Activation of Leucocytes, Fibroblasts, Endothelial Cells
Release of IL-1, IL-6, TNFα, IFNγ,...

↓

SYSTEMIC REACTION

Further Generation of Arachidonate Metabolites
Further Activation of Phagocytic Cells
Increased Circulating Granulocyte Pool
Release of Hormones and/or Cytokines

Fever
Pain
Immune System
Endocrine System
Hematopoiesis
Metabolic Changes in Various Tissues
Changes in the Metabolism of the Liver

THE ACUTE PHASE RESPONSE

THE ACUTE PHASE RESPONSE

Systemic Response

Influenced by, and Interacts with the Neurologic, Endocrine, and Immune Responses

It involves dramatic changes in the synthesis and secretion of plasma proteins by the liver, called the

ACUTE PHASE REACTANTS

Collectively these changes help to minimize damage and to repair, with the purpose being to re-establish homeostasis and to maintain the integrity of the host

THE ACUTE PHASE REACTANTS

Albumin
α_1 Acid Glycoprotein
α_1 Antichymotrypsin
α_2 Antiplasmin
α_1 Inhibitor III
α_1 Lipoprotein
α_1 Macroglobulin
α_2 Macroglobulin
α_1 Proteinase Inhibitor
C Reactive Protein
Ceruloplasmin
Complement Components, C3, C4, and C5
Cysteine Proteinase Inhibitor
Fibrinogen
Haptoglobin
Hemopexin
Inter α Antitrypsin
Lipopolysaccharide Binding Protein
Prealbumin
Prekallikrein
Prothrombin
Serum Amyloid A
Serum Amyloid P
$\alpha 2 \rightarrow 6$ Sialyltransferase
Transferrin

THE RESPONSE OF THE ACUTE PHASE REACTANTS

(De Beer *et al*, 1982; Fey & Gauldie, 1990; Whicher *et al*, 1989)

	Plasma	Concentration	(mg/ml)
Group	**Species**	**Normal**	**Injury**
1. Concentration increases by ~ 50%			
α_1 macroglobulin	Rat	1.8	2.2-3.6
C3	Man	0.55-1.2	2.2-3.0
C4	Man	0.2-0.5	1.0
2. Concentration increases two- to four-fold			
α_1 AGP	Man	0.5-1.4	3.0
	Rat	2.8	5.3
α_1 antitrypsin	Man	1.0-2.0	7.0
α_1 antichymotrypsin	Man	0.3-0.6	3.0
C reactive protein	Rat	0.3-0.6	0.9
Fibrinogen	Man	2-4.5	10
	Rat	3.2	5.6
Haptoglobin	Man	1-3	6.0
	Rat	1.3	2.6
3. Concentration increases 100- to 1000-fold			
CRP	Man	0.00007-0.008	0.4
	Rabbit	0.02-0.1	2-3
Serum amyloid A	Man	0.001-0.03	2.5
	Mouse	$\leq$ 0.01	$\geq$ 0.2
Serum amyloid P	Mouse	$\leq$ 0.04	$\leq$ 0.2
α_2 macroglobulin	Rat	$\leq$ 0.04	$\geq$ 2-8
4. Negative acute phase reactants			
$\alpha_1 I_3$	Rat	7-8	1-2

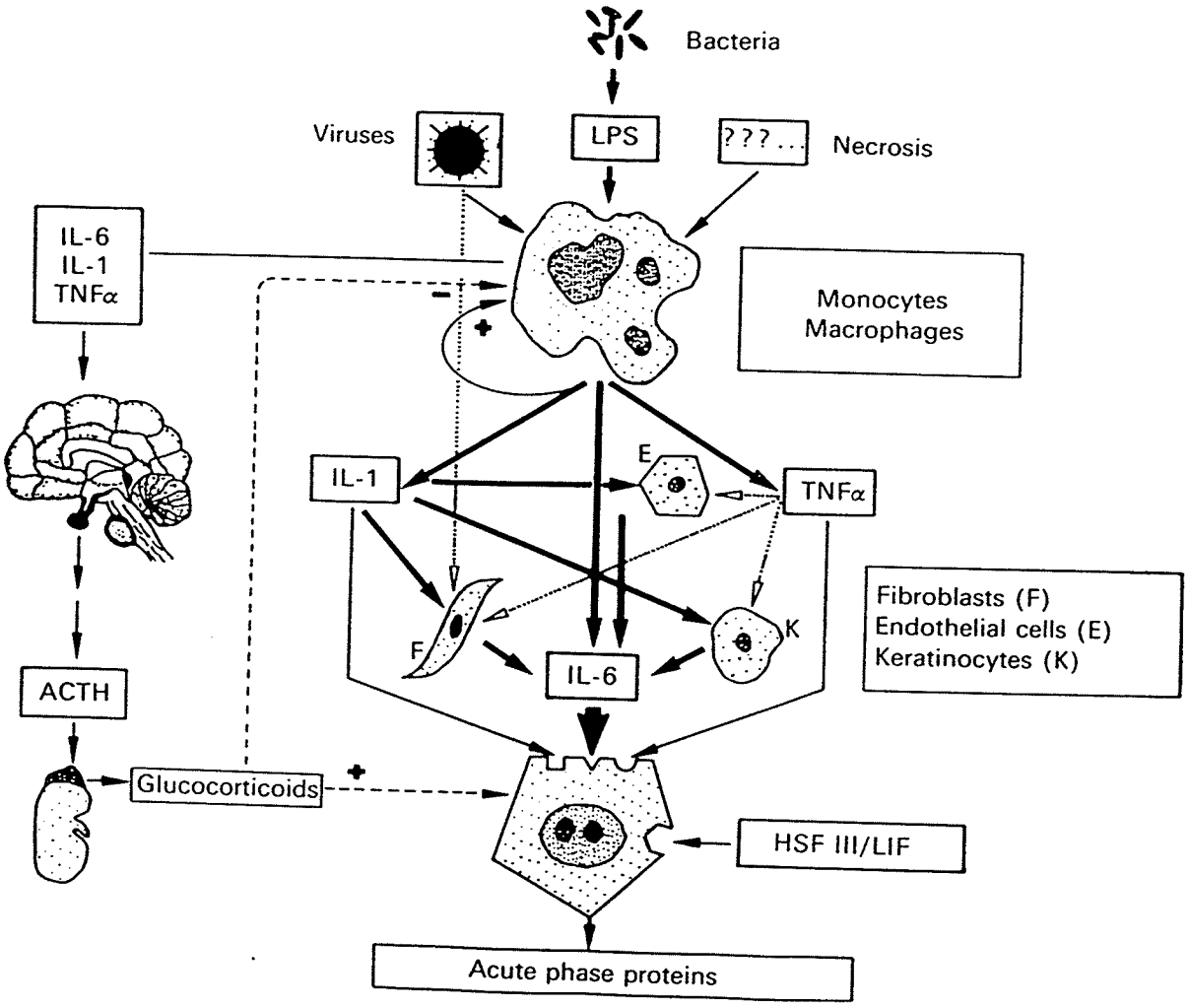
THE PURPOSE OF THE ACUTE PHASE REACTANTS

(Gauldie *et al*, 1985; Thompson *et al*, 1992; Whicher *et al*, 1989)

ACUTE PHASE PROTEIN	POSTULATED FUNCTION(S)
Albumin	Transport protein
α_1 acid glycoprotein	Promotes fibroblast growth; interacts with collagen; transport protein; expressed on lymphocyte cell membrane surfaces
α_1 antichymotrypsin	Protease inhibitor; cathepsin G inhibitor; binds to new elastic fibres; inhibits remodelling by leucocytic proteases
α_1 antitrypsin	Protease inhibitor; deposits on newly formed elastic fibres
α_1 macroglobulin	Broad spectrum protease inhibitor
α_2 macroglobulin	Broad spectrum protease inhibitor; transports growth factors and cytokines
C reactive protein	Binds phosphorylcholine in all membranes with complement activation and opsonization; interacts with T and B lymphocytes; scavenges DNA
Ceruloplasmin	Transport protein; O_2^- scavenger
Complement Components	Opsonisation; chemotaxis, mast cell degranulation
Fibrinogen	Coagulation; clotting, and formation of matrix for repair
Haptoglobin	Binds and removes hemoglobin; inhibits cathepsins B, H, L
Kallikrein	Promotes vascular permeability and vasodilation
Serum amyloid A	Scavenges and clears cell-membrane derived cholesterol from macrophage
Transferrin	Transport protein

THE REGULATION OF THE ACUTE PHASE RESPONSE

(Copied from Heinrich *et al*, 1990)



ACUTE PHASE PROTEINS ARE SECRECTED FROM HEPATOCYTES. ACTH, adrenocorticotrophic hormone; HSF, hepatocyte stimulating factor; IL-1, interleukin-1; IL-6, interleukin-6; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide.

APPENDIX B

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MINI REVIEW

SIALYLTRANSFERASE: A NOVEL ACUTE-PHASE REACTANT

J. C. JAMIESON,* G. MCCAFFREY† and P. G. HARDER
Department of Chemistry, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2
(Tel. 204 474 9253; Fax 204 275 0905)

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Abstract—1. Proteins that are released into the circulation in elevated amounts in injured mammals are referred to as acute-phase reactants. Most are liver synthesized glycoproteins of the secretable type. However, Gal-β(1→4)-GlcNAc-α(2→6)-sialyltransferase (EC 2.4.99.1) is a novel acute-phase reactant since it is a Golgi membrane-bound enzyme rather than a secretable glycoprotein.

2. The role of glucocorticoids and cytokines in the control of synthesis and expression of acute-phase glycoproteins, including sialyltransferase, is discussed.

3. The acute-phase behaviour of Gal-β(1→4)-GlcNAc-α(2→6)-sialyltransferase is dependent on the release of the enzyme from the Golgi in the acute-phase state. The mechanism of release of a catalytically active form of the enzyme is described.

