THE UNIVERSITY OF MANITOBA

STUDIES ON THE MECHANISM OF RELEASE OF A NOVEL ACUTE PHASE REACTANT, GALβ1,4GlcNAc α2,6-SIALYLTRANSFERASE, FROM LIVER GOLGI MEMBRANES BY A CATHEPSIN D-LIKE PROTEINASE

By

Gwen McCaffrey

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ABSTRACT

The liver Golgi membrane-bound Galβ1,4GlcNAc α2,6-sialyltransferase exhibits acute phase behaviour in the rat, with serum levels increasing approximately 5-fold within 48 hr of the onset of turpentine-induced inflammation (Kaplan *et al.*, 1983). This thesis examines the mechanism of release of the α2,6-sialyltransferase in the rat, mouse and guinea pig. Studies using a Golgi vesicle system show that approximately 70% of rat, mouse and guinea pig membrane-bound α2,6-sialyltransferase is released from ultrasonicated Golgi membranes at pH 5.6, 4.6 and 5.2, respectively. The importance of reduced pH in the release of the α2,6-sialyltransferase is also demonstrated by the significant inhibitory effects of lysosomotropic agents on the secretion of this glycosyltransferase from liver slices. Release of the α2,6-sialyltransferase from liver slices and ultrasonicated Golgi membranes at reduced pH is substantially inhibited by pepstatin A, a potent inhibitor of cathepsin D-like proteinases. Antiserum raised against rat liver lysosomal cathepsin D inhibits release of the α2,6-sialyltransferase from ultrasonicated Golgi membranes at reduced pH. The cathepsin D-like proteinase activity, which is shown to be present at the luminal face of the Golgi membrane, may be released from ultrasonicated Golgi membranes upon incubation with 5 mM mannose 6-phosphate. Immunoblot analysis of SDS-polyacrylamide gels shows that rat, mouse and guinea pig membrane-bound α2,6-sialyltransferases have molecular weights of 49,000, 49,000 and 42,000 Da, respectively. Proteolytic cleavage by cathepsin D-like proteinase activities results in the generation of biologically-active, solubilized forms of rat, mouse and guinea pig α2,6-sialyltransferases which have molecular weights of 42,000, 38,000 and 38,000 Da, respectively. The acute phase behaviour of the

liver Golgi α2,6-sialyltransferase is therefore the result of a pH-dependent, proteolytic event involving a cathepsin D-like proteinase, possibly the lysosomal enzyme, which generates a lower molecular weight form of the α2,6-sialyltransferase containing the catalytic domain which is secreted into the extracellular space.

INTRODUCTION

ACUTE PHASE RESPONSE

Assault to the body in the form of physical or thermal trauma, bacterial or parasitic infection, rheumatoid arthritis, malignant proliferation, ischaemic necrosis or injection of chemical irritants is countered by a series of nonspecific yet highly complex cellular, metabolic, endocrine and physiologic alterations which collectively function to restore homeostasis. Characteristic events initiating the local inflammatory response to tissue injury include arteriole vasodilation, increase in venule and capillary permeabilities, exudation of plasma fluids and solutes, leukocyte migration into the injury site and subsequent phagocytosis of foreign material by neutrophils and activated macrophages (Gallin *et al.*, 1988). Important metabolic and physiologic alterations which may occur are fever, leukocytosis, activation of the clotting process, initiation of complement, kinin-forming and fibrinolytic pathways, negative nitrogen balance, increased protein catabolism, increased gluconeogenesis, and a change in plasma concentrations of copper, zinc and iron (Beisel, 1980; Kushner, 1982; Gauldie *et al.*, 1985; Gordon and Koj, 1985; Heinrich *et al.*, 1990). Endocrine changes involve the increased secretion of cortisol, glucagon, insulin, adrenocorticotropic hormone, vasopressin, aldosterone, growth hormone, thyroid stimulating hormone, thyroxine and adrenal catecholamines (Jamieson *et al.*, 1986).

The most significant homeostatic systemic alteration, which directly influences many of the changes in physiological biochemistry that take place during inflammation, is the dramatically increased synthesis and secretion of certain plasma proteins by liver parenchymal cells (Koj, 1974; Beisel, 1980;

Gauldie *et al.*, 1985; Gordon and Koj, 1985; Jamieson *et al.*, 1986; Kushner and Mackiewicz, 1987; Koj *et al.*, 1988; Heinrich *et al.*, 1990). By definition, any hepatocyte-derived protein whose plasma concentration increases at least 25% during inflammation is referred to as an "acute phase reactant", and the combined effect of the heterogeneous actions of acute phase reactants is called the "acute phase response" (Kushner, 1982). The majority of acute phase reactants are glycoproteins with N-linked oligosaccharide chains containing up to approximately 40% carbohydrate (Montreuil, 1980; Berger *et al.*, 1982; Jamieson, 1983; Hatton *et al.*, 1983; Gordon and Koj, 1985; Jamieson *et al.*, 1986).

Examples of acute phase reactants whose plasma concentration during inflammation may be increased 100- to 1000-fold are C-reactive protein (CRP), serum amyloid A protein (SAA), α_2-macroglobulin (α_2-M) and lipopolysaccharide binding protein (van Gool *et al.*, 1974; Hudig and Sell, 1978; Kushner *et al.*, 1981; McAdam *et al.*, 1982; Tobias *et al.*, 1986). Fibrinogen, α_1-acid glycoprotein (α_1-AGP), haptoglobin, α_1-antichymotrypsin (α_1-ACh), complement component C4b-binding protein, α_1-proteinase inhibitor (α_1-PI), and serum amyloid P component (SAP) represent acute phase reactants that demonstrate changes in plasma concentration of approximately 2- to 5-fold. Moderate increases of approximately 50% in plasma concentration are exhibited by the acute phase reactants ceruloplasmin and C3 component of complement (Jamieson *et al.*, 1972; Aronsen *et al.*, 1972; Koj, 1974; Voelkel *et al.*, 1978; Urban *et al.*, 1979; Pepys *et al.*, 1979; Pepys, 1981; Le *et al.*, 1982; Saeki *et al.*, 1989). Proteins such as albumin, transferrin, α_1-inhibitor III, inter-α-antitrypsin and α_1-lipoprotein, whose plasma concentrations are decreased during inflammation, are described as "negative" acute phase reactants (Billingham and Gordon, 1976;

Woloski *et al.*, 1983a; Koj, 1984; Koj *et al.*, 1984; Gordon and Koj, 1985; Geiger *et al.*, 1987; Heinrich *et al.*, 1990).

Investigation into the biological activities of individual acute phase reactants has revealed a marked diversity in function. Fibrinogen, which performs a vital role in control of excessive blood loss (Koj, 1974), has been shown to facilitate the clumping and subsequent removal of staphylococci and streptococci (Duthie, 1955; Lipinski *et al.*, 1967), to inhibit the casein-induced chemotactic response of blood monocytes (Samak *et al.*, 1982), and in concert with fibrinopeptides to promote a reduction in carrageenin-induced edema (Ruhenstroth-Bauer *et al.*, 1981). Ability of CRP to bind to phosphorylcholine residues exposed on membranes of necrotic cells, or present within bacterial and parasitic polysaccharides, facilitates clearance of damaged host tissue and invading microorganisms, respectively, through complement activation and generation of chemotactic and opsonic activities which result in enhanced phagocytosis (Pepys, 1981; Uhlenbruck *et al.*, 1981; Mold *et al.*, 1982; Volanakis, 1982; Pepys and Baltz, 1983). In addition, CRP has been found to act as a scavenger for chromatin and nucleosome core particles released from injured cells (Robey *et al.*, 1984), and to promote platelet activation (Fiedel *et al.*, 1982). Although the main biological activity of α_1-AGP remains obscure, it has been shown to have antiheparin activity (Anderson *et al.*, 1980), to inhibit platelet aggregation (Snyder and Coodley, 1976; Anderson and Eika, 1979; Costello *et al.*, 1979), and to participate in the binding and transport of pteridine, phenothiazine neuroleptics, and various basic drugs such as the tricyclic antidepressant amitriptyline (Piafsky *et al.*, 1978; Javaid *et al.*, 1983; Muller *et al.*, 1983; Tinguely *et al.*, 1985). Evidence of significant sequence homology between α_1-AGP and the lipocalins, serum retinol-binding protein and β-lactoglobulin, combined with its observed

ability to bind lipophilic drugs and certain steroids such as progesterone, has promoted the hypothesis that α_1-AGP may function during inflammation as a scavenger for potentially harmful lipophiles (Ganguly and Westphal, 1968; Pervaiz and Brew, 1987).

Binding of hemoglobin by haptoglobin results in formation of a complex that possesses true peroxidase activity (Jayle, 1951), and rapid clearance from plasma of hemoglobin-haptoglobin complexes simultaneously serves to prevent loss of iron and to inhibit bacterial growth (Weinberg, 1974; Eaton *et al.*, 1982). Ceruloplasmin, which functions as a copper transport protein (Hsieh and Frieden, 1975; Linder and Moor, 1977; Frieden, 1979) and as a scavenger of superoxide anion radicals (Frieden, 1979; Goldstein *et al.*, 1979), also enhances the incorporation of iron into apotransferrin and apoferritin due to its capacity to act as a ferroxidase (Frieden, 1973; Frieden and Hsieh, 1976; Boyer and Schori, 1983). Inhibition of the leukocyte-derived neutral proteinases, cathepsin G and elastase, by α_1-ACh and α_1-PI, respectively, serves to prevent secondary tissue damage at the site of injury (Travis, 1986). The proteinase inhibitor α_2-M, which is effective against a variety of serine, cysteine, aspartate and metalloproteinases (Barrett, 1977; Travis and Salvesen, 1983), has in addition been found to bind interleukin-1, interleukin-6, transforming growth factor β, and basic fibroblast growth factor (Borth and Luger, 1989; Fey and Gauldie, 1989; Dennis *et al.*, 1989), and to be responsible for the rapid plasma clearance of platelet-derived growth factor (Huang *et al.*, 1984). Moreover, α_2-M, α_1-PI and α_1-ACh have been demonstrated to inhibit, in a concentration-dependent manner, both the activity of natural killer cells and antibody-dependent cell-mediated cytotoxicity (Ades *et al.*, 1982; Gravagna *et al.*, 1983).

Participation of acute phase reactants in such processes as blood clotting and fibrinolysis, removal of foreign materials, and binding and transport of metals and biologically-active compounds demonstrates the direct assistance provided by the acute phase response to the local inflammatory reaction. In addition, involvement of acute phase reactants in anti-inflammatory activities, proteinase inhibition and modulation of the immunological response reveals the value of this systemic response in ensuring that the inflammation-induced physiological processes do not inadvertently cause unnecessary tissue damage either at the site of injury or elsewhere. Importance of the acute phase response as a homeostatic mechanism is underscored by its apparent evolutionary phylogenetic conservation. However, although the acute phase response *per se* appears to be an inter-species phenomenon in higher animals, the acute phase behaviour of several plasma proteins has been found to be species-specific (Gauldie *et al.*, 1985; Gordon and Koj, 1985; Kushner and Mackiewicz, 1987; Mackiewicz *et al.*, 1988). Although elevations in plasma concentration of at least 100-fold are exhibited by SAA in man, rabbit and mouse, this protein cannot be detected in rat plasma during inflammation. The proteinase inhibitor α_2-M, which is a major acute phase reactant in the rat, exhibits no change in human plasma and an elevation of only 2- to 10-fold in rabbit plasma in response to injury. Similarly, the plasma concentration of CRP, which is dramatically enhanced in man, monkey, dog and rabbit during the acute phase response, demonstrates only a moderate increase of approximately 2-fold in the mouse and rat. The considerable variation displayed by acute phase reactants with respect to magnitude of response to inflammation may be speculated to reflect the evolutionary development of the acute phase response. Alternatively, diversity of acute phase behaviour may be a direct consequence of the

pleiotropic nature of acute phase reactants. Differences in evolutionary selection of a particular plasma protein for acute phase inducibility therefore could be the result of a species variation in its primary function during inflammation.