INTRODUCTION

Inflammation in humans and experimental animals, caused by a variety of agents including chemical irritants, bacterial infection and neoplastic disease, results in significant biochemical and physiological changes known as the acute-phase response (Koj, 1974; Kaplan *et al.*, 1983; Jamieson *et al.*, 1983). A major change is an increase in the levels of circulating glycoproteins; these are usually referred to as the acute-phase reactants (Koj, 1974). The acute-phase reactants that are responsible for most of the increase in protein-bound carbohydrate in inflammation are secretable glycoproteins like α_1-acid glycoprotein, haptoglobin and fibrinogen, which typically increase by 5–10-fold. These glycoproteins are present in normal serum in milligram quantities. Other acute-phase reactants, like *C*-reactive protein and serum amyloid A, are present in trace quantities, but these often increase by more than 100-fold in inflammation (Kushner and Feldman, 1978; MacIntyre *et al.*, 1985). Regulation of acute-phase protein synthesis occurs mainly as a result of increased transcriptional events involving the action of glucocorticoids coupled with specific cytokines, particularly interleukin 6 (e.g. Harder *et al.*, 1990; see Heinrich *et al.*, 1990 for a review). A common feature of most major acute-phase reactants is that they are liver-synthesized glycoproteins of the secretable type which pass into blood in larger quantities in inflammation. However, there has been considerable recent interest in another type of acute-phase reactant that does not fall into this category. The enzyme Gal-β(1→4)-GlcNAc-α(2→6)-sialyltransferase was first shown to be an acute-phase reactant in 1983 (Kaplan *et al.*, 1983). The enzyme attaches NeuAc to terminal positions of the oligosaccharide chains of *N*-linked glycoproteins. The enzyme is bound to the luminal side of the Golgi membrane, but during the acute-phase state the enzyme is released into the extracellular space in a catalytically active form. Recent work in our laboratory and by others has thrown some light on control of synthesis of sialyltransferase during the acute-phase response as well as the mechanism of release of the enzyme from the membrane-bound form.

Factors influencing expression of Gal-β(1→4)-GlcNAc-α(2→6)-sialyltransferase

There has been considerable recent interest in identifying factors that can control the expression of the sialyltransferase gene. The approach has been to utilize primary hepatocytes or a variety of hepatoma cell lines grown in culture. Primary hepatocytes were found to synthesize and secret more sialyltransferase when challenged with a cytokine-containing preparation in presence of glucocorticoids (Woloski *et al.*, 1986). The response of the cells was very similar to the effect of cytokines and glucocorticoids on synthesis and secretion of fibrinogen and other acute-phase reactants from both primary hepatocytes and hepatoma cell lines (e.g. Baumann *et al.*, 1983; Fuller *et al.*, 1985; VanDijk *et al.*, 1986; see Jamieson *et al.*, 1987, for a review). The role of cytokines, particularly interleukins 1 and 6, coupled with glucocorticoid action in the acute-phase response has recently been

*To whom all correspondence should be addressed.
†Current address: Department of Medicine and Biochemistry, Washington University School of Medicine, St Louis, MO, U.S.A.

Abbreviations—NeuAc; *N*-acetylneuraminic acid; CMP-NeuAc; CMP-N-acetylneuraminic acid; GlcNAc; *N*-acetylglucosamine.

reviewed (Heinrich *et al.*, 1990). Glucocorticoid treatment of FAZA hepatoma cells grown in culture produced a 6-fold increase in the amount of sialyltransferase secreted from the cells (Harder *et al.*, 1990); the effect was magnified in the presence of phorbol ester. The effect of glucocorticoids and cytokines appears to be directly linked to increased expression of mRNA for acute-phase reactants including sialyltransferase (Baumann *et al.*, 1984, 1990, 1991; Fey *et al.*, 1991; O'Hanlon *et al.*, 1989; Richards *et al.*, 1992; VanDijk *et al.*, 1986; Wang *et al.*, 1989, 1990). However, increased expression of the enzyme protein cannot in itself explain the acute-phase response since the enzyme must be secreted in increased quantities to qualify as an acute-phase reactant; this aspect of the acute-phase response of the enzyme is discussed below.

Mechanism of release of Gal-β(1→4)-GlcNAc-α(2→6)-sialyltransferase from the Golgi during the acute-phase response

Although increased expression of sialyltransferase protein can be explained by increased levels of mRNA in the cell, it is clear that the behaviour of sialyltransferase as an acute-phase reactant depends on a release mechanism which allows the active enzyme to pass into the extracellular space. Other liver enzymes (Jamieson *et al.*, 1987) are elevated in the liver in inflammation, including other glycosyltransferases (Lombard *et al.*, 1980), but sialyltransferase appears to be the only one that is secreted in a catalytically active form, implying that it may have a specific function to play as an active enzyme in the extracellular space in the inflammatory process. The mechanism of release of sialyltransferase from the Golgi during the acute-phase response has now been elucidated. Sialyltransferase has been purified (Weinstein *et al.*, 1982a,b) and a sequence has been elucidated from the cDNA for the enzyme (Weinstein *et al.*, 1987; Paulson and Colley, 1989; Paulson *et al.*, 1989). The enzyme contains three polypeptide domains (Weinstein *et al.*, 1987; Paulson *et al.*, 1989). There is a total of 403 amino acids, the major portion being the catalytic unit which is suspended from the intraluminal face of the Golgi by a linker or stem region containing 35 amino acids; this is attached to a membrane spanning domain containing the *N*-terminus which has a nine amino acid cytoplasmic tail and a 17 amino acid signal anchor region (Weinstein *et al.*, 1987; Colley *et al.*, 1989, 1992). The anchor linker domain has been found to be critical for the retention of sialyltransferase in the Golgi membrane (Colley *et al.*, 1992; Wen *et al.*, 1992). Thus, the catalytic unit is favourably oriented in the Golgi to attach sialic acid to secretable glycoproteins that are *in transit* through the luminal channels of the Golgi complex on their way out of the cell. Using an *in vitro* system in which Golgi membranes were disrupted with ultrasonic vibrations to expose the sialyltransferase to the incubation medium, it was found that it was possible to study factors that were required for the release of a catalytically active form of the enzyme from the membrane (Lammers and Jamieson, 1988, 1990; McCaffrey and Jamieson, 1993). Table 1 shows that sialyltransferase could be released from the disrupted membrane by incubating at a reduced pH. Over 70% of the Golgi activity was typically released after 30 min incubation at pH 5.6 (Table 1). Similar behaviour was found with the mouse and guinea-pig Golgi enzymes which also behave as acute-phase reactants (Lammers and Jamieson, 1986; Lammers and Jamieson, 1990; McCaffrey and Jamieson, 1992) although the pH for maximum release in these two species was found to differ from the rat enzyme (Table 1). There was also a difference in M_r between rat, mouse and guinea-pig enzymes as shown in Table 1. The cleavage of the rat enzyme resulted in an M_r loss of 7000, whereas the mouse and guinea-pig showed a reduction in M_r of 11,000 and 4000, respectively, when released from the membrane (Lammers and Jamieson, 1988, 1990). The release of sialyltransferase from the Golgi membrane in all

Table 1. Conditions for the release of Galβ1-4GlcNAcα2-6sialyltransferase from disrupted Golgi membranes*

Species	Optimum pH for release	Sialyltransferase released (%)	M_r of sialyltransferase	
			Bound	Released
Rat	5.6	72.0	49,000	42,000
Mouse	4.6	85.5	49,000	38,000
Guinea-pig	5.2	71.7	42,000	38,000

*Golgi membranes were sonicated for 30 sec and incubated at reduced pH for 30 min (see Lammers and Jamieson, 1988, 1990, for details). Particulate material was sedimented and sialyltransferase activity in the supernatant fraction was assayed using the standard procedure (Lammers and Jamieson, 1988). Sialyltransferase is measured in units of enzyme activity where one unit is defined as equal to the transfer of 1 pmol of NeuAc from CMP-NeuAc to the rat asialoα_1 acid glycoprotein acceptor/hr/mg protein. The pH values given were those where maximum release of enzyme activity was observed; these values are given in % terms relative to the total Golgi sialyltransferase activity. The total units of activity in the Golgi membranes in the three species were: rat, 3177; mouse, 2800; and guinea-pig, 3250. Values for M_r were determined by immunoblot analysis as previously described (Lammers and Jamieson, 1988). The experiments were carried out on animals suffering from inflammation for 36 hr, 72 hr and 96 hr for rat, mouse and guinea-pig, respectively. These times were where the acute-phase response of sialyltransferase was at a maximum (Kaplan *et al.*, 1983; Lammers and Jamieson, 1986).

Table 2. Effect of the presence of proteinase inhibitors on the release of sialyltransferase from sonicated rat Golgi membranes*

Additions	Sialyltransferase activity (Units)
None	2830 ± 250
Leupeptin (10^{-3} M)	2895 ± 242
Antipain (10^{-3} M)	2928 ± 234
Aprotinin (10^{-3} M)	2870 ± 144
Bestatin (10^{-3} M)	3000 ± 207
Pepstatin A (10^{-7} M)	250 ± 50
Dimethylsulfoxide	2195 ± 165
Soybean trypsin inhibitor (100 μg/ml)	3376 ± 271

*Experiments were as described in Table 1 with sonicated Golgi membranes from 36-hr inflamed rats. Inhibitors were added at the concentrations indicated prior to incubation at pH 5.6. At the end of incubation, pH was adjusted upwards to 7.0 for sialyltransferase assay. Results are expressed in units of enzyme activity as defined in the footnote to Table 1. The dimethylsulfoxide was a control since pepstatin is insoluble in water; it was added as a stock solution in dimethylsulfoxide. The final dimethylsulfoxide concentration was 1%. Results represent means from three experiments ± SD.

three species under the conditions described in Table 1 could be blocked only by pepstatin A, a potent inhibitor of the aspartate proteinase cathepsin D; the results of these studies are given for the rat in Table 2. Similar results were found with mouse and guinea-pig Golgi. Subsequent work, using an immunological approach employing antiserum containing antibodies against lysosomal cathepsin D, strongly suggested that the lysosomal enzyme was responsible for the proteolytic clipping of the linker region of sialyltransferase in all three species (Lammers and Jamieson, 1988, 1990). The role of an acidic proteinase in the clipping of sialyltransferase in rats and mice during the acute-phase response has been confirmed in whole cell experiments using liver slices (Lammers and Jamieson, 1989; McCaffrey and Jamieson, 1993) and under *in vivo* conditions in the mouse (McCaffrey and Jamieson, 1993). The model that is emerging predicts that there is an alteration in trafficking of lysosomal cathepsin D during the acute-phase state in such a way that the proteinase is brought into contact with the sialyltransferase, resulting in cleavage of the catalytic unit from the membrane-bound enzyme. The M_r of 7000 for the clipped portion of sialyltransferase in the rat (Table 1) would indicate a cleavage site at some position between amino acid residues 60 and 70 (the numbering of amino acids is based on the complete amino acid sequence of the linker anchor containing the form of sialyltransferase as deduced from the nucleotide sequence of the rat cDNA (see Weinstein *et al.*, 1987). This correlates well with the predicted terminal Ser-64 for purified rat sialyltransferase which has lost its linker anchor domain (Weinstein *et al.*, 1987). However, it remains to be determined if cathepsin D is responsible for cleavage of the membrane-bound enzyme between residues Asn 63 and Ser 64 of the rat enzyme. This would confirm that the action of cathepsin D in clipping sialyltransferase is likely to be a key physiological event in the acute-phase state. Since the sequences of the mouse and guinea-pig enzymes are unknown it is not possible, at this time, to specify the cleavage sites for these two enzymes. However, in view of the M_r values found for the clipped portion of mouse and guinea-pig enzymes, it is likely that a similar mechanism exists to explain the acute-phase response of sialyltransferase in rat, mouse and guinea-pig.

THE FUTURE

Although there have been significant advances in our understanding of the control of synthesis and mechanism of release of Gal-β(1→4)-GlcNAc-α(2→6)-sialyltransferase from the Golgi two key questions remain to be answered. Is sialyltransferase the only Golgi enzyme that can be proteolytically clipped from the membrane in the acute-phase state? So far, sialyltransferase is the only one that has been identified, but it is conceivable that there will be other examples in the future. In addition, it is also possible that other Golgi enzymes are released, but they may have escaped detection because of the destruction of catalytic activity during proteolytic clipping.

What is the function of sialyltransferase in the extracellular space? It is unlikely that α2-6 sialyltransferase is functional in blood because of the low level of CMP-NeuAc in the circulation (Kaplan *et al.*, 1984). A more likely scenario is that sialyltransferase is utilized at the site of injury of glycosylation reactions, but there is no evidence for this at this time. Studies on the role of sialyltransferase in the circulation in inflammation will clearly be a high priority for the future.

Sialyltransferases are key enzymes in glycosylation since sialic acid imparts a negative charge to macromolecules. This is important for the function of the molecule; a good recent example of the importance of sialic acid in an oligosaccharide is the cellular recognition molecule sialyl-Lex which is found on neutrophil, monocyte and some tumour cell surfaces. Sialyl-Lex acts as the ligand for recognition between the selectin family of adhesion receptors found on endothelial cells and lymphocytes (Polley *et al.*, 1991; Berg *et al.*, 1992; Foxall *et al.*, 1992; Majuri *et al.*, 1992). Although the sialic acid is present in sialyl-Lex in α2-3 linkage it does point out the importance of sialic acid in this type of structure in a molecule that is involved in the inflammatory process. Clearly, sialic acid and the sialyltransferases have an important role to play in glycobiology which should lead to a greater understanding of cellular recognition reactions and glycoprotein function.

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APPENDIX C

Reprinted from the International Journal of Biochemistry, volume 22, Harder, G., Woloski, B.M.R.N.J., and Jamieson, J.C., "Stimulation of Release of Galβ1-4GlcNAcα2-6 Sialyltransferase from the FAZA Hepatoma Cell Line by Dexamethasone and Phorbol Ester," pages 11-14, ©1990, with kind permission from Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.

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STIMULATION OF RELEASE OF Galβ1-4GlcNAcα2-6 SIALYLTRANSFERASE FROM THE FAZA HEPATOMA CELL LINE BY DEXAMETHASONE AND PHORBOL ESTER

G. HARDER and J. C. JAMIESON*
Department of Chemistry, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2
[*Tel.* (204) 474-9321; *Fax* (204) 269-6629]

B. M. R. N. J. WOLOSKI
ABI Biotechnology Inc., Winnipeg, Manitoba, Canada

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Abstract—1. Galβ1-4GlcNAcα2-6 sialyltransferase was assayed in FAZA hepatoma cells and the cell culture medium following growth of cells in presence of dexamethasone and phorbol ester.

2. There was about a seven-fold increase in sialyltransferase activities in cells and medium in presence of dexamethasone with the maximum effect occurring at 10^{-6}–10^{-7} M dexamethasone.

3. The presence of 10^{-6} M phorbol ester in the culture medium increased sialyltransferase activities in cells and medium by *ca* 40% over the values found with dexamethasone alone.

4. The use of the FAZA hepatoma cell line for studies on sialyltransferase is compared with the primary hepatocyte system reported on earlier (Woloski *et al.*, 1986).

INTRODUCTION

Inflammation in humans and experimental animals results in a variety of biochemical and physiological changes known as the acute phase response (Koj, 1974). One of the main changes is an increase in the circulating levels of certain serum glycoproteins which are usually referred to as the acute phase reactants (Jamieson, 1983). Recently, we have characterized Galβ1-4GlcNAcα2-6 sialyltransferase as a new type of acute phase reactant (Lammers and Jamieson, 1988). Sialyltransferase is a Golgi membrane bound enzyme which is involved in the attachment of NeuAc in α2-6 linkage to terminal positions of oligosaccharide chains of glycoproteins of the N-linked type. The enzyme is released from the membrane by the action of a cathepsin D-like proteinase which cleaves the catalytic site of the enzyme from a membrane anchor, and this allows the enzyme to exit the cell (Lammers and Jamieson, 1988). The cathepsin D-like proteinase is believed to be the lysosomal enzyme which is diverted into the Golgi complex during the acute phase response, and acts to release sialyltransferase (Lammers and Jamieson, 1988).

In a previous publication we described the use of a primary hepatocyte system to study factors that can control the release of sialyltransferase (Woloski *et al.*, 1986). It was found that hepatocyte stimulating factor (HSF), a monokine preparation, could stimulate the release of sialyltransferase from the hepatocyte by about two-fold; secretion of fibrinogen, another acute phase reactant, was stimulated about five-fold under the same conditions. However, the primary hepatocyte system suffered from the disadvantages that it can be maintained for only relatively short times and is technically difficult to reproduce. Recent studies have shown that the FAZA hapatoma cell line can be induced to synthesize a variety of acute phase reactants, including fibrinogen, on treatment with monokines (Ott *et al.*, 1984; Evans *et al.*, 1987), but the release of sialyltransferase from this cell line has never been examined. In addition, the FAZA cell line has been shown to respond to the presence of phorbol ester which mimics the action of monokines (Evans *et al.*, 1987). In this work we have examined the conditions needed for the maximum release of sialyltransferase from the FAZA cell line. It was found that dexamethasone could stimulate release of sialyltransferase from the cells by about seven-fold and the presence of phorbol ester caused an additional stimulation. The results show that the FAZA cell line is a good permanent cell system to study factors that affect the release of sialyltransferase from the hepatoma. The work provides us with a new cell system for use in studies on sialyltransferase as an acute phase reactant.

*To whom all correspondence should be addressed.

Abbreviations: CMP-NeuAc, CMP-*N*-acetylneuraminic acid; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate (phorbol ester).

MATERIALS AND METHODS

Materials

CMP-[4,5,6,7,8,9-^{14}C] NeuAc (247 mCi/mmol) was from New England Nuclear Corp., Lachine Quebec, Canada.

Streptomycin sulfate, penicillin-G, dexamethasone, insulin and Triton X-100, Sigma Chemical Co., St Louis, Mo. Delbecco's modification of Eagle's Medium (DMEM), Hanks balanced salt solution, and Hams F12, Flow Laboratories, Rockville, Md; TPA, Calbiochem, San Diego, Calif.

Methods

Cell culture. The FAZA hepatoma cell line was that described by Woloski and Fuller (1988). Cells were cultured in a 1:1 mixture of DMEM and Ham's F12, with 100 mg streptomycin sulfate and 6 mg/l^{-1} penicillin and 10% fetal bovine serum. Cells were incubated in a humid atmosphere of 5% CO_2 at 37°C. Cells were subcultured at 10^5 cells per plate for 24 hr to allow the cells to adhere to the plates. Cells were then washed with warm medium to remove non-adherent cells and adherent cells were then grown in medium containing insulin, dexamethasone and TPA as appropriate (Woloski and Fuller, 1988). Cells were grown for up to 80 hr and medium was aspirated and assayed for sialyltransferase. Cells were washed with 1.0 ml of PBS and scraped from the plates with a fresh 1.0 ml sample of PBS (Spearman *et al.*, 1987). Cell suspensions were sonicated for 10 sec at 60 W using a 1 mm dia probe in an Artek sonic dismembrator as before (Spearman *et al.*, 1987) and assayed for sialyltransferase.

Sialyltransferase assay. Sialyltransferase was assayed based on methods described previously (Kaplan *et al.*, 1983). The standard assay contained 250 μg asialo-α_1-acid glycoprotein as acceptor, 7.5 mM imidazole buffer, pH 7.0 and 5 nmol. CMP-[^{14}C]NeuAc adjusted to 4 nCi/nmol. Enzyme was in the form of up to 100 μl culture medium or hepatocyte homogenates; 1% Triton X-100 was present in the sialyltransferase assay of the homogenates (Woloski *et al.*, 1986). In all assays, conditions were such that product formation was linear with time and amount of enzyme protein used. Incubation time was for up to 2 hr as appropriate. Results are expressed as units of enzyme activity where 1 U represents the transfer of 1 pmol. NeuAc from CMP-NeuAc to acceptor protein per hr as before (Kaplan *et al.*, 1983; Woloski *et al.*, 1986; Lammers and Jamieson, 1988). The specificity of the sialyltransferase released from hepatocytes was determined by an inhibition assay utilizing antiserum raised against Galβ1-4GlcNAcα2-6 sialyltransferase (Woloski *et al.*, 1986). Briefly, these experiments involved preincubation with up to 2 μl antiserum for 45 min prior to assay for sialyltransferase; controls utilized normal rabbit serum. In all medium samples assayed it was found that preincubation with antiserum caused >90% inhibition of enzyme activity showing that the enzyme activity being assayed was the α2-6 sialyltransferase. Protein was assayed by the BioRad method as described before (Spearman *et al.*, 1987).