Enhanced hepatic synthesis of plasma glycoproteins during inflammation correspondingly has been found to be accompanied by dilation of the endoplasmic reticulum (ER), enlargement and proliferation of the Golgi complex, and an increase in biosynthetic glycosyltransferase activity (Turchen *et al.*, 1977; Lombart *et al.*, 1980; Jamieson *et al.*, 1983; Kaplan *et al.*, 1983; Fraser *et al.*, 1984; Woloski *et al.*, 1983a, 1985a, 1986). In addition, increases in glucosamine-6-phosphate synthase and UDP-N-acetyl-glucosamine-2-epimerase activities, and in liver pool sizes of UDP-GlcNAc, UDP-GalNAc, GDP-Man, CMP-NeuAc and UDP-Gal have been observed (Jamieson *et al.*, 1983; Kaplan *et al.*, 1984). Other alterations in liver biochemistry during inflammation include an elevation in cAMP (Jamieson *et al.*, 1983), accumulation of plasma-derived iron and zinc (Pekarek *et al.*, 1972), reduced receptor-mediated endocytosis of asialoglycoproteins (Wong and Jamieson, 1979), increased glycogenolysis (Jamieson *et al.*, 1983), enhanced uptake of free amino acids released from muscle (Shibata *et al.*, 1970; Powanda *et al.*, 1973; Beisel, 1980; Woloski *et al.*, 1983b), and increased secretion of lysosomal enzymes (Jamieson *et al.*, 1983; Woloski *et al.*, 1983a, 1985a).

Identification of the liver as the primary site of plasma protein synthesis (John and Miller, 1969), combined with evidence that injury at a distant site promotes an increase in plasma protein synthesis, initiated the proposal that blood-borne mediators arising from the site of injury were responsible for induction of the acute phase response (Koj, 1974). Consistent

with this hypothesis were a variety of immunofluorescence studies which demonstrated the sequential recruitment of hepatocytes, from periportal to midlobular and then to centrilobular zones, in the inflammation-induced synthesis of CRP in rabbits, and of fibrinogen, α_1-AGP, α_2-M and haptoglobin in rats (Kushner and Feldmann, 1978; Courtoy *et al.*, 1981). Moreover, evidence of a simultaneous time-dependent increase in intracellular levels of fibrinogen, α_1-AGP, α_2-M and haptoglobin in all stimulated cells revealed the lack of specialization among liver parenchymal cells in plasma protein synthesis (Kushner and Feldmann, 1978; Courtoy *et al.*, 1981; Macintyre *et al.*, 1982).

Early investigative studies focussed on leukocytes as a source of mediators of the acute phase response because leukocyte migration into the injury site is an important event in the initial local inflammatory reaction. *In vivo* administration of leukocyte supernatants, prepared from glycogen-induced rabbit peritoneal exudates, was found to promote hepatic synthesis of plasma proteins in both rat and rabbit (Kampschmidt *et al.*, 1973; Powanda *et al.*, 1973; Kampschmidt and Upchurch, 1974; Wannemacher *et al.*, 1975). Purification and further characterization of the "leukocyte endogenous mediator" contained within leukocyte supernatants revealed that it was indistinguishable from endogenous pyrogen, lymphocyte-activating factor, mononuclear cell factor, catabolin, osteoclast-activating factor and hemopoietin 1. All of these cell-derived mediators (cytokines) have now been collectively renamed "interleukin-1" (IL-1) (Merriman *et al.*, 1977; Krane *et al.*, 1985; Saklatvala *et al.*, 1985; Dinarello, 1984, 1988; Mizel, 1989).

Peripheral blood monocytes and activated tissue macrophages represent the primary sources of IL-1 during inflammation (Gordon and Koj, 1985; Fey and Gauldie, 1989; Mizel, 1989), and two distinct IL-1 molecules have

been identified (IL-1α and IL-1β) and cloned from human, mouse and rat sources (Auron *et al.*, 1984; Lomedico *et al.*, 1984; Marsh *et al.*, 1985; Gray *et al.*, 1986; Nishida *et al.*, 1988). Use of purified and recombinant IL-1 (rIL-1) in numerous *in vivo* and *in vitro* studies has revealed that it functions as an important, but not a universal, mediator of acute phase protein synthesis. Evidence that this cytokine exerts a direct action on acute phase protein genes was provided by the demonstration that exposure of transfected mouse fibroblasts to murine rIL-1 allowed the expression of both SAA and complement factor B (Perlmutter *et al.*, 1986; Woo *et al.*, 1987). Ability of IL-1 to promote an elevation of fibrinogen synthesis *in vivo* in rats, mice and rabbits however stands in contradiction to the finding that this cytokine is ineffective *in vitro* in inducing an elevated expression of fibrinogen, at either the mRNA or protein level, in rat, rabbit, mouse or human hepatocytes or hepatoma cells (Woloski and Fuller, 1985; Darlington *et al.*, 1986; Evans *et al.*, 1987; Koj *et al.*, 1987; Baumann *et al.*, 1987).

Limited regulation of acute phase protein synthesis has also been demonstrated to be a property of another pleiotropic cytokine, cachectin or tumor necrosis factor-α (TNFα), that is secreted by peripheral blood monocytes, macrophages, natural killer cells and T cells in response to inflammation (Le and Vilcek, 1987; Dinarello, 1987; Carswell *et al.*, 1975; Degliantoni *et al.*, 1985; Cuturi *et al.*, 1987). TNFα has been shown both to enhance the expression of complement factors B and C3, and α_1-ACh, and to depress the synthesis of albumin and transferrin, in human hepatocytes and hepatoma cells (Darlington *et al.*, 1986; Perlmutter *et al.*, 1986; Gauldie *et al.*, 1987a; Daveau *et al.*, 1988; Koj *et al.*, 1988; Castell *et al.*, 1988, 1989). However, although TNFα exerts a regulatory influence on the synthesis of a similar subset of acute phase proteins as IL-1, it has been shown, both *in vivo* and *in*

vitro, to be less potent than IL-1 (Darlington *et al.*, 1986; Koj *et al.*, 1987; Sipe *et al.*, 1987).

Discovery that normal rat hepatocytes exhibit increased fibrinogen synthesis and secretion upon being maintained in medium supplemented with supernatants obtained from either rabbit peritoneal cells, or from lipopolysaccharide-stimulated human peripheral blood monocytes, allowed detection and subsequent characterization of another monocyte/macrophage-derived cytokine, termed "hepatocyte-stimulating factor" (HSF), which has proved to be a potent inducer of acute phase protein synthesis (Ritchie and Fuller, 1981, 1983; Woloski and Fuller, 1985; Baumann *et al.*, 1984; Gauldie *et al.*, 1985). Establishment of co-identity between hepatocyte-stimulating factor and B-cell stimulatory factor-2, interferon-β_2, hybridoma-plasmacytoma growth factor, 26 kDa protein and myeloid blood cell differentiation-inducing protein has resulted in the collective reclassification of these cytokines under the name "interleukin-6" (IL-6) (Billiau, 1986, 1987; Sehgal *et al.*, 1987a; Poupart *et al.*, 1987; Shabo *et al.*, 1988).

Availability of recombinant B-cell stimulatory factor-2 allowed the initial demonstration that HSF/IL-6 was capable of promoting the *in vitro* synthesis of fibrinogen, α_1-ACh, α_1-AGP and haptoglobin in human hepatoma HepG2 cells (Gauldie *et al.*, 1987b). Effective at the picomolar level, IL-6 has in addition exhibited a dose- and time-dependent regulation of the synthesis of α_2-M, fibrinogen, cysteine proteinase inhibitor, α_1-AGP, angiotensinogen and albumin in rat hepatocytes and hepatoma Fao and H-35 cells, and the synthesis of CRP and SAA in human hepatocytes (Andus *et al.*, 1987, 1988a, b; Baumann *et al.*, 1987; Castell *et al.*, 1988; Itoh *et al.*, 1989). Moreover, comparative studies using IL-1, IL-6 and TNFα have revealed that only IL-6, in synergy with glucocorticoids, is capable of inducing the *in vitro*

synthesis by human hepatocytes of the full spectrum of human acute phase proteins (Castell *et al.*, 1988, 1989; Moshage *et al.*, 1988).

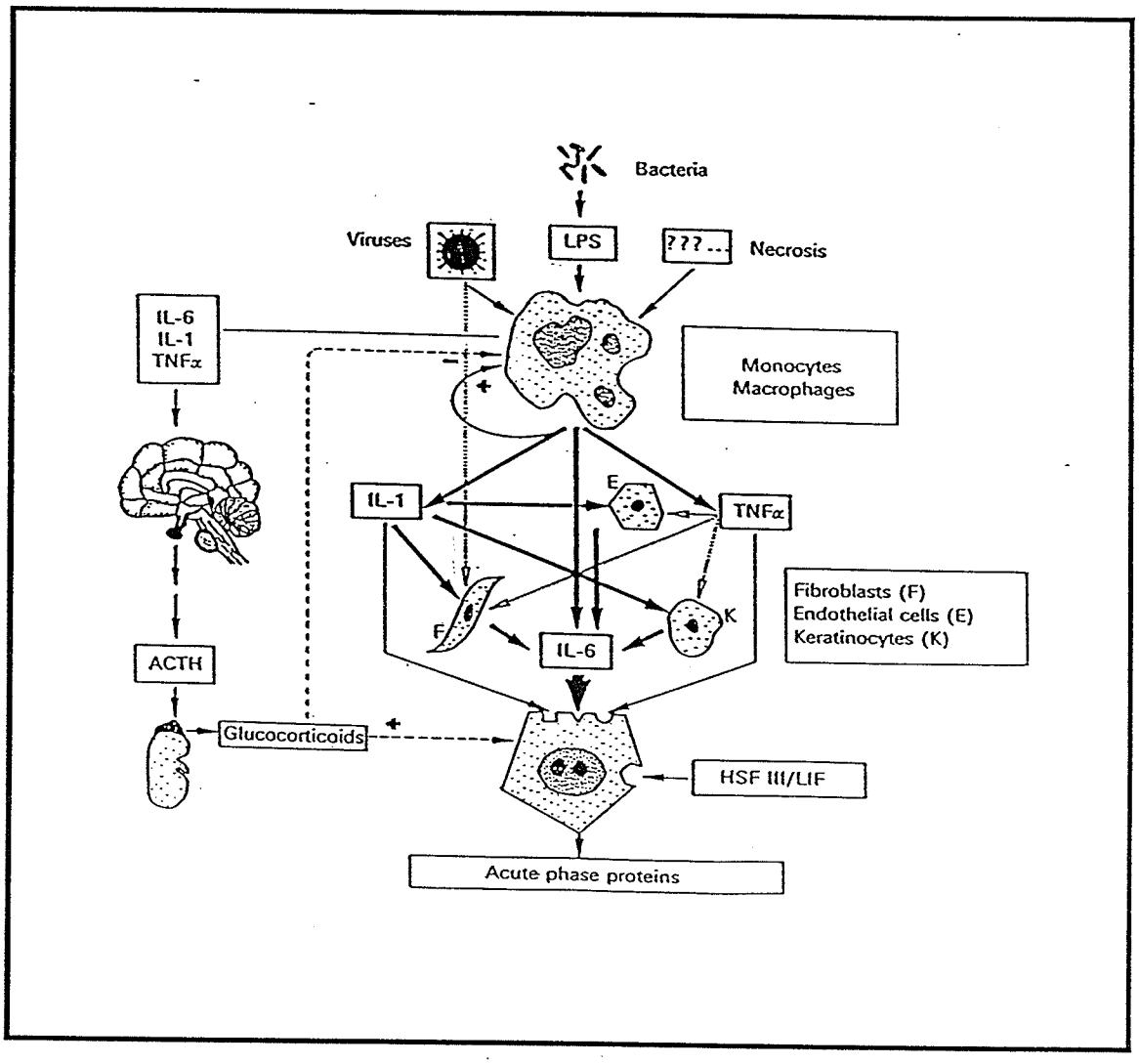
Evidence that hepatocytes are a primary physiological target of IL-6 has been provided by the demonstration that, within 20 min of injection, 80% of human radiolabelled recombinant IL-6 was found to be associated with the liver. Furthermore, the radiolabelled cytokine was exclusively localized on the surface of liver parenchymal cells, apparently bound to plasma membrane IL-6 receptors (Castell *et al.*, 1988). Although signal transduction by IL-1 and TNFα has been shown to involve the second messenger systems protein kinase C and cAMP (Sehgal *et al.*, 1987b; Zhang *et al.*, 1988a, b), IL-6-mediated signal transduction appears to be independent of protein kinase C, inositol triphosphate and adenylate cyclase (Baumann *et al.*, 1988; Heinrich *et al.*, 1990). The consensus sequence CTGGGAA has been demonstrated to be present within promoter regions of α_1-AGP, α_2-M, haptoglobin, fibrinogen, T-kininogen, CRP, SAA and α_1-PI, and the importance of this sequence motif in IL-6-mediated acute phase protein induction has been shown by the fact that its fusion to the chloramphenicol acetyltransferase gene resulted in significant enhancement of protein expression in transfected human hepatoma HepG2 cells in response to IL-6 (Folwkes *et al.*, 1984; Fung and Schreiber, 1987; Arcone *et al.*, 1988; Lowell *et al.*, 1986; Kunz *et al.*, 1989).

In summary, current weight of evidence supports a model for the regulation of hepatic plasma protein synthesis during inflammation that emphasizes IL-1, IL-6, TNFα and glucocorticoids as the principal mediators (Figure 1). Given that IL-6 is capable of promoting expression of the full spectrum of acute phase reactants, it is presently considered to be the major mediator in the hepatic acute phase response. However, there recently has been identified another hepatocyte-stimulating factor, HSF-III, that appears to

FIGURE 1

Regulation of Hepatic Acute Phase Protein Synthesis

Stimulation of monocytes/macrophages by lipopolysaccharide, bacteria, viruses, IL-1 and other unknown factors results in the synthesis and secretion of IL-1, IL-6, and TNFα, all three of which are capable of inducing the release of adrenocorticotropic hormone (ACTH) from pituitary cells. The cytokines IL-1, IL-6, TNFα and hepatocyte-stimulating factor HSF-III/LIF, each of which acts directly on the liver through a specific hepatic receptor, selectively promote plasma protein production. Ability of IL-1 and TNFα to stimulate the synthesis and secretion of IL-6 by endothelial cells, fibroblasts and keratinocytes serves to amplify the biological effects of IL-6. Increased secretion of glucocorticoids through action of ACTH simultaneously serves to promote acute phase protein production and to inhibit cytokine synthesis. (Diagram taken from Heinrich *et al.*, 1990.)


Bacteria
Viruses
LPS
???...
Necrosis
IL-6
IL-1
TNFα
Monocytes
Macrophages
IL-1
E
TNFα
F
K
IL-6
Fibroblasts (F)
Endothelial cells (E)
Keratinocytes (K)
ACTH
Glucocorticoids
HSF III/LIF
Acute phase proteins

elicit the same response as IL-6 with respect to acute phase protein synthesis in human hepatoma HepG2 cells. Synthesized by human squamous carcinoma COLO 16 cells, HSF-III is physically and immunologically distinct from IL-6, binds to a different hepatic receptor, and has been found to be identical to leukemia inhibitory factor (LIF) (Baumann *et al.*, 1989; Baumann and Wong, 1989). As more information becomes available not only on the participation of HSF-III in promoting plasma protein synthesis and its importance within the cytokine network, but also on the individual structures of the specific hepatic cytokine receptors and the mechanisms of cytokine-mediated signal transduction, a clearer picture will emerge as to the biochemical and physiological changes that take place within liver parenchymal cells during inflammation which induce expression of the acute phase reactants.

GLYCOPROTEINS

Glycoproteins are proteins that contain covalently-bound carbohydrate. Initially considered to be a minor class of "protein-carbohydrate complexes", glycoproteins were dismissed by many as being of relatively limited interest. Increased awareness of both the ubiquity and the diversity of function of glycoproteins in animals, plants and microorganisms expanded the horizons of glycoprotein biochemistry, and discovery of the importance of protein-bound carbohydrate in biological recognition rightfully catapulted glycoproteins from obscurity. Attachment of carbohydrate is now recognized

to be a very common, and very important, co- or post-translational modification of proteins. Glycosylated proteins function as enzymes, hormones, lectins, receptors, structural or storage proteins, transporters, mucins of epithelial secretions or antibodies and are present in soluble and membrane-bound forms within cells, in the intercellular matrix and in extracellular fluids.

Considerable variation exists among glycoproteins in the extent of glycosylation, in the composition and structure of covalently-bound carbohydrate, and in the influence of attached carbohydrate on intramolecular and intermolecular characteristics. Physicochemical properties (e.g. viscosity, charge, solubility and thermal stability) may be substantially altered by the presence of carbohydrate. Addition of bulky, hydrophilic carbohydrate substituents may facilitate protein folding, provide resistance to proteolytic degradation and significantly expand the surface area of a protein molecule. Attachment of carbohydrate may be a prerequisite for biological activity, correct biosynthetic proteolytic processing and intracellular targeting, insertion into lipid bilayers or secretion.

Covalently-bound carbohydrate of a protein may be in the form of a monosaccharide, disaccharide, oligosaccharide, polysaccharide or their sulfo- or phospho-substituted derivatives; an individual polypeptide may contain one or more glycosylation sites. Polyfunctional molecules such as monosaccharides have tremendous potential for producing elaborately complex structures due to their ability to combine in an α or β configuration at different linkage positions (e.g. α2,6, β1,4) which may result in the formation of branched chains. Structural analysis of covalently-bound carbohydrate substituents of proteins has revealed that an individual polypeptide may contain carbohydrate chains of different linkage and

composition at different glycosylation sites. In addition, reproducible variation in carbohydrate structure may occur at the same glycosylation site, and patterns of site heterogeneity may be altered in response to changes in physiological condition or biosynthetic cell-type.

Glycoprotein microheterogeneity, which is significantly influenced by the differential activities of the Golgi N-acetylglucosaminyltransferases (Schachter *et al.*, 1983, 1985; Schachter, 1986), is predicated on the manner of synthesis of protein-bound carbohydrate. Carbohydrate substituents of proteins are secondary gene products whose mature form reflects the selective participation of glycosidases and glycosyltransferases, the availability of substrates and cofactors, the influence of adjacent carbohydrate and protein architecture, and the route taken through the membranous ER and Golgi apparatus organelles in which assembly and processing of covalently-bound carbohydrate takes place.

STRUCTURE

N- linked and O- linked Glycoproteins

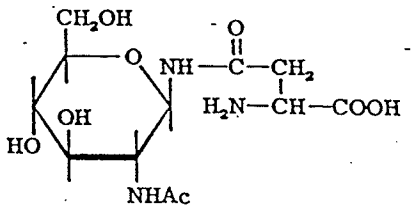
Glycoproteins may be classified according to the nature of the glycosidic bond linking protein and carbohydrate (Kornfeld and Kornfeld, 1980; Sharon and Lis, 1982). The locant "O-" designates glycosyl linkages between either N-acetylgalactosamine (GalNAc), galactose (Gal), xylose (Xyl), mannose (Man), arabinose (Ara) or N-acetylglucosamine (GlcNAc), and either serine (Ser), threonine (Thr), hydroxylysine (Hyl) or hydroxyproline (Hyp). The anomeric carbon of the reducing sugar residue of the carbohydrate moiety is attached in an α or β configuration to the hydroxyl group of the amino acid residue (Figure 2). The GalNAc-Ser/Thr, Xyl-Ser and Gal-Hyl linkages are the most

FIGURE 2

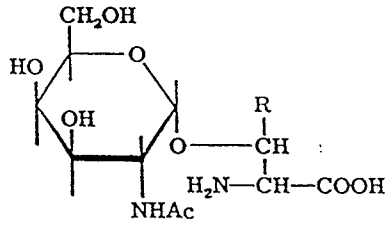
N- and O-Glycosidic Linkages

(Taken from Sharon and Lis, 1982)

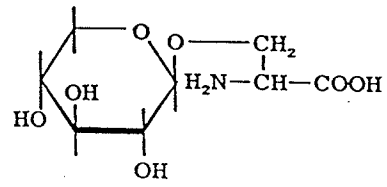
N-Glycosidic
β-*N*-Acetylglucosaminyl-asparagine (GlcNAc-Asn)



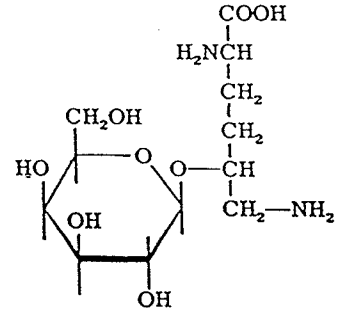
O-Glycosidic
α-*N*-Acetylgalactosaminyl-serine/threonine (GalNAc-Ser/Thr)



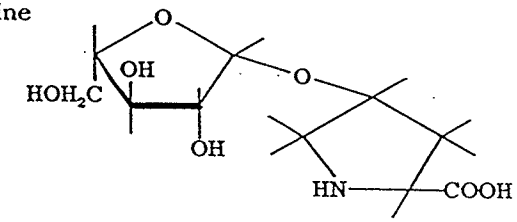
β-Xylosyl-serine
(Xyl-Ser)



β-Galactosyl-hydroxylysine
(Gal-Hyl)



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



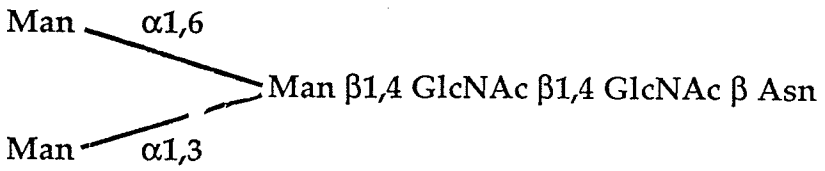
common. Examples of GalNAc-Ser/Thr linkages are found in submaxillary mucins, keratin sulphate type II, IgA immunoglobulin, and blood group active glycoproteins. Xyl-Ser and Gal-Hyl linkages are found in proteoglycans and collagens, respectively. GlcNAc-Ser/Thr linkages have recently been found in proteins of the nuclear pore, chromatin and cytoskeleton (Hart *et al.*, 1988).

The locant "N-" designates the glycosyl linkage between N-acetylglucosamine (GlcNAc) and asparagine (Asn). The anomeric carbon of the reducing N-acetylglucosamine residue of the carbohydrate moiety is attached in a β configuration to the nitrogen of the amide group of asparagine (Figure 2). GlcNAc-Asn linkages are found in numerous animal and plant glycoproteins including plasma glycoproteins, hormones, enzymes, immunoglobulins, keratan sulphate type I, lectins and basement membrane glycoproteins. Both types of glycosyl linkages may be present in the same glycoprotein, and examples of "N-, O-glycoproteins" include fetuin, procollagen, erythrocyte membrane glycophorin and chorionic gonadotropin.

N-linked Oligosaccharides

Carbohydrate attached in N-glycosyl linkage to asparagine may be classified "high-mannose", "complex" or "hybrid" according to sugar composition and structure (Kornfeld and Kornfeld, 1985; Schachter *et al.*, 1985). High-mannose N-linked oligosaccharides are comprised of N-acetylglucosamine and mannose, whereas complex and hybrid oligosaccharides may contain galactose, fucose and N-acetylneuraminic acid in addition to N-acetylglucosamine and mannose. Common to all N-glycosyl

oligosaccharides is the presence of an inner pentasaccharide core of the following structure:



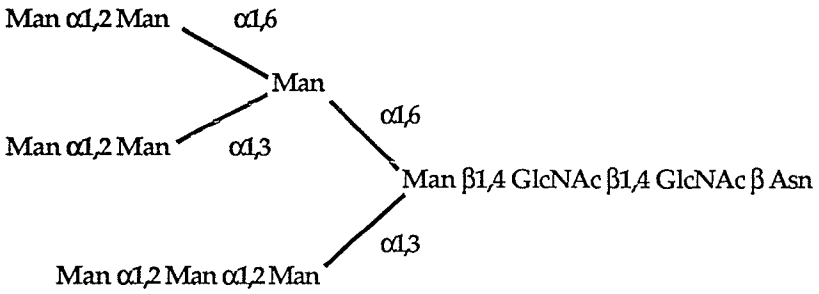
High-mannose N-linked oligosaccharides are characterized by the presence of additional mannose residues that are attached to the inner $Man_3GlcNAc_2$ core (Figure 3). Typically two to four mannose residues are found linked to the pentasaccharide core, however, as in the case of yeast mannans, dozens of mannose residues may be attached. Terminal mannose residues are attached in an α1,2 linkage, and outer chain branching of α-mannosyl residues is restricted to the mannose residue that is linked β1,6 to the core β-mannosyl residue. N-acetylglucosamine may be attached β1,4 to the mannose residue that is linked β1,6 to the inner β-mannosyl residue to form an "intersected" high-mannose oligosaccharide (Couso *et al.*, 1987).