RESULTS

Effect of dexamethasone and TPA on sialyltransferase in hepatocytes and culture medium

Figure 1 shows a typical growth curve for FAZA hepatoma cells grown in culture. Cells were subcultured at *ca* 10^5 cells per plate and incubated for 24 hr in medium in absence of insulin or dexamethasone to allow the cells to attach to the plates. Cells were then washed and adherent cells were grown in culture medium in presence of insulin with added dexamethasone or TPA as appropriate (Woloski and Fuller, 1988; see Materials and Methods). Cell protein and cellular and medium sialyltransferase activities were then monitored as a function of culture time. Figure 1 shows a typical growth curve obtained in presence of 10^{-7} M dexamethasone; similar growth curves were obtained in absence of dexamethasone. Cells

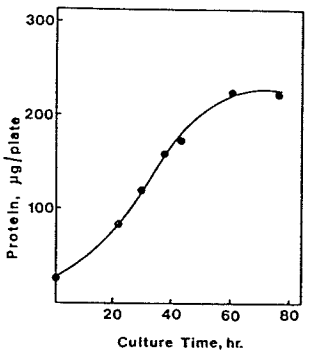

Fig. 1. Growth curve for FAZA cells grown in culture in presence of 10^{-7} M dexamethasone. Cells typically reached confluence between 40–50 hr of growth, but by 60 hr cells were detaching from the plates (see Materials and Methods). Results represent means from 4–6 determinations with reproducibility of *ca* ± 12%.

typically reached confluence after *ca* 40 hr, but by *ca* 60 hr cells were no longer viable and were detaching from the plates. Sialyltransferase activities in the cells and release of the enzyme into the medium was found to be sensitive to the presence of dexamethasone. Dexamethasone at 10^{-6}–10^{-7} M caused maximum sialyltransferase activities to appear in the cells and medium. Figure 2 shows the results from experiments in which sialyltransferase was assayed in cells and medium in presence of 10^{-7} M dexamethasone as a function of culture time. Sialyltransferase activities increased substantially in the cells in presence of dexamethasone with the maximum increase occurring at confluence where activities were increased *ca* six-fold compared to cells cultured in absence of dexamethasone (Fig. 2, panel A). Sialyltransferase activities in the cells decreased rapidly after confluence most likely due to cell death. Release of sialyltransferase into medium was rapid and fairly linear until the cells achieved confluence. The presence of dexamethasone resulted in about a

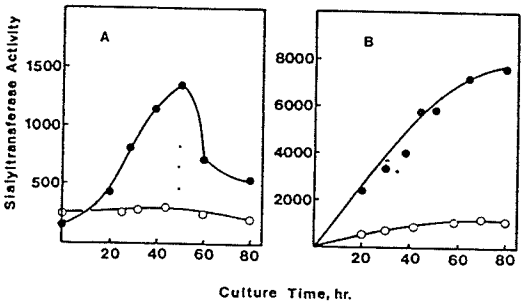

Fig. 2. Effect of 10^{-7} M dexamethasone on sialyltransferase activity in FAZA cells and release of sialyltransferase into culture medium as a function of culture time. Panel A, sialyltransferase activity in cells: ●, in presence of dexamethasone; ○, in absence of dexamethasone. Panel B, sialyltransferase activity in culture medium: ●, in presence of dexamethasone; ○, in absence of dexamethasone. Sialyltransferase activity is expressed as units of enzyme activity per mg cell protein (see Materials and Methods). Results represent means from 4–6 determinations with reproducibility of ±10%.

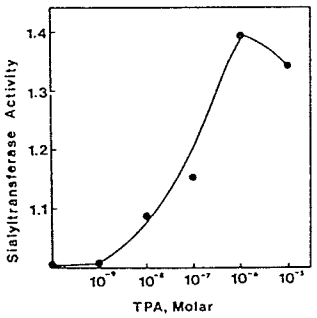

Fig. 3. Effect of TPA on the release of sialyltransferase into the culture medium. FAZA cells were cultured to confluence (see Fig. 1) in presence of 10^{-7} M dexamethasone and 10^{-5}–10^{-9} M TPA. Results are expressed as ratios of sialyltransferase activities in the medium in presence of dexamethasone and TPA compared to sialyltransferase activities in presence of dexamethasone alone. Results were from three experiments with reproducibility of ±12%.

seven-fold increase in medium sialyltransferase at confluence (Fig. 2, panel B).

The synthesis of other acute phase reactants by the FAZA cell line has been shown to be stimulated by the presence of TPA which mimics the effect of monokines (Evans *et al.*, 1987). Figure 3 shows that 10^{-5}–10^{-7} M TPA caused a further stimulation of release of sialyltransferase into medium beyond that found in presence of dexamethasone alone.

DISCUSSION

Rat Galβ1-4GlcNAcα2-6 sialyltransferase is a new type of acute phase reactant which is normally membrane bound at the luminal side of the *trans* Golgi membrane. However, during the acute phase response a major part of the enzyme containing the catalytic site is cleaved from the membrane by a cathepsin D-like proteinase (possibly the lysosomal enzyme) and passed into the extracellular space explaining why this enzyme behaves as an acute phase reactant (Lammers and Jamieson, 1988). In a previous study we showed that sialyltransferase was capable of being synthesized in a primary hepatocyte system in presence of dexamethasone (Woloski *et al.*, 1986). Moreover, there was stimulation of synthesis and secretion of the enzyme on treatment of the cells with HSF, a monokine preparation isolated from peripheral blood monocytes (Woloski *et al.*, 1986). It has been shown that other factors variously described as interferon β-2 (IFN-2), B-cell stimulating factor 2 (BSF-2) and hybridoma growth factor (HGF) are similar or identical to HSF and the term interleukin-6 (IL-6) has now been accepted as a suitable description of the polypeptide that is responsible for stimulation of acute phase reactant synthesis in the hepatocyte (Gauldie *et al.*, 1987; Sehgal *et al.*, 1987). It has been shown that the IL-6 can stimulate the synthesis of acute phase reactants in the FAZA cell line and that this effect can be mimicked by phorbol ester (Evans *et al.*, 1987; Baumann *et al.*, 1988). This makes the FAZA cell line particularly useful for studies on the synthesis of acute phase reactants since it is a permanent cell line which overcomes many of the difficulties associated with experimental variability found with the primary hepatocyte system used earlier (Woloski *et al.*, 1986).

The synthesis of sialyltransferase in the FAZA cell line was stimulated about seven-fold by the presence of dexamethasone at 10^{-7} M. This is in agreement with our own work (Woloski *et al.*, 1986) and that by van Dijk *et al.* (1986) who showed that synthesis and release of sialyltransferase from primary hepatocytes was dependent on the presence of 1 μM dexamethasone. In a more recent study by Wang *et al.* (1989) using the rat liver hepatoma cell line H35, it was found that exposure to 1 μM dexamethasone was sufficient to cause a 3–4-fold enrichment of sialyltransferase mRNA; however, the presence of cytokines did not stimulate sialyltransferase mRNA levels further. In the studies of Wang *et al.* (1989) only sialyltransferase mRNA and hepatocyte enzyme activities were measured, but the release of sialyltransferase activity into the culture medium was not followed. The current studies show that although phorbol ester caused minimal stimulation of sialyltransferase activity in the hepatocytes beyond that found on treatment with dexamethasone as described by Wang *et al.* (1989), the release of the enzyme from the hepatocytes into the culture medium was stimulated further.

The results obtained in this work show that FAZA hepatoma cells behave in a comparable way to the primary hepatocyte system described earlier (Woloski *et al.*, 1986). Sialyltransferase is stimulated in both cell lines by the presence of dexamethasone and synthesis and release into medium can be stimulated further by the action of a monokine in the case of the primary cell line, or by phorbol ester which has been shown to mimic some actions of monokines in the FAZA cell line. The FAZA cell line would appear to be a preferable alternative to the primary hepatocyte system for studies on sialyltransferase as an acute phase reactant since it can be maintained in culture over long time periods. Also, the seven-fold stimulation of sialyltransferase in FAZA cells and medium is greater than the two–three-fold stimulation of sialyltransferase found with the primary hepatocyte system. As indicated earlier, sialyltransferase only behaves as an acute phase reactant because of the cleavage of the catalytic site from the Golgi membrane anchor so that an important aspect of study as far as this enzyme is concerned is to do with factors that can control the release of the enzyme from the hepatocyte. This would include studies on the cathepsin D-like enzyme that releases sialyltransferase as well as factors such as intra-Golgi pH and the importance of the mannose-6-phosphate receptor for lysosomal enzymes (Lammers and Jamieson, 1988) which can affect the activity of this proteinase. The greater response of sialyltransferase found with FAZA cells compared with primary hepatocytes provide us with a superior system for use in studying factors that can control the release of sialyltransferase from the hepatocyte. This should lead to a greater understanding of the acute phase behaviour of this enzyme.