The distinguishing feature of complex N-glycosyl oligosaccharides is the presence of branches or antennae that are initiated by attachment of N-acetylglucosamine in β1,2, β1,4 or β1,6 linkages to inner core mannose residues (Figure 4). Bi-, tri- or tetra-antennary chains are typically found, and these may be extended by addition of galactose and N-acetylneuraminic acid (Kornfeld and Kornfeld, 1985; Schachter *et al.*, 1985). Galactose is characteristically attached to N-acetylglucosamine in a β1,4 linkage, and terminal N-acetylneuraminic acid residues are typically linked α2,3 or α2,6.

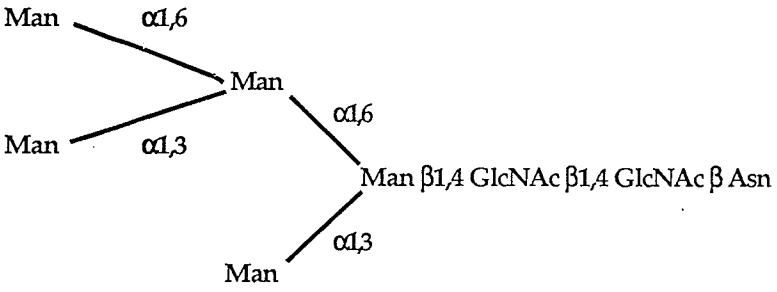
FIGURE 3

High Mannose N-linked Oligosaccharides

a) Man9



b) Man5



c) Intersected

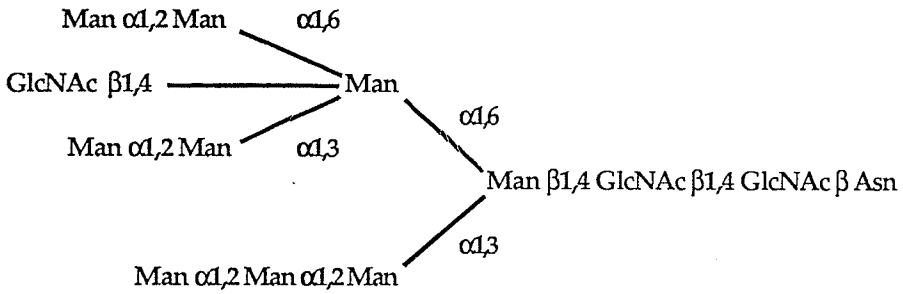
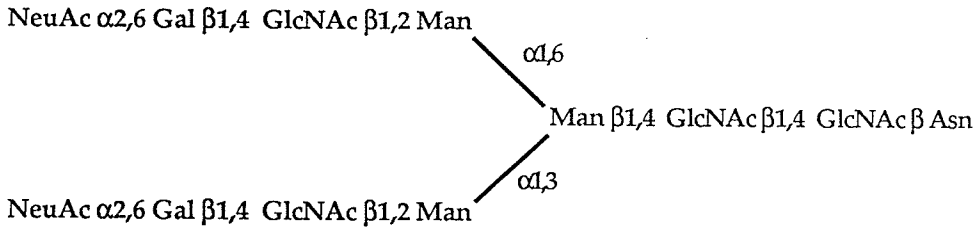


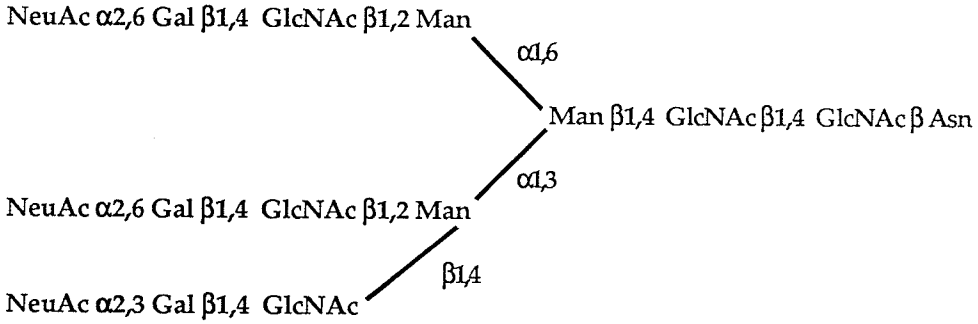
FIGURE 4

Complex N-linked Oligosaccharides

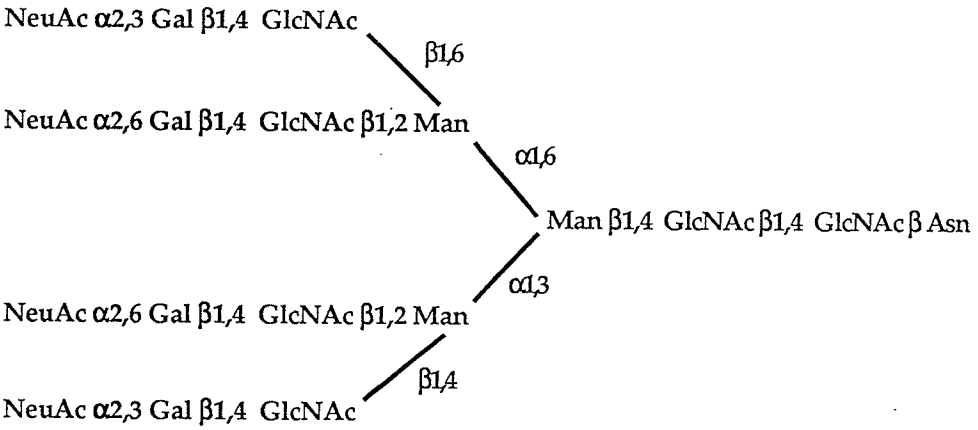
a) Bi-antennary



b) Tri-antennary



c) Tetra-antennary



However, galactose has been found linked β1,3 to N-acetylglucosamine, and N-acetylneuraminic acid may be attached to this Galβ1,3GlcNAc disaccharide sequence in an α2,3 linkage to galactose, and in an α2,6 linkage to N-acetylglucosamine. Complex N-glycosyl oligosaccharides are frequently "bisected" by the addition of N-acetylglucosamine in a β1,4 linkage to the core β-mannosyl residue, and fucose is commonly found attached in an α1,6 linkage to the innermost N-acetylglucosamine residue. Attachment of fucose in an α1,3 linkage to chain-initiating N-acetylglucosamine residues of outer-most branches may occur if the galactose residue of the Galβ1,4GlcNAc sequence is not substituted with N-acetylneuraminic acid. Unusual variations in N-glycosyl oligosaccharide structure have been found such as the presence both of the repeat unit $(Gal\beta1,4GlcNAc\beta1,3)_n$ in poly-N-acetyl-lactosamino-glycans of embryonic tissue (Li *et al.*, 1980), and of the developmentally-regulated polysialosyl sequence NeuAcα2,8$(NeuAc\alpha2,8)_n$-NeuAcα2,8NeuAc-α2,3Gal in the neural cell adhesion molecule (Rutishauser *et al.*, 1988).

Hybrid N-glycosyl oligosaccharides (Figure 5) are so-named because the Manα1,6 and Manα1,3 arms extending from the $Man_3GlcNAc_2$ core incorporate features of high-mannose and complex structures, respectively. "Bisected" hybrid oligosaccharides are frequently found in which N-acetyl-glucosamine is attached in a β1,4 linkage to the core β-mannosyl residue.

BIOSYNTHESIS OF N-LINKED GLYCOPROTEINS

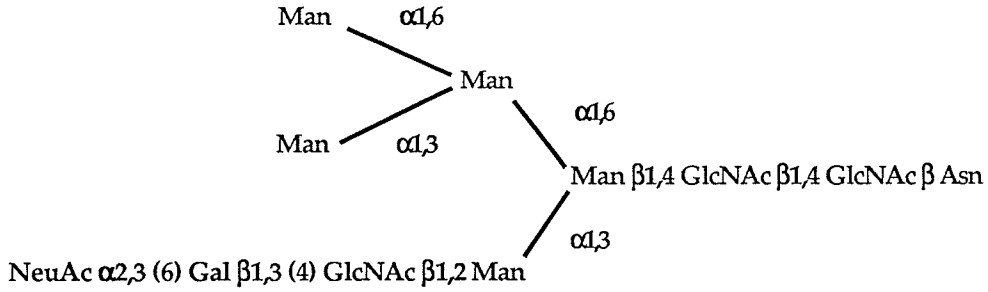
Assembly of Lipid-linked Oligosaccharide Precursor

Presence of the $Man_3(GlcNAc)_2$ inner core arrangement of sugars in all N-linked oligosaccharides reflects their derivation from a common oligo-saccharide precursor, $Glc_3Man_9(GlcNAc)_2$, which is synthesized in step-wise

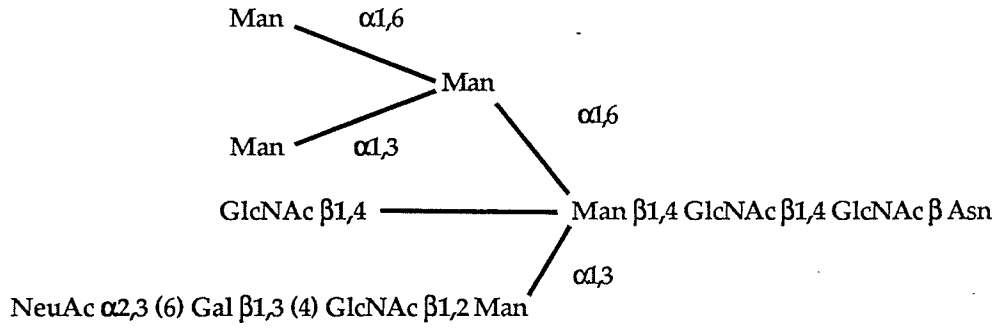
FIGURE 5

Hybrid N-linked Oligosaccharides

a) Non-bisected Bi-antennary



b) Bisected Bi-antennary



fashion on the lipid-carrier dolichol phosphate (Dol-P), transferred *en bloc* to protein and subsequently modified by action of specific glycosidases and glycosyltransferases. The biosynthetic pathway leading to the formation of the lipid-linked oligosaccharide precursor is now known as the "dolichol phosphate cycle" (Sharon and Lis, 1982), and oligosaccharide assembly takes place on both the cytoplasmic and luminal faces of the rough ER membrane (Hirschberg and Snider, 1987; Roth, 1987).

Dolichol phosphate is a polyisoprenoid that contains an α-saturated isoprene residue and typically 16-23 isoprene units, with 19 units being most common in eukaryotic cells (Chojnacki and Dallner, 1988):

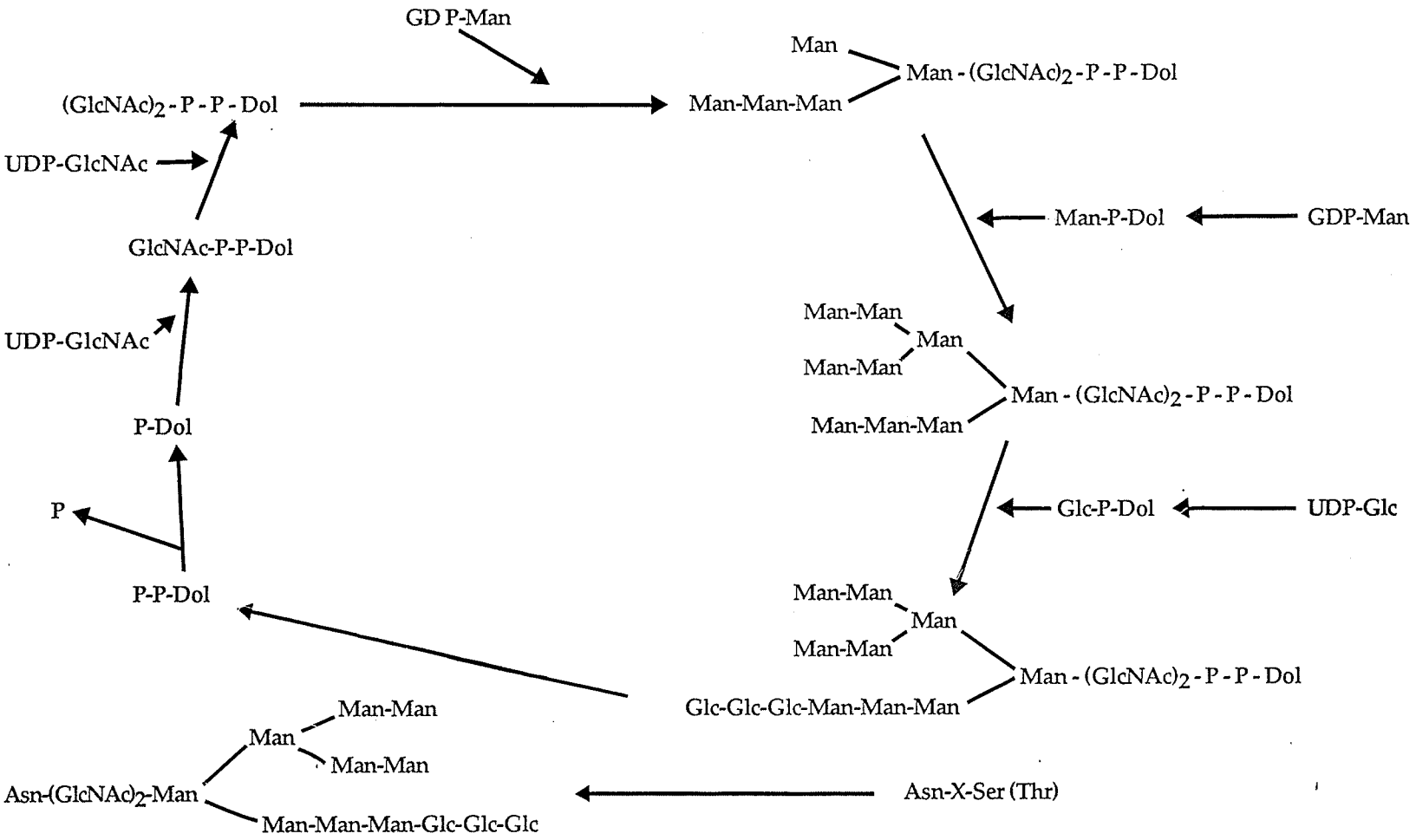
$$(CH_3)_2C{=}CH\text{-}CH_2\text{-}[CH_2\text{-}(CH_3)C{=}CH\text{-}CH_2]_{15\text{-}22}\text{-}CH_2\text{-}(CH_3)CH\text{-}(CH_2)_2\text{-}O\text{-}P\text{-}O_3^{\,2-}$$

Dolichol phosphates which act as obligatory intermediates in N-linked oligosaccharide biosynthesis are embedded in the hydrophobic centre of the ER membrane such that their phosphate groups extend beyond the hydrophilic outer surface of the lipid bilayer and their extended polyisoprenes are maintained parallel with the fatty acid chains (van Duijn *et al.*, 1987; Chojnacki and Dallner, 1988). Localization of dolichol phosphate within the membrane causes destabilization of the lipid bilayer which facilitates transmembrane movement of lipid-linked oligosaccharide intermediates (Valtersson *et al.*, 1985; van Duijn *et al.*, 1986).

The dolichol phosphate cycle (Figure 6) is initiated by transfer to Dol-P of GlcNAc-1-P from UDP-GlcNAc, and the product, GlcNAc-α-P-P-Dol, is subsequently elongated to GlcNAcβ1,4GlcNAc-α-P-P-Dol by addition of a second GlcNAc residue from UDP-GlcNAc (Struck and Lennarz, 1980). Formation of $(GlcNAc)_2$-P-P-Dol is believed to occur at the luminal face of the

FIGURE 6

Dolichol Phosphate Cycle



ER membrane, and protein carrier-mediated import of UDP-GlcNAc is coupled with exit of luminal UMP (Perez and Hirschberg, 1985). Translocation of $(GlcNAc)_2$-P-P-Dol to the cytoplasmic face of the membrane is then believed to take place, and this is followed by transfer of five mannose residues from GDP-Man to form the branched heptasaccharide Manα1,2Manα1,2Manα1,3(Manα1,6)Man$\beta$$(GlcNAc)_2$-P-P-Dol (Prakash *et al.*, 1984). Current weight of evidence indicates that the nucleotide sugar donor GDP-Man is not transported into the lumen of the ER (Hanover and Lennarz, 1982; Perez and Hirschberg, 1986a), which supports the conclusion that assembly of $Man_5(GlcNAc)_2$-P-P-Dol occurs at the cytoplasmic face of the membrane. $Man_5(GlcNAc)_2$-P-P-Dol is elongated to $Man_9(GlcNAc)_2$-P-P-Dol by attachment of four additional mannose residues in α1,2 linkage. The donor for these last four mannosylation reactions is Dol-P-Man (Chapman *et al.*, 1980) which is synthesized on the cytoplasmic face of the membrane by transfer of mannose to Dol-P from GDP-Man. Therefore, formation of $Man_9(GlcNAc)_2$-P-P-Dol is believed to be preceded by translocation of both $Man_5(GlcNAc)_2$-P-P-Dol and Dol-P-Man to the luminal face of the membrane, although the mechanism by which this occurs remains to be clarified (Snider and Robbins, 1982; Snider and Rogers, 1984). The last three steps in the dolichol phosphate cycle involve addition of terminal glucose residues to form the sequence Glcα1,2Glcα1,3Glc which is attached in an α1,3 linkage to the Manα1,3 arm of the oligosaccharide core (Liu *et al.*, 1979; Chapman *et al.*, 1979). Dol-P-Glc is the sugar donor for these glucosylation reactions and they occur at the luminal face of the membrane. It remains to be clarified whether Dol-P-Glc is synthesized at the luminal face of the membrane by transfer of glucose from imported UDP-Glc to Dol-P, or is formed by a similar mechanism at the cytoplasmic face of the membrane and then translocated

(Hirschberg and Snider, 1987). Glucosylation of the oligosaccharide precursor prevents its degradation by a phosphodiesterase (Hoflack *et al.*, 1981) and enhances efficiency of its transfer to protein (Huftaker and Robbins, 1983; Ballou *et al.*, 1986).

Translocation of Protein into the Endoplasmic Reticulum and N-Glycosylation

Transfer of the oligosaccharide precursor $Glc_3Man_9(GlcNAc)_2$ to protein is believed to be mediated by an oligosaccharyltransferase which is an integral component of the rough ER membrane and appears to possess a cytoplasmically-oriented domain that binds the Dol-P-P-oligosaccharide donor and a luminally-oriented domain that attaches to the polypeptide acceptor (Welply *et al.*, 1986). Translocation of protein into the ER therefore is a necessary prerequisite for N-glycosylation. Proteins targeted to the ER typically contain within the NH_2-terminus a "signal sequence" that is essentially a consensus domain of 15 to 30 amino acids comprised of a core of hydrophobic residues preceded by one or more basic residues (Rapoport and Wiedmann, 1985; Warren, 1987). Emergence of the signal sequence beyond the confines of the 60S ribosomal subunit of the mRNA-translation complex allows it to be recognized by a cytoplasmic "signal recognition particle" (SRP) whose primary function is to maintain the nascent polypeptide in a translocation-competent configuration and direct its attachment to an integral membrane protein of the ER known as the "signal recognition particle receptor" (SRP receptor) or "docking protein" (Gilmore *et al.*, 1982; Meyer *et al.*, 1982; Grossman, 1988). Once attached to SRP receptor, SRP dissociates from both ribosome and signal sequence (Gilmore and Blobel, 1983; Wiedmann *et al.*, 1987a), and the signal sequence then interacts with the

membrane-bound "signal sequence receptor" (SSR) (Wiedmann *et al.*, 1987b). The SSR is believed to be a component of the protein-translocation complex or "translocon", and phosphorylation of SSR at its cytoplasmic tail has been suggested as a means of inducing formation of a transmembrane aqueous tunnel through which the nascent protein moves in rachet-like units of about 20 amino acids into the lumen of the ER (Singer *et al.*, 1987; Prehn *et al.*, 1990). Protein translocation is an energy-dependent process involving nucleotide triphosphates (Perara and Lingappa, 1985; Mueckler and Lodish, 1986), and it is believed that an ATP-requiring protein is a component of the translocon (Chen and Tai, 1987). Presence of charged residues at specific sites within the signal sequence appears to be necessary for complete translocation (Szczesna-Skorupa *et al.*, 1988; Green *et al.*, 1989), and shortly after the signal sequence has penetrated into the lumen of the ER it is cleaved by a membrane-bound signal peptidase (Rapoport and Wiedmann, 1985).

N-glycosylation is typically a co-translational event (Kornfeld and Kornfeld, 1985; Kaplan *et al.*, 1987), however post-translational attachment of carbohydrate has been demonstrated to occur in the biosynthesis of rat liver α_1-AGP *in vivo* (Jamieson, 1977), and of prepro-α-factor *in vitro* (Waters and Blobel, 1986). The asparagine residue to which the oligosaccharide precursor is attached occurs within the tripeptide sequence Asn-X-Ser/Thr known as the "asparagine sequon" in which X may be any amino acid except proline (Marshall, 1972, 1974; Bause and Lehle, 1979). Presence of the primary sequence Asn-X-Ser/Thr is both necessary and sufficient for *in vitro* glycosylation of COOH- and NH_2-terminally blocked tripeptides (Struck and Lennarz, 1980). However, proteins contain asparagine sequons that are not glycosylated, and accessibility of the tripeptide sequence appears to be a major contributing factor in determining attachment of carbohydrate (Kronquist and

Lennarz, 1978). In addition, presence of proline at the COOH-terminal of the asparagine sequon inhibits N-glycosylation (Roitsch and Lehle, 1989). Recognition of the asparagine sequon is performed by a glycosylation site binding protein that is a luminal component of the ER which associates with the oligosaccharyltransferase (Welply *et al.*, 1985; Kaplan *et al.*, 1988; Geetha-Habib *et al.*, 1988).