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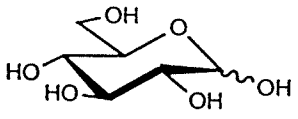
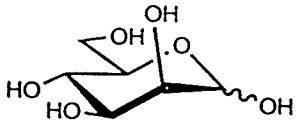
APPENDIX D

THE NINE MOST COMMON MAMMALIAN MONOSACCHARIDES

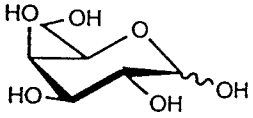
(Copied from Ding *et al*, 1995)



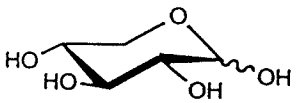
Glucose (**Glc**)



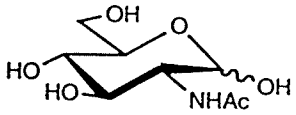
Mannose (**Man**)



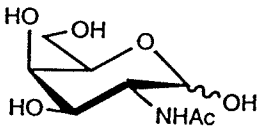
Galactose (**Gal**)



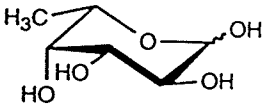
Xylose (**Xyl**)



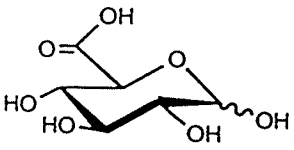
N-Acetylglucosamine (**GlcNAc**)



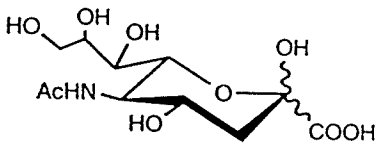
N-Acetylgalactosamine (**GalNAc**)



Fucose (**Fuc**)



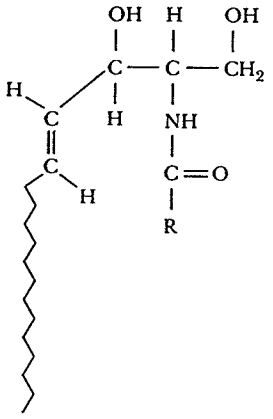
Glucuronic acid (**GlcA**)



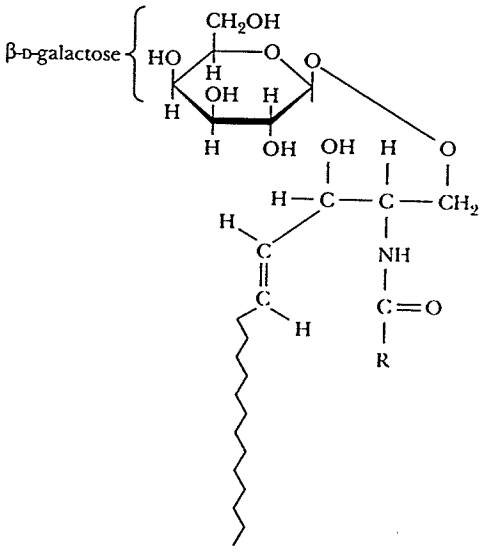
N-Acetylneuraminic acid

GLYCAN-LIPID LINKAGES

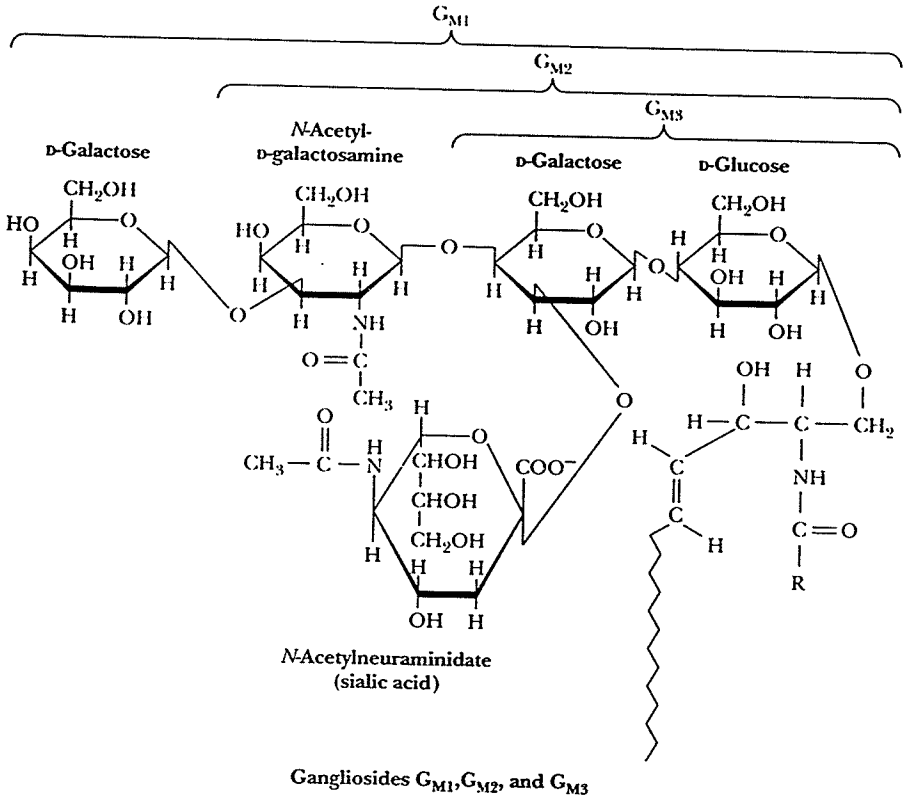
(Copied from Garrett & Grisham, 1995)



Ceramide



A cerebroside



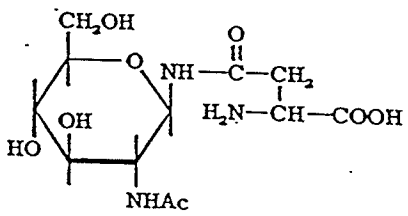
Gangliosides G_{M1}, G_{M2}, and G_{M3}

R = fatty acid (varying lengths and degrees of saturation)

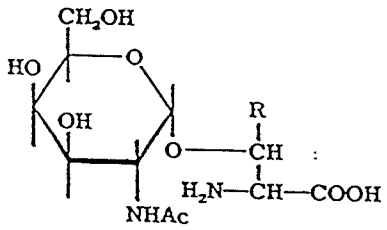
GLYCAN-PROTEIN LINKAGES

(Copied from Sharon & Lis, 1982)

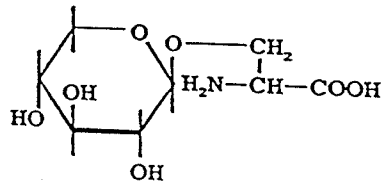
N-Glycosidic
β-*N*-Acetylglucosaminyl-asparagine (GlcNAc-Asn)



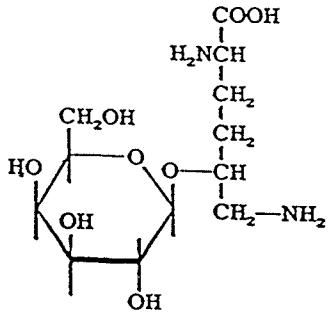
O-Glycosidic
α-*N*-Acetylgalactosaminyl-serine/threonine
(GalNAc-Ser/Thr)



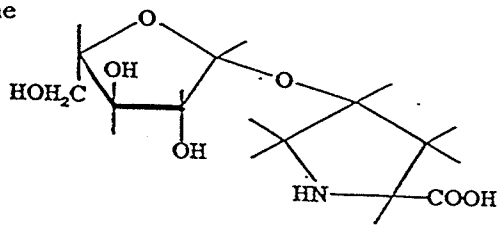
β-Xylosyl-serine
(Xyl-Ser)



β-Galactosyl-hydroxylysine
(Gal-Hyl)

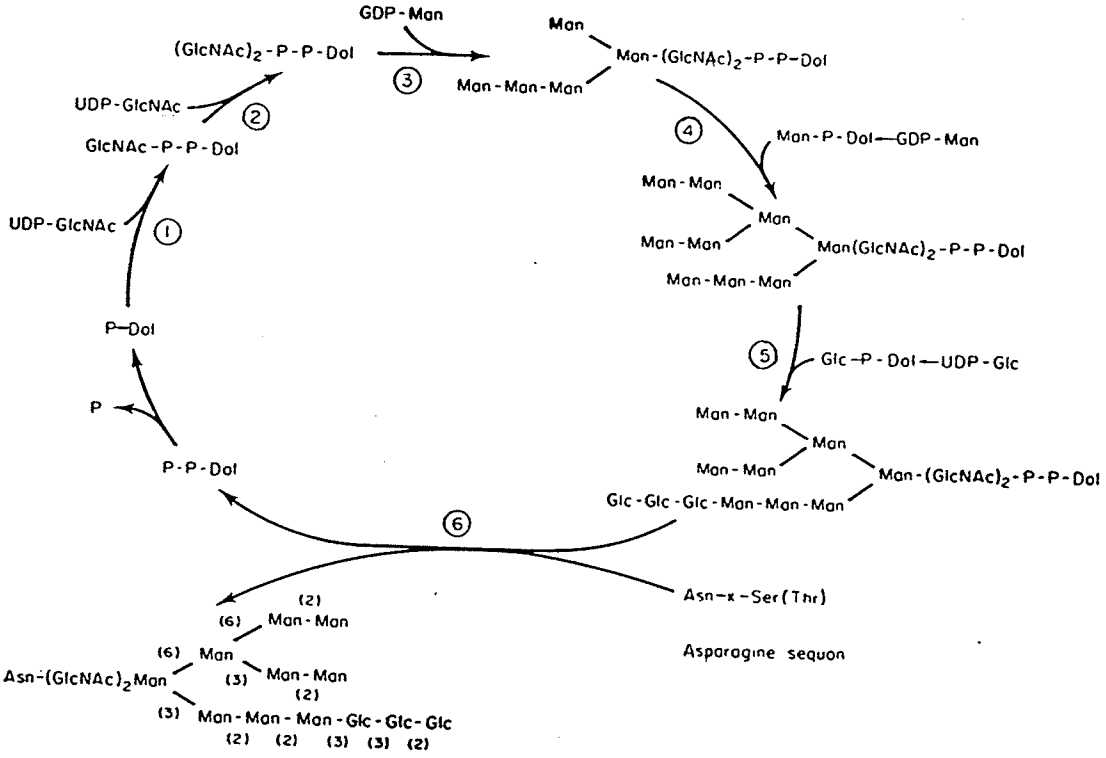


α-L-Arabinosyl-hydroxyproline
(Ara-Hyp)



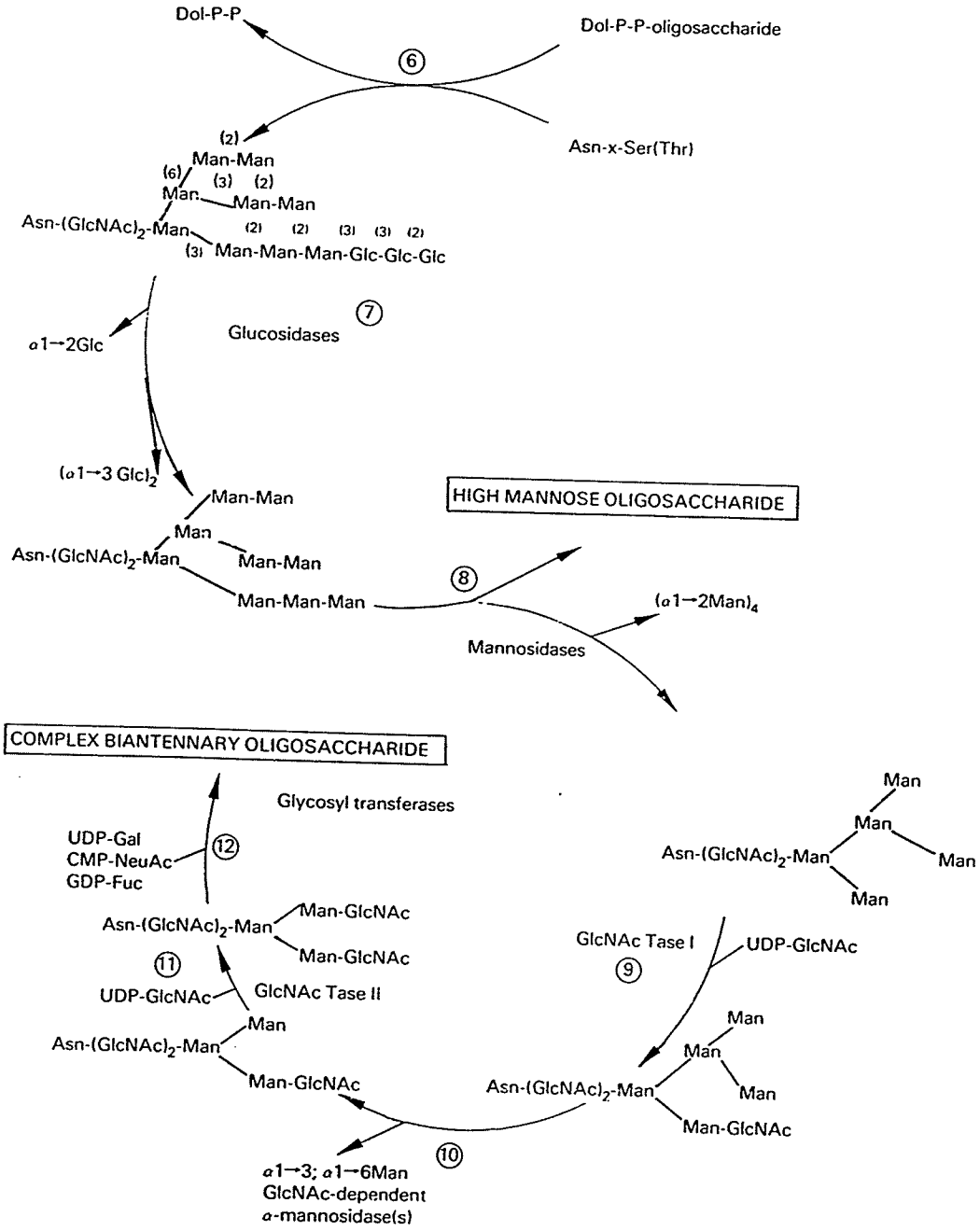
THE DOLICHOL CYCLE

(Copied from Jamieson, 1983)



THE TRIMMING AND PROCESSING REACTIONS

(Copied from Jamieson, 1983)

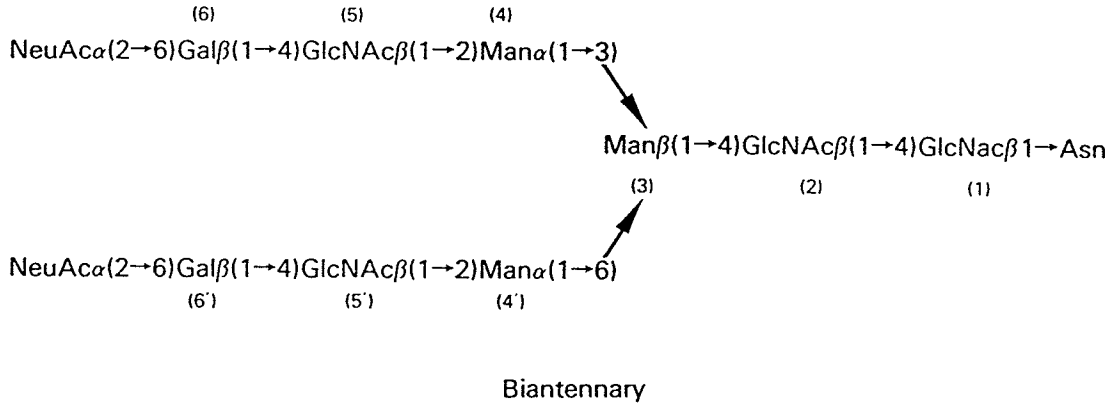


CIRCLED NUMBERS INDICATE ENZYMATIC REACTIONS

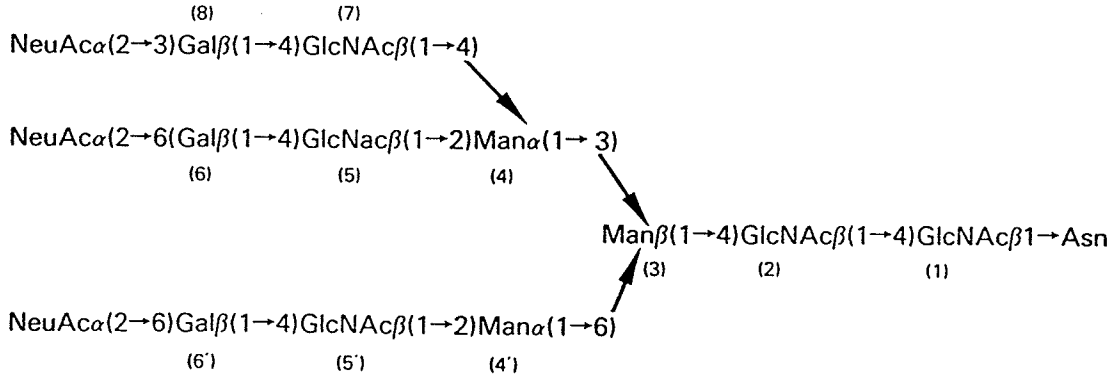
1. N-acetylglucosamine-1-phosphate is transferred from UDP-N-acetylglucosamine to dolichol phosphate
2. UDP-N-acetylglucosamine is used as donor to transfer another GlcNAc to the GlcNAc-Dol
3. GDP-mannose is used as the donor to transfer mannose to the GlcNAc-GlcNAc-Dol
4. Further elongation with the addition of another mannose
5. The UDP-Glc donor is used for the addition of glucose residues
6. Transfer of the oligosachharide from dolichol to the protein through the linkage between GlcNAc and asparagine
7. Glucosidases remove the terminal Glc residues
8. Mannosidases remove various Man residues
9. GlcNAc transferase I adds a GlcNAc to a core Man
10. Mannosidases remove the terminal two Man residues
11. GlcNAc transferase II adds another GlcNAc to the other core Man
12. Various sequential glycosyltransferases add Gal, Fuc, and NeuAc

VARIOUS N-LINKED GLYCAN-PROTEIN CONJUGATES

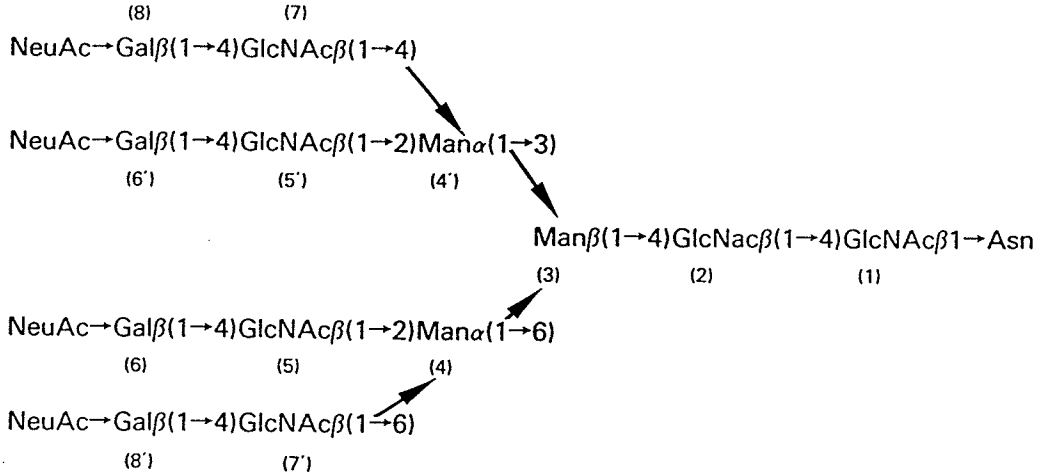
(Copied from Jamieson, 1983)