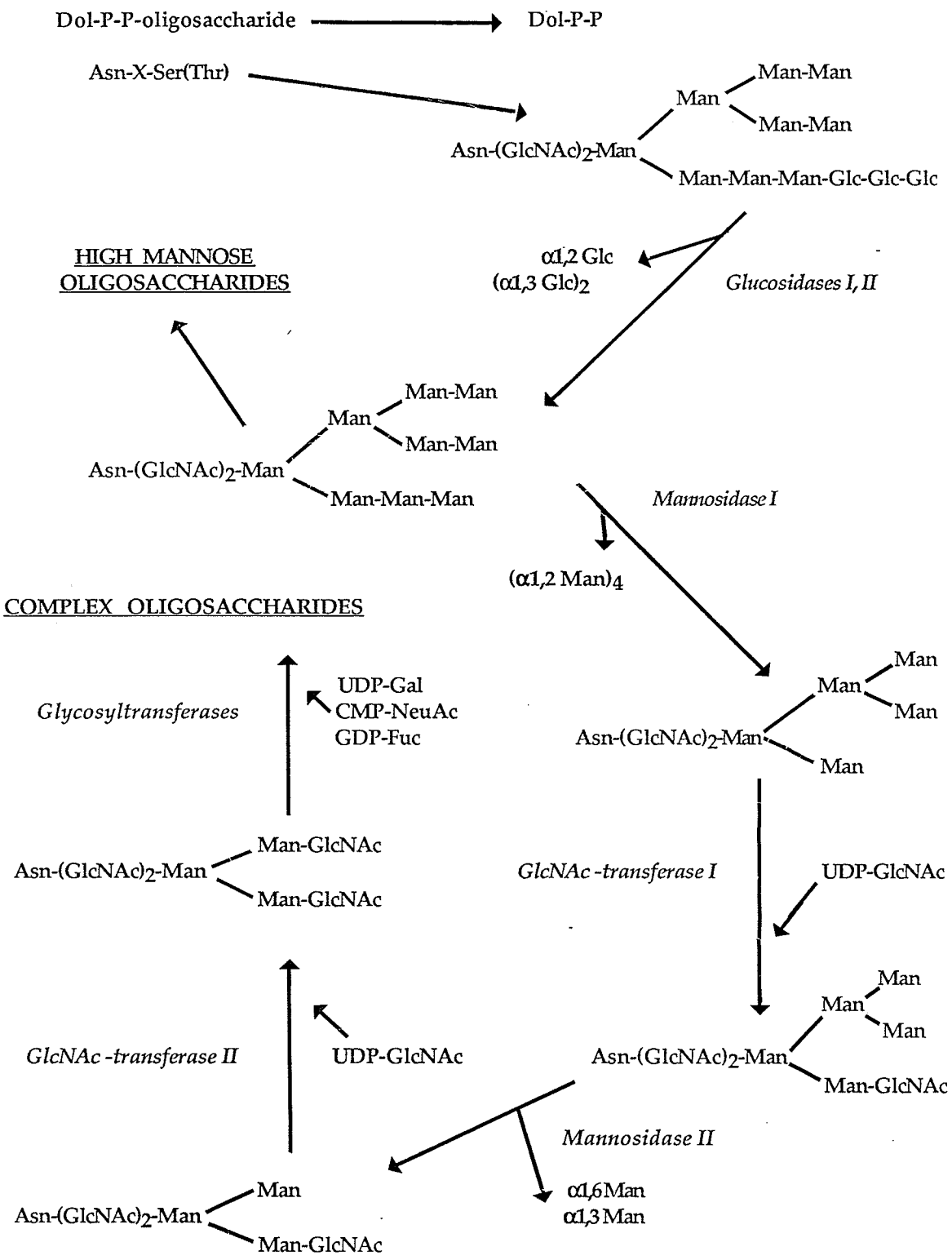
Processing of N-linked Oligosaccharides

Concomitant with the migration of newly-formed N-glycosylated proteins through the organelles of the biosynthetic pathway is the modification of protein-bound carbohydrate to generate high-mannose, complex or hybrid structures. Strategic location of specific glycosidases and glycosyltransferases within the rough, smooth and transitional ER, and the *cis*, *medial* and *trans* compartments of the Golgi apparatus, in large part dictates the sequence of oligosaccharide processing. The major processing steps in the biosynthetic pathway leading to the formation of complex, bi-antennary structures from the precursor $Glc_3Man_9GlcNAc_2$ are shown in Figure 7.

Processing is initiated by glucosidase I which removes the terminal α1,2-linked glucose residue on the α1,3-mannosyl branch, and this is followed by sequential cleavage of the inner two α1,3-linked glucose residues by glucosidase II (Elting *et al.*, 1980; Hubbard and Robbins, 1979; Michael and Kornfeld, 1980; Kornfeld *et al.*, 1978; Ugalde *et al.*, 1979, 1980). Glucosidases I and II have been purified from several sources and characterized (Hettkamp *et al.*, 1984; Schweden *et al.*, 1986; Bause *et al.*, 1986, 1989; Brada and Dubach, 1984; Burns and Touster, 1982; Hino and Rothman, 1985; Strous *et al.*, 1987). Glucosidase II has been localized by immunoelectron microscopy to the

FIGURE 7

N-linked Oligosaccharide Processing Reactions



rough, smooth and transitional ER in pig hepatocytes (Lucocq *et al.*, 1986). Glucosidase processing may occur co-translationally (Atkinson and Lee, 1984), or after synthesis of the polypeptide chain has been completed (Hubbard and Robbins, 1979). Pulse chase studies using virus-infected Chinese hamster ovary (CHO) cells have shown that removal of the first two glucose residues is very rapid (within 5 minutes), whereas cleavage of the innermost glucose residue is significantly slower (within 20 - 30 minutes) (Kornfeld *et al.*, 1978). Rates of deglucosylation may be different for secretory and nonsecretory glycoproteins (Ronin *et al.*, 1984). Deglucosylation may be a prerequisite for efficient transport of certain N-linked glycoproteins from the ER to the Golgi apparatus. Inhibition of glucosidases I and II by 1-deoxynojirimycin was shown to significantly retard entry into the Golgi apparatus of the lysosomal enzymes cathepsin D and β-hexosaminidase (Lemansky *et al.*, 1984) and the secretory proteinase inhibitors α_1-PI and α_1-ACh (Lodish and Kong, 1984).

Deglucosylation of the $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor is followed by sequential cleavage of α-1,2-linked mannose residues to generate the $Man_{5-8}GlcNAc_2$ structures. Trimming of mannose residues is pivotal in N-linked oligosaccharide biosynthesis because it determines whether the oligosaccharide will remain as high-mannose (Man_{5-9}) or be further processed to hybrid or complex forms. Presence of processing α-mannosidase activities in the ER allows initial trimming of mannose residues to take place prior to transport of the newly-formed N-linked glyco-protein to the Golgi apparatus. An ER α-mannosidase that specifically removes the terminal mannose of the middle branch of $Man_9GlcNAc_2$ to form the "A" isomer of $Man_8GlcNAc_2$ has been purified from rat liver and characterized (Bischoff and Kornfeld, 1983, 1986). This ER α-mannosidase is insensitive to inhibition by the mannose analogue 1-deoxymannojirimycin (dMM) which

allows it to be distinguished from the Golgi α-mannosidase I which can remove four α1,2-linked mannose residues from $Man_9GlcNAc_2$ to generate $Man_5GlcNAc_2$. Use of dMM to differentiate between these two α-mannosidases showed that in rat hepatocytes approximately one-third and one-half of the oligosaccharides on secreted and cellular glycoproteins, respectively, were initially processed by the ER α-mannosidase. Characterization of the covalently-bound carbohydrate present on α_1-AGP isolated from rat liver rough ER membranes revealed the presence of equivalent amounts of oligosaccharide chains of the $Man_9GlcNAc_2$ structure and the "A" isomer of $Man_8GlcNAc_2$, which demonstrated that the ER α-mannosidase was the sole participant in the *in vivo* processing of high mannose oligosaccharides on nascent α_1-AGP within the rough ER (Silvanovich and Jamieson, 1989).

Trimming of high-mannose oligosaccharides to generate the $Man_5GlcNAc_2$ structure is performed in the Golgi apparatus by two neutral α1,2-specific mannosidases that differ with respect to physical properties and collectively are called "mannosidase I" (Tabas and Kornfeld, 1979; Tulsiani *et al.*, 1982). Biochemical studies with CHO cells and rat liver Golgi membranes have localized mannosidase I activity to the *cis* or *medial* compartments of the Golgi apparatus (Dunphy *et al.*, 1981; Pohlmann *et al.*, 1982; Dunphy and Rothman, 1983; Rothman *et al.*, 1984 a, b). Mannosidase IA has been purified from rat liver Golgi membranes and has been shown to remove mannose residues from $Man_9GlcNAc_2$ oligosaccharides in a specific sequence (Tulsiani and Touster, 1988). First, the terminal mannose residue of the middle branch was removed to generate a single $Man_8GlcNAc_2$ isomer. Then either of the terminal mannose residues on the outer two branches were cleaved to form two $Man_7GlcNAc_2$ isomers, which were subsequently processed to a single

$Man_6GlcNAc_2$ isomer. Lastly, removal of a mannose residue from the α1,3-mannosyl branch produced a single $Man_5GlcNAc_2$ isomer.

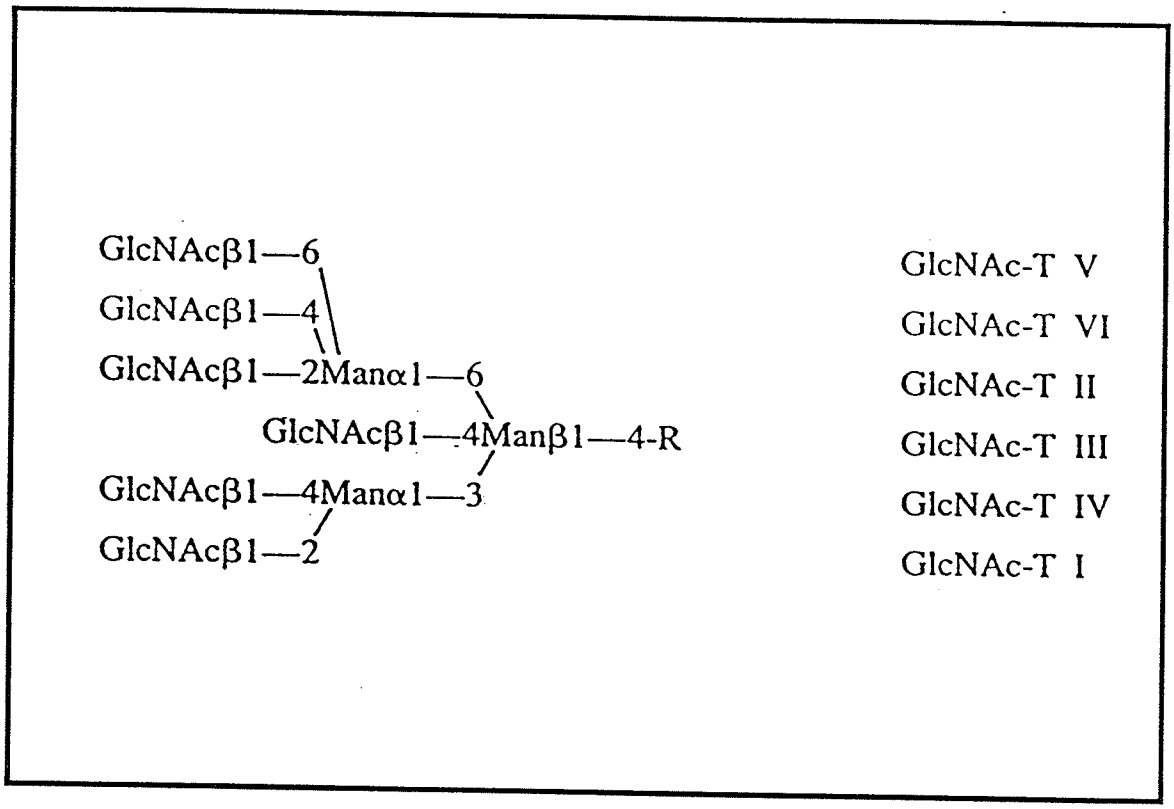
A novel neutral endo-α-mannosidase has been identified in rat liver Golgi membranes which is capable of cleaving the α1,2 linkage between the glucose-substituted mannose residue and the remainder of the α1,3-mannosyl branch of $Glc_{1-3}Man_9GlcNAc_2$ oligosaccharides, and therefore it provides an alternative processing route to the sequential actions of glucosidases I and II, and mannosidase I (Lubas and Spiro, 1987, 1988). This endo-α-mannosidase has been characterized to be insensitive to inhibitors of N-linked oligosaccharide processing exoglycosidases such as 1-deoxynojirimycin, dMM and swainsonine. Presence in the Golgi apparatus of a mannosidase capable of accepting glucosylated high-mannose oligosaccharides as substrates would allow processing of selected oligosaccharide species that either were not suitable substrates for glucosidases I and II, or were transiently reglucosylated by a glucosyltransferase that has been shown to be present in the ER (Parodi *et al.*, 1984) and perhaps may be located in a compartment distal to glucosidase II.