Biantennary



Triantennary

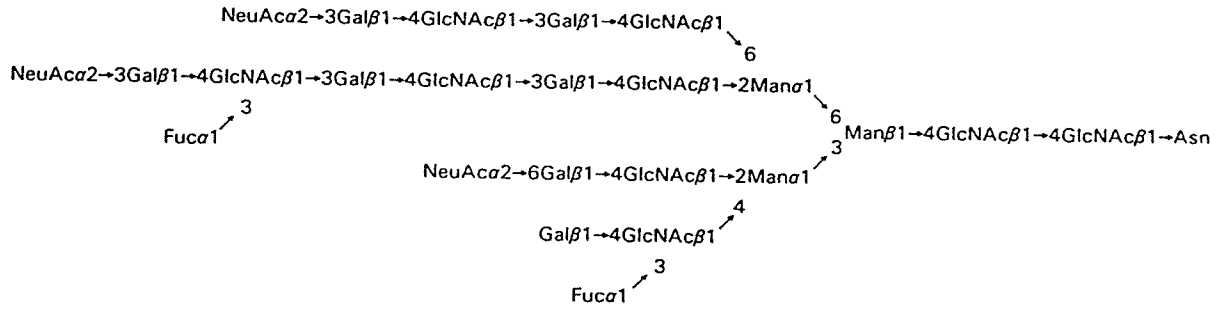


Tetra-antennary

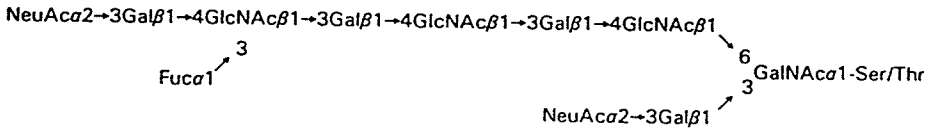
THE TERMINAL, BACKBONE, AND CORE STRUCTURES OF GLYCOCONJUGATES

(Copied from Van den Eijnden *et al*, 1995)

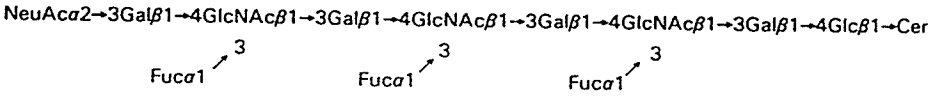
N-linked polylactosaminoglycan



O-linked polylactosaminoglycan



Lipid-linked polylactosaminoglycan

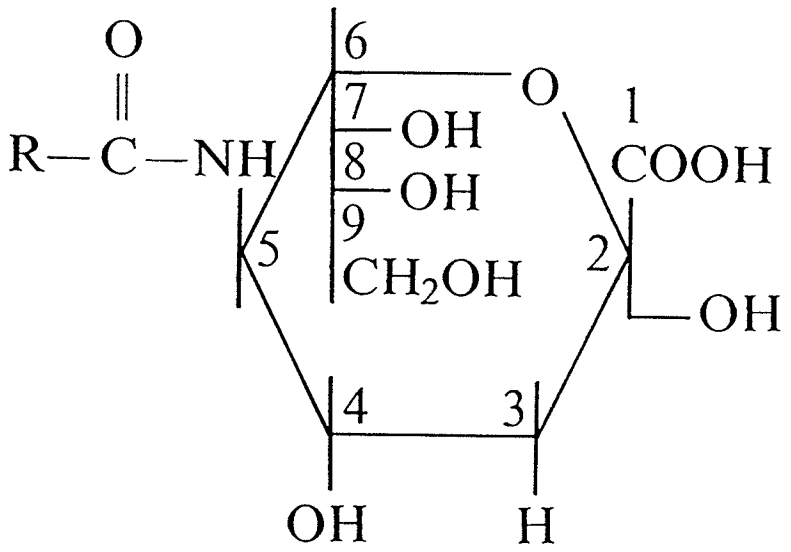


Terminal structure *Backbone* *Core*

APPENDIX E

THE SIALIC ACIDS

(Copied from Culling & Reid, 1982)



$R = CH_3$ = N-acetylneuraminic acid
$R = CH_2OH$ = N-glycolylneuraminic acid

Sialic acids include the N-acetyl- and N-glycolyl- derivatives, each of which can be have substituent groups added at C4, C5, C7, C8, and C9

NATURALLY OCCURRING SIALIC ACIDS

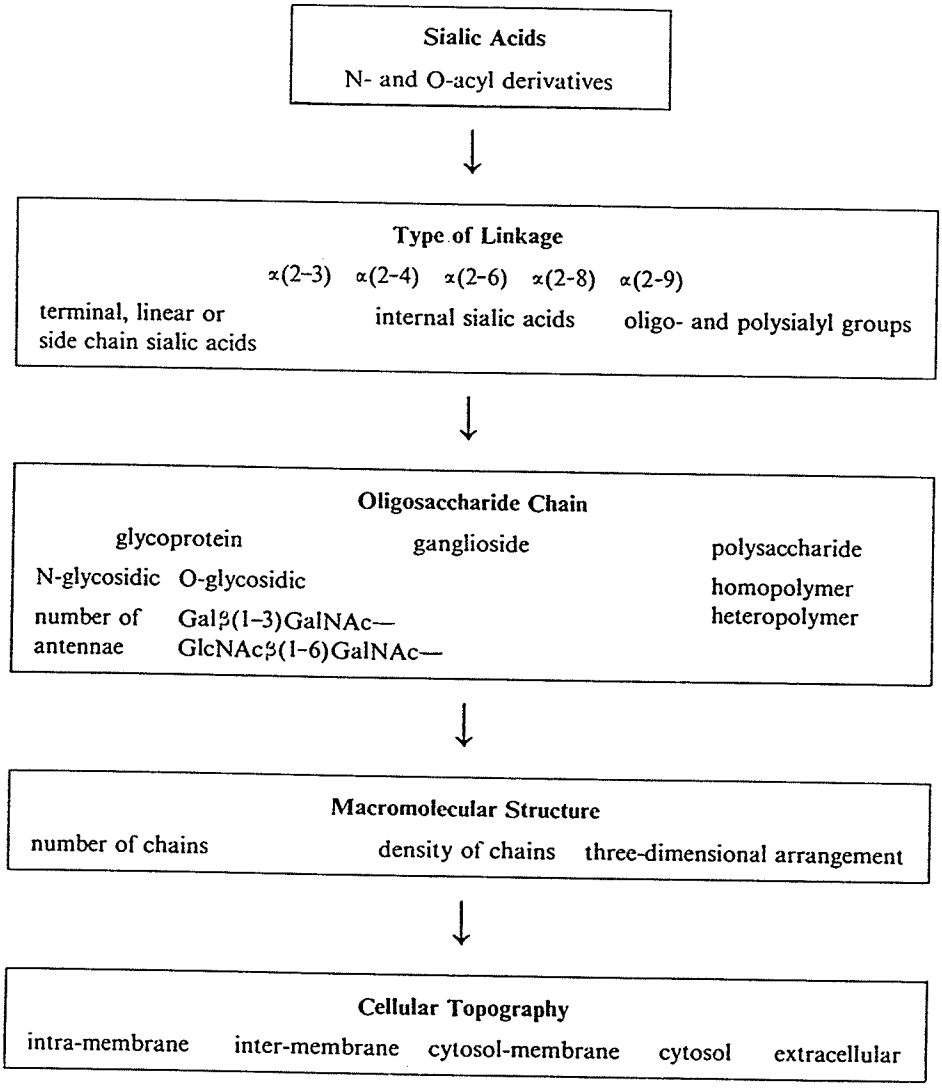
(Copied from Corfield & Schauer, 1982a)

Established structures of naturally occurring sialic acids

Name	Abbreviation	Substituent				
		R^4	R^5	R^7	R^8	R^9
N-Acetylneuraminic acid	Neu5Ac	H	acetyl	H	H	H
N-Acetyl-4-O-acetylneuraminic acid	Neu4,5Ac$_2$	acetyl	acetyl	H	H	H
N-Acetyl-7-O-acetylneuraminic acid	Neu5,7Ac$_2$	H	acetyl	acetyl	H	H
N-Acetyl-8-O-acetylneuraminic acid	Neu5,8Ac$_2$	H	acetyl	H	acetyl	H
N-Acetyl-9-O-acetylneuraminic acid	Neu5,9Ac$_2$	H	acetyl	H	H	acetyl
N-Acetyl-4,9-di-O-acetylneuraminic acid	Neu4,5,9Ac$_3$	acetyl	acetyl	H	H	acetyl
N-Acetyl-7,9-di-O-acetylneuraminic acid	Neu5,7,9Ac$_3$	H	acetyl	acetyl	H	acetyl
N-Acetyl-8,9-di-O-acetylneuraminic acid	Neu5,8,9Ac$_3$	H	acetyl	H	acetyl	acetyl
N-Acetyl-7,8,9-tri-O-acetylneuraminic acid	Neu5,7,8,9Ac$_4$	H	acetyl	acetyl	acetyl	acetyl
N-Acetyl-9-O-L-lactylneuraminic acid	Neu5Ac9Lt	H	acetyl	H	H	L-lactyl
N-Acetyl-4-O-acetyl-9-O-lactylneuraminic acid	Neu4,5Ac$_2$9Lt	acetyl	acetyl	H	H	lactyl
N-Acetyl-8-O-methylneuraminic acid	Neu5Ac8Me	H	acetyl	H	methyl	H
N-Acetyl-8-O-sulphoneuraminic acid	Neu5Ac8S	H	acetyl	H	sulphate	H
N-Acetyl-9-O-phosphoroneuraminic acid	Neu5Ac9P	H	acetyl	H	H	phosphate
N-Acetyl-2-deoxy-2,3-dehydroneuraminic acid	Neu5Ac2en	H	acetyl	H	H	H
N-Glycolylneuraminic acid	Neu5Gc	H	glycolyl	H	H	H
N-Glycolyl-4-O-acetylneuraminic acid	Neu4Ac5Gc	acetyl	glycolyl	H	H	H
N-Glycolyl-7-O-acetylneuraminic acid	Neu7Ac5Gc	H	glycolyl	acetyl	H	H
N-Glycolyl-9-O-acetylneuraminic acid	Neu9Ac5Gc	H	glycolyl	H	H	acetyl
N-Glycolyl-7,9-di-O-acetylneuraminic acid	Neu7,9Ac$_2$5Gc	H	glycolyl	acetyl	H	acetyl
N-Glycolyl-8,9-di-O-acetylneuraminic acid	Neu8,9Ac$_2$5Gc	H	glycolyl	H	acetyl	acetyl
N-Glycolyl-7,8,9-tri-O-acetylneuraminic acid	Neu7,8,9Ac$_3$5Gc	H	glycolyl	acetyl	acetyl	acetyl
N-Glycolyl-8-O-methylneuraminic acid	Neu5Gc8Me	H	glycolyl	H	methyl	H
N-Glycolyl-8-O-sulphoneuraminic acid	Neu5Gc8S	H	glycolyl	H	sulphate	H