Formation of hybrid and complex asparagine-linked oligosaccharides is initiated by N-acetylglucosaminyltransferase I (GlcNAc-transferase I), which belongs to a family of *medial* Golgi N-acetylglucosaminyltransferases that are being extensively researched because these processing enzymes are responsible for initiation of the antennae of hybrid and complex asparagine-linked oligosaccharides (Schachter *et al.*, 1983, 1985; Schachter, 1986; Brockhausen *et al.*, 1988). Linkages of N-acetylglucosamine catalyzed by hen oviduct Golgi GlcNAc-transferases are shown in Figure 8. GlcNAc-transferase I, which has recently been cloned using rabbit liver cDNA (Hull *et al.*, 1990), transfers an N-acetylglucosamine residue from UDP-GlcNAc to

FIGURE 8

Linkages Catalyzed by Hen Oviduct N-acetylglucosaminyltransferases Involved in N-linked Glycoprotein Biosynthesis

Sequence of GlcNAc-transferase processing of newly-synthesized glycoprotein N-linked oligosaccharides is to a great extent influenced by enzyme substrate specificities and by relative enzyme activities. Presence of the GlcNAcβ1,2Manα1,3Manβ1,4 sequence is an absolute requirement for reactions catalyzed by α-mannosidase II and GlcNAc-transferases II, III, IV and V, and therefore action by these processing enzymes must be preceded by that of GlcNAc-transferase I. Incorporation of a "bisecting" GlcNAc residue causes this recognition sequence on the Manα1,3 arm to be sterically covered, and therefore action by GlcNAc-transferase III precludes further processing by α-mannosidase II and GlcNAc-transferases II, IV and V. Although GlcNAc-transferase VI may act on both bisected and non-bisected N-linked oligosaccharides, it displays a minimum structural requirement for the trisaccharide structure GlcNAcβ1,6(GlcNAcβ1,2)Manα- and therefore action by GlcNAc-transferase VI on the Manα1,6 arm must be preceded by that of GlcNAc-transferases I, II and V (Schachter *et al.*, 1983; Schachter *et al.*, 1985; Schachter, 1986; Brockhausen *et al.*, 1988, 1989). (Diagram taken from Brockhausen *et al.*, 1988).


GlcNAcβ1—6
GlcNAcβ1—4
GlcNAcβ1—2Manα1—6
GlcNAcβ1—4Manβ1—4-R
GlcNAcβ1—4Manα1—3
GlcNAcβ1—2
GlcNAc-T V
GlcNAc-T VI
GlcNAc-T II
GlcNAc-T III
GlcNAc-T IV
GlcNAc-T I

$Man_5GlcNAc_2$ to form the hybrid $GlcNAcMan_5GlcNAc_2$ oligosaccharide (Harpaz and Schachter, 1980a). Kinetic studies using synthetic substrate analogues and purified enzyme have shown that GlcNAc-transferase I exhibits a strict specificity towards the Manα1,3 terminus of the tri-mannosyl core of $Man_5GlcNAc_2$ (Vella *et al.*, 1984). Presence of GlcNAc-transferase I is essential for the formation of hybrid and complex N-linked oligosaccharides, and this was demonstrated by the finding that a mutant CHO cell line which is deficient in GlcNAc-transferase I synthesizes glycoproteins whose N-linked oligosaccharides are not processed beyond the $Man_5GlcNAc_2$ stage (Li and Kornfeld, 1978; Tabas *et al.*, 1978).

Complex, bi-antennary N-linked oligosaccharides are generated from the biosynthetic intermediate $GlcNAcMan_5GlcNAc_2$ by the sequential actions of α-mannosidase II and GlcNAc-transferase II. Mannose processing by α-mannosidase II of hybrid $GlcNAcMan_5GlcNAc_2$ oligosaccharides is strictly predicated on previous GlcNAc-transferase I activity, and involves the removal of the terminal α1,3 and α1,6 mannose residues attached to the α1,6-mannosyl branch to form $GlcNAcMan_3GlcNAc_2$ (Tabas and Kornfeld, 1978; Harpaz and Schachter, 1980b). Initiation of the second antenna is performed by GlcNAc-transferase II, which adds an N-acetylglucosamine residue to the Manα1,6 terminus of $GlcNAcMan_3GlcNAc_2$ to form the bi-antennary $GlcNAc_2Man_3GlcNAc_2$ oligosaccharide (Tabas and Kornfeld, 1978; Harpaz and Schachter, 1980a). GlcNAc-transferase II activity is predicated on the previous actions of GlcNAc-transferase I and α-mannosidase II, given its strict specificity for both the terminal Manα1,6 residue and the presence of a terminal GlcNAcβ1,2Manα1,3 branch (Bendiak and Schachter, 1987a). Golgi mannosidase II and GlcNAc-transferase II have been purified to homogeneity from rat liver (Tulsiani *et al.*, 1977; Moremen and Touster, 1985,1986; Bendiak

and Schachter, 1987b). Immunocytochemical studies have shown that in rat liver Golgi mannosidase II is present throughout the Golgi apparatus with the exception of the *trans* - most cisternae (Novikoff *et al.*, 1983). However, biochemical studies with CHO cells have shown that mannosidase II cofractionates with GlcNAc-transferases I and II, which supports the *medial* Golgi location of this processing enzyme (Dunphy and Rothman, 1983).

Antennae of N-linked oligosaccharides initiated by GlcNAc-transferase action may be elongated by attachment of galactose and N-acetylneuraminic acid residues. In addition, both chain-initiating and asparagine-linked N-acetylglucosamine residues are suitable acceptors for the attachment of fucose. Transfer of fucose to the innermost N-acetylglucosamine residue of nascent glycoprotein N-linked oligosaccharides is believed to occur in the *medial* Golgi due to the cofractionation on sucrose density gradients of "core fucosyltransferase" activity with GlcNAc-transferase I, II, IV and α-mannosidase II activities (Goldberg and Kornfeld, 1983). A porcine liver fucosyltransferase that catalyzes the attachment of fucose in an α1,6 linkage to the asparagine-linked N-acetylglucosamine residue was found to display a strict specificity for both the absence of antennae elongated by addition of galactose or N-acetylneuraminic acid residues, and the presence of the GlcNAcβ1,2Manα1,3 sequence (Longmore and Schachter, 1982; Schachter *et al.*, 1983). Therefore, α1,6-fucosyltransferase activity must precede GlcNAc-transferase III, galactosyltransferase and sialyltransferase activities, and be subsequent to GlcNAc-transferase I activity. Accordingly, N-linked glycoproteins typically do not contain high-mannose oligosaccharides that are fucosylated at the innermost N-acetylglucosamine residue (Schachter *et al.*, 1985). An exception to this rule is porcine spleen lysosomal cathepsin D which has been found to contain at one glycosylation site an N-linked high-

mannose oligosaccharide of the structure $Man_5GlcNAc\beta1,4(Fuc\alpha1,6)GlcNAc$ (Takahashi *et al.*, 1983).

Attachment of galactose to chain-initiating N-acetylglucosamine residues of N-linked oligosaccharides is usually through a β1,4 linkage, although occasionally it may be either β1,3 or β1,6 (Beyer *et al.*, 1981). Addition of galactose in a β1,4 linkage is catalyzed by a β1,4-galactosyltransferase that in lactating breast tissue is the catalytic component of lactose synthetase (Brew *et al.*, 1968), and in HeLa and human hepatoma HepG2 cells has been localized by immunocytochemical methods to a few *trans* Golgi cisternae (Roth and Berger, 1982; Slot and Geuze, 1983). Studies with purified rat liver β1,4-galactosyltransferase have shown that this enzyme preferentially attaches galactose to the GlcNAcβ1,2Manα1,3 branch of complex, bi-antennary asparagine-linked oligosaccharides (Paquet *et al.*, 1984).

Terminal sialylation of the antennae of complex N-linked oligosaccharides may be performed by sialyltransferases which specifically attach N-acetylneuraminic acid in either an α2,3, α2,4 or α2,6 linkage to penultimate galactose residues. In addition, N-acetylneuraminic acid may be attached in an α2,6 linkage to chain-initiating N-acetylglucosamine residues, or in an α2,8 linkage to terminal N-acetylneuraminic acid residues (Beyer *et al.*, 1981). The α2,6-sialyltransferase which attaches N-acetylneuraminic acid in an α2,6 linkage to penultimate galactose residues is present in a soluble form in goat, bovine and human colostrum and human, pig and rat serum, and in a membrane-bound form in rat, bovine, pig, guinea pig and human liver (Bartholomew *et al.*, 1973; Beyer *et al.*, 1981; Schachter *et al.*, 1985). It has been purified to homogeneity from bovine colostrum (Paulson *et al.*, 1977a) and cloned from rat liver (Paulson *et al.*, 1987; Weinstein *et al.*, 1987). It exhibits a substrate specificity for galactose residues that are linked β1,4 to

chain-initiating N-acetylglucosamine residues (Paulson *et al.*, 1977b; Weinstein *et al.*, 1982a). Recently however, the rat liver α2,6-sialyltransferase has been found capable of catalyzing the *in vitro* sialylation of the mannose-terminated disaccharide Manβ1,4GlcNac and trisaccharide Manβ1,4GlcNAc-β1,4GlcNAc (van Pelt *et al.*, 1989). Studies with bovine colostrum α2,6-sialyltransferase using bi-, tri- and tetra-antennary N-linked glycopeptides and oligosaccharides have shown that this sialyltransferase displays a branch specificity for the Manα1,3 branch which is dependent upon the presence of the outermost N-acetylglucosamine residue of the $Man_3GlcNAc_2$ core. In bi- and tri-antennary oligosaccharide structures, the first and second residues of N-acetylneuraminic acid are preferentially attached to the galactose residues at the Galβ1,4GlcNAcβ1,2Manα1,3 and the Galβ1,4GlcNAcβ1,2Manα1,6 branches, respectively. The Galβ1,4GlcNAcβ1,6Manα1,6 branch was found to be extremely resistant to α2,6-sialylation, and to direct the incorporation in tetra-antennary structures of the second residue of N-acetylneuraminic acid towards the Galβ1,4GlcNAcβ1,4Manα1,3 branch (van den Eijnden, 1980; Joziasse *et al.*, 1985, 1987). The α2,6-sialyltransferase has been shown by immunoelectron microscopy to codistribute with β1,4-galactosyltransferase within *trans* Golgi cisternae and the *trans* Golgi network (TGN) in rat hepatocytes and hepatoma cells (Roth *et al.*, 1985; Taatjes *et al.*, 1987). However, evidence that the α2,6-sialyltransferase in rat intestinal cells is present throughout the Golgi apparatus with the exception of the first *cis* cisterna demonstrates that polarized distribution of processing glycosyl-transferases within the Golgi apparatus may be characteristic only of selected cell types (Roth *et al.*, 1986; Taatjes *et al.*, 1988).

Attachment of N-acetylneuraminic acid in an α2,3 linkage to penultimate galactose residues of N-linked oligosaccharides is catalyzed by an

α2,3-sialyltransferase which has been purified to homogeneity from rat liver (Weinstein *et al.*, 1982b). In contrast to the α2,6-sialyltransferase, the α2,3-sialyltransferase accepts both Galβ1,3GlcNAc and Galβ1,4GlcNAc as substrates to form the terminal sequences NeuAcα2,3Galβ1,3GlcNAc and NeuAcα2,3-Galβ1,4GlcNAc, respectively (Weinstein *et al.*, 1982a). Competition between the α2,3- and the α2,6-sialyltransferases for the common substrate sequence Galβ1,4GlcNAc allows terminal sialylation of N-linked oligosaccharides to be influenced by differential enzyme expression (Paulson *et al.*, 1989; Lee *et al.*, 1989). The sequence Galβ1,4GlcNAc is in addition a suitable substrate for an α1,3-fucosyltransferase that attaches a fucose residue in an α1,3 linkage to the N-acetylglucosamine residue (Schachter *et al.*, 1985). Fucosylation of terminal Galβ1,4GlcNAc sequences inhibits action by the α2,6-sialyltransferase, and therefore extent of terminal sialylation may be influenced by competition between these two glycosyltransferases (Paulson *et al.*, 1978).

Mannose 6-phosphate Modification of Lysosomal Enzymes

N-linked high-mannose oligosaccharide side-chains of lysosomal enzymes may be modified by the addition of a mannose 6-phosphate signal that facilitates recognition of lysosomal enzymes by specialized mannose 6-phosphate receptors which mediate their translocation to the lysosome (Kornfeld, 1986; von Figura and Hasilik, 1986). Mannose 6-phosphate modification of newly-formed lysosomal enzymes is a post-translational event that involves the sequential action of two processing enzymes, a phosphotransferase and a phosphoglycosidase, which have been shown to be associated with dense membranes of *cis* Golgi cisternae (Pohlmann *et al.*, 1982). N-acetylglucosaminylphosphotransferase (phosphotransferase) acts first to catalyze the transfer of N-acetylglucosamine-phosphate from UDP-

GlcNAc to the hydroxyl of carbon 6 of a mannose residue to form a phosphodiester intermediate (Tabas and Kornfeld, 1980; Reitman and Kornfeld, 1981; Hasilik *et al.*, 1981). N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (phosphoglycosidase) then cleaves the "blocking" N-acetylglucosamine residue to expose the mannose 6-phosphate monoester recognition marker (Varki and Kornfeld, 1980; Waheed *et al.*, 1981).

The phosphotransferase, which has been partially purified from rat liver Golgi membranes, selectively phosphorylates native lysosomal enzymes (Waheed *et al.*, 1982). Phosphorylation at different N-linked glycosylation sites within a single lysosomal enzyme is random (Goldberg and Kornfeld, 1981; Hasilik and von Figura, 1981). Studies with chimeric aspartic proteinases derived from human pepsinogen and cathepsin D have demonstrated that insertion of two regions of cathepsin D into the pepsinogen backbone allowed this secretory protein to be phosphorylated. Moreover, site-directed mutagenesis has provided evidence that phosphorylation of the chimera is dependent upon the presence of lysine residues within these two sequence inserts. Use of the three-dimensional model of the aspartic proteinase pepsin has revealed that upon folding of the chimera, these critical lysine residues are brought together in close proximity so as to form a "signal patch" of lysine residues that apparently is specifically recognized by the phosphotransferase (James and Siedecki, 1986; Kornfeld and Mellman, 1989).

Structural studies of phosphorylated N-linked high-mannose oligosaccharide side-chains of lysosomal enzymes have revealed that at the time of phosphorylation the bulk of oligosaccharides contain six to nine mannose residues, and that a particular oligosaccharide side-chain may contain one or two mannose 6-phosphate monoesters (Tabas and Kornfeld, 1980; Varki and Kornfeld, 1980). Mannose residues which are most

commonly phosphorylated are those which occupy the terminal and penultimate positions on the Manα1,6 branch of the Manα1,6 arm, and phosphorylation of either of these residues precludes phosphorylation of the other. The second most commonly phosphorylated site is the penultimate mannose residue on the Manα1,3 arm (Varki and Kornfeld, 1980).

Presence of glucose residues not only inhibits phosphorylation of mannose residues on the Manα1,3 arm but also retards phosphorylation of mannose residues on the Manα1,6 arm, and therefore glucosidase processing of N-linked high-mannose oligosaccharides is a prerequisite for efficient lysosomal enzyme phosphorylation (Gabel and Kornfeld, 1982). In addition, presence of at least one Manα1,2Man sequence has been found to be a requirement for phosphorylation, and therefore complete processing by mannosidase I of high-mannose oligosaccharides to $Man_5GlcNAc_2$ structures precludes phosphotransferase activity (Couso *et al.*, 1986).

Inhibition of inter-organelle movement from the ER to the Golgi apparatus in mouse lymphoma cells by incubation at low temperature led to the discovery that initial phosphorylation of nascent lysosomal enzymes may occur within the ER. Transport-impaired mouse lymphoma cells produced monophosphorylated lysosomal enzymes in which the phosphomonoester was exclusively located on the Manα1,6 arm of N-linked high-mannose oligosaccharides. Reversal of the low temperature block was found to result in the appearance of diphosphorylated lysosomal enzymes that contained a single phosphomonoester on both the Manα1,3 and the Manα1,6 arms (Lazzarino and Gabel, 1988). Existence of a single phosphotransferase has been demonstrated by the fact that fibroblasts isolated from patients suffering from the autosomal recessive lysosomal disorder I-cell disease (mucolipidosis II) are deficient in phosphotransferase activity, and secrete lysosomal enzymes

that are not phosphorylated (von Figura and Hasilik, 1986). Accordingly, pre-Golgi and Golgi phosphorylation of nascent lysosomal enzymes is believed to be performed by a single phosphotransferase whose ability to phosphorylate mannose residues on the Manα1,3 arm is dependent upon prior limited mannose processing by Golgi mannosidase I.

The phosphodiesterase has been partially purified from rat liver (Varki and Kornfeld, 1981). It specifically cleaves N-acetylglucosamine residues that are α-linked to a phosphate group, and in contrast to the phosphotransferase, the phosphodiesterase will accept as substrates not only phosphorylated glycopeptides and isolated phosphorylated oligosaccharides but also molecules such as UDP-N-acetylglucosamine, N-acetylglucosamine-P-α-methylmannoside and N-acetylglucosamine-1-P. Although "blocking" N-acetylglucosamine residues may be removed from mannose residues located at various positions within a particular high-mannose oligosaccharide, the phosphodiesterase displays a preference for phosphodiesters that are located on the Manα1,3 arm and therefore the uncovering of mannose 6-phosphate signals in diphosphorylated oligosaccharides is not random. The phosphodiesterase is believed to be located in a Golgi compartment that is distal to the one which contains the phosphotransferase because in sucrose density gradients it fractionates with Golgi membranes of slightly lighter densities (Goldberg and Kornfeld, 1983).

Targeting and Transport within the Central Vacuolar System

In eukaryotic cells the ER, the Golgi apparatus, the TGN, secretory vesicles and granules, endosomes, lysosomes and the plasma membrane collectively constitute what is known as the "central vacuolar system" (Figure

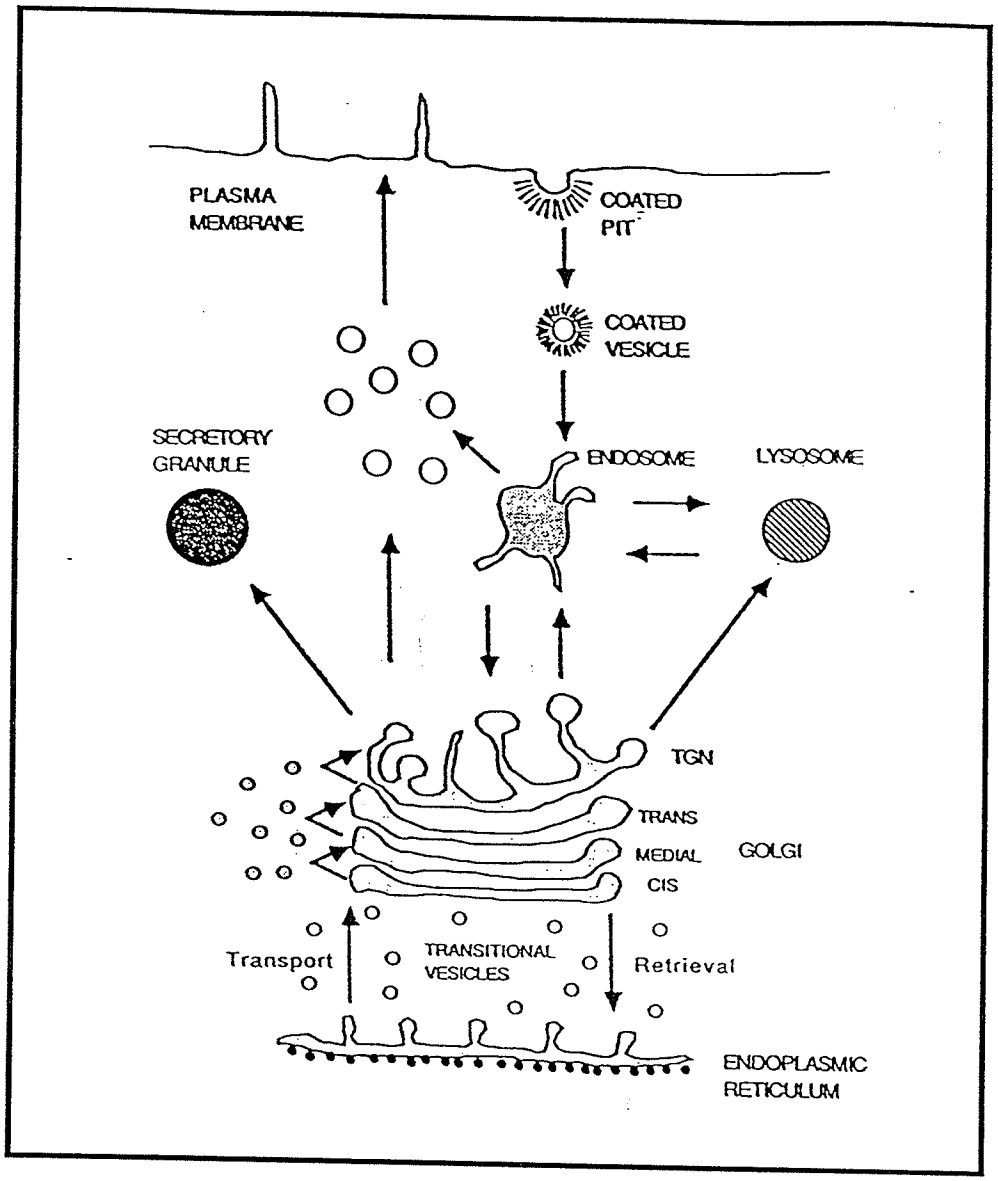
9). Access to this multi-organelle network may be through the ER or through endocytic vesicles that form upon invagination of the plasma membrane, and transport within the central vacuolar system accordingly may be along biosynthetic or endocytic pathways (Palade, 1975; Farquhar, 1985; Kelly, 1985; Griffiths and Simons, 1986; Burgess and Kelly, 1987; Pfeffer and Rothman, 1987; Goda and Pfeffer, 1989; Klausner, 1989).

The ER is an intricate network of tubules and cisternae that extends throughout the cytoplasm and that is dependent for its structural integrity upon an underlying microtubule system (Terasaki *et al.*, 1986; Lee and Chen, 1988; Dabora and Sheetz, 1988). Characterized by the presence of bound ribosomes, the rough ER is comprised of parallel arrays of broad flattened cisternae, membranes of which in the vicinity of the nucleus merge with the nuclear envelope. The ribosome-free smooth ER consists of widely dispersed tubules that extend towards the proximal or *cis* face of the Golgi apparatus. Transport of newly synthesized proteins from the ER to the Golgi apparatus is mediated by transitional vesicles which continuously bud from specialized regions of the smooth ER and fuse with membranes of *cis* Golgi cisternae (Palade, 1975). Studies with "semi-intact" CHO cells in which portions of the plasma membrane have been removed have revealed that budding and fusion of ER-to-Golgi transport vesicles require the participation of ATP, GTP, calcium and an N-ethylmaleimide-sensitive factor (NSF) in an ordered sequence (Beckers *et al.*, 1987; Beckers and Balch, 1988; Beckers *et al.*, 1989; Balch, 1989). In addition, fusion of transport vesicles with *cis* Golgi cisternal membranes has been shown in a number of different cell systems to be temperature-sensitive (Saraste and Kuismanen, 1984; Brand *et al.*, 1985; Fries and Lindstrom, 1986; Saraste *et al.*, 1986; Tartakoff, 1986; Roarke *et al.*, 1989). Vesicles which accumulate at the *cis* face of the Golgi apparatus upon

FIGURE 9

Central Vacuolar System

Entrance of nascent proteins to the central vacuolar system is facilitated by the presence of an appropriate signal sequence within the NH_2-terminus of the polypeptide which allows translation to be coupled with translocation into the lumen of the rough ER. Newly synthesized N-linked glycoproteins destined to be secreted, inserted into the plasma membrane or incorporated into lysosomes are transported sequentially through the ER and the *cis, medial* and *trans* Golgi cisternae to the TGN. The TGN is the site where the common biosynthetic pathway diverges into the constitutive and regulated secretory pathways, and the lysosomal pathway. The constitutive secretory pathway is traversed by proteins that are routinely secreted or inserted into the plasma membrane, and transport to the cell surface is mediated by secretory vesicles which continuously bud from membranes of the TGN, migrate to and fuse with the plasma membrane. Proteins targeted to the regulated secretory pathway are first concentrated within the lumen of the TGN and subsequently incorporated into secretory granules which remain in the cytoplasm until induced by an external stimulus to fuse with the plasma membrane. Lysosomal N-linked glycoproteins that possess mannose 6-phosphate monoesters are bound by mannose 6-phosphate receptors within the TGN, and this facilitates their transport to the lysosome along the lysosomal pathway which involves passage through an intermediary acidic pre-lysosomal compartment. (Diagram taken from Klausner, 1989).


PLASMA
MEMBRANE
COATED
PIT
COATED
VESICLE
SECRETORY
GRANULE
ENDOSOME
LYSOSOME
TGN
TRANS
MEDIAL
CIS
GOLGI
Transport
TRANSITIONAL
VESICLES
Retrieval
ENDOPLASMIC
RETICULUM

incubation at temperatures of 18 °C or below collectively