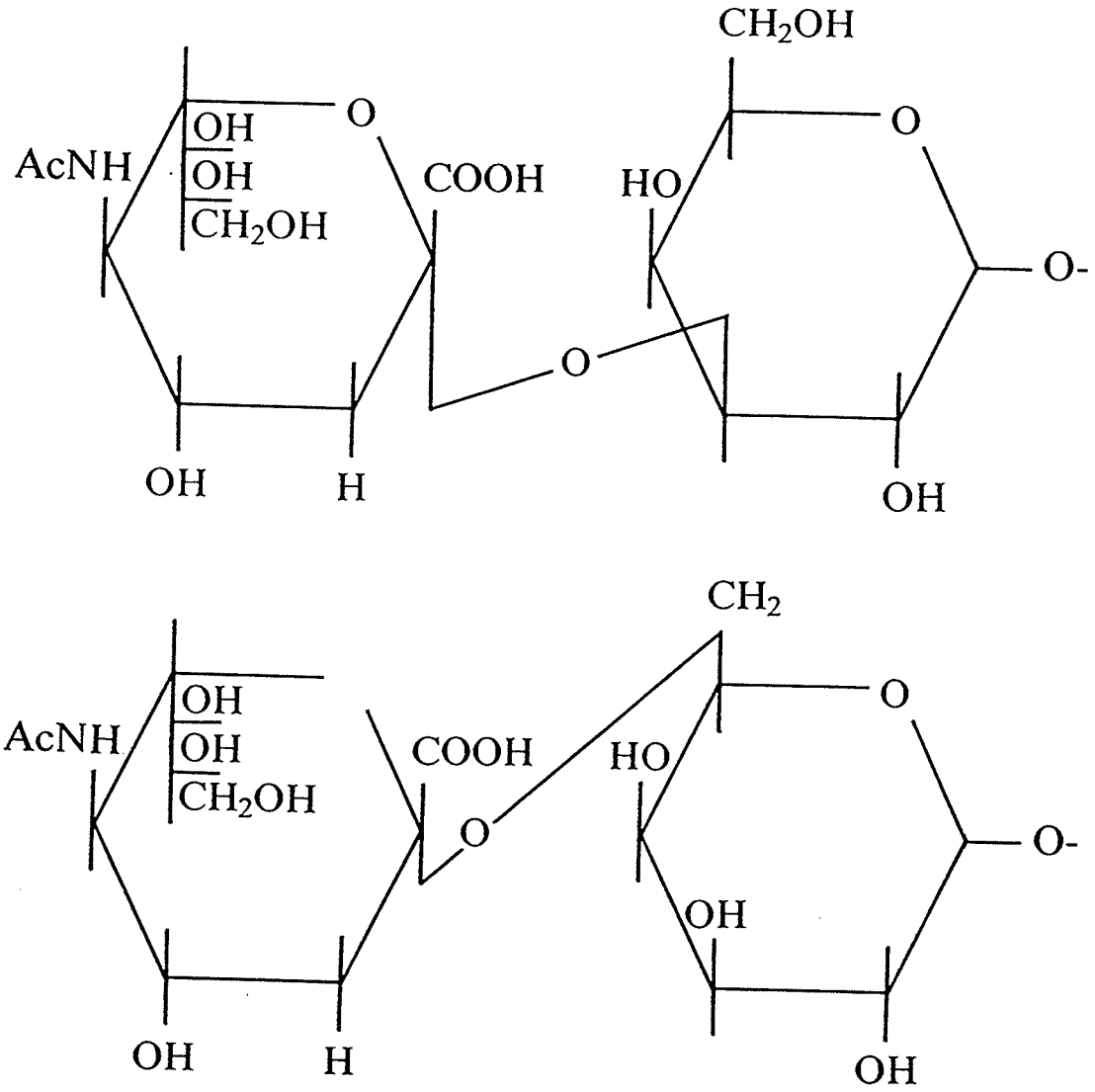
LEVELS OF COMPLEXITY IN THE STRUCTURE OF GLYCOCONJUGATES CONTAINING SIALIC ACIDS

(Corfield & Schauer, 1982a)



α2,3 AND α2,6 LINKAGES BETWEEN NeuAc AND Gal

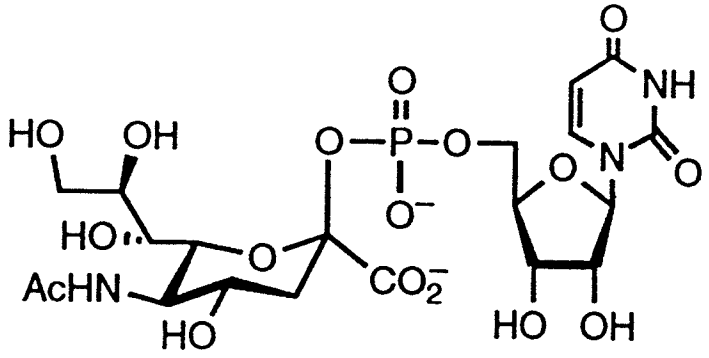
(Copied from Culling & Reid, 1982)



Ac = acetyl group, $-COCH_3$

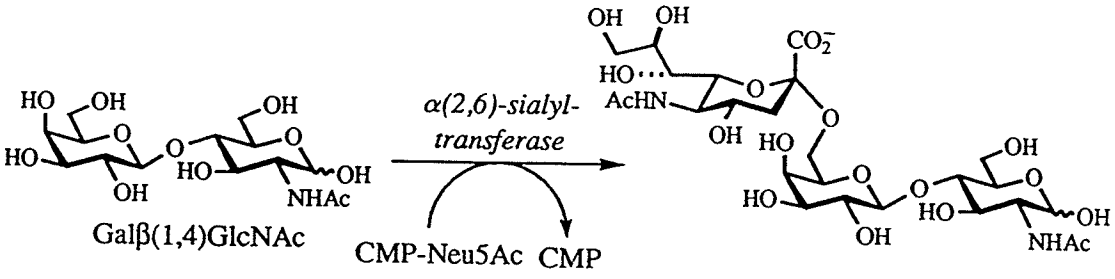
THE Galβ1-4GlcNAcα2-6 SIALYLTRANSFERASE (EC 2.4.99.1) REACTION

(Copied from Ichikawa *et al*, 1994)



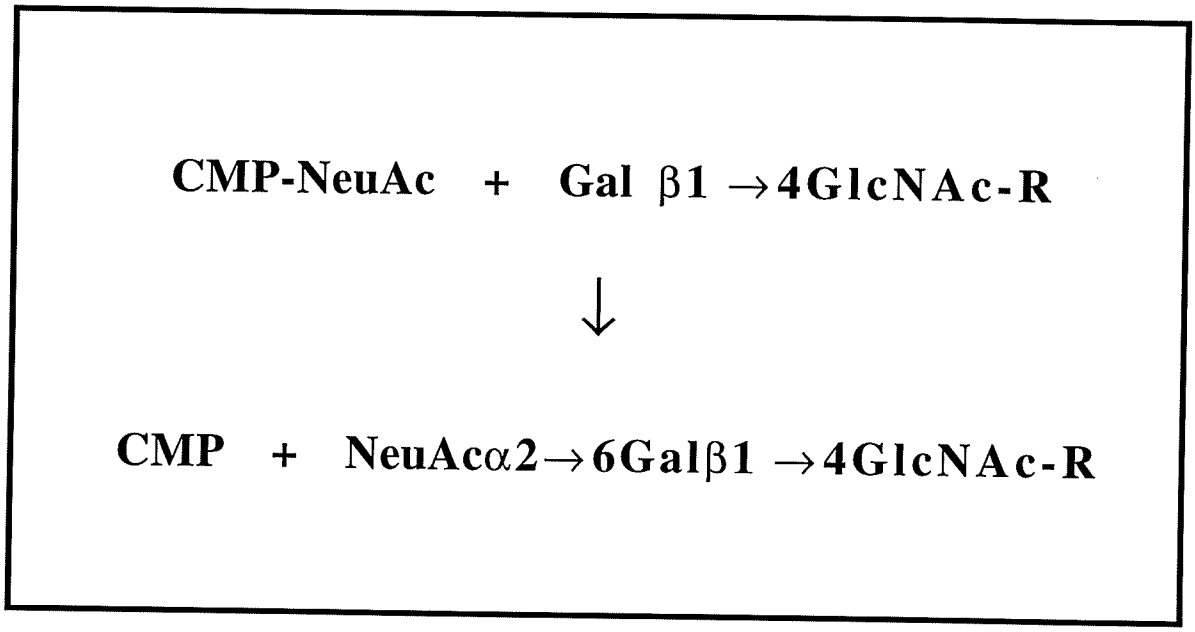
β-CMP-*N*-Acetylneuraminic acid

(CMP-NeuAc)



Note: CMP-NeuAc and CMP-Neu5Ac refer to the same compound.

THE Galβ1-4GlcNAcα2-6 SIALYLTRANSFERASE (EC 2.4.99.1) REACTION



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EPILOGUE

I've Given My Life To Thee

I've given my life to Thee, dear Lord,
To use where Thou hast planned,
Whether it be at home, dear Lord,
or in a distant land;
So let me place my hand in Thine,
And may I know Thy will,
My heart will follow Thy leading, Lord,
Thy purpose to fulfill.

The way may be marked with pain, dear Lord,
But Thou endurest the cross,
Honor and wealth of the world, dear Lord,
Oh may I count but loss,
To have Thy love is all I ask,
Thy will, to be mine own,
And though to ends of the earth I go,
I will not go alone.

Author Unknown

THE UNIVERSITY OF MANITOBA DEPARTMENT OF CHEMISTRY

Winnipeg, Manitoba
Canada R3T 2N2
Tel: (204) 474-9321
FAX: (204) 275-0905
TELEX: 07-587721

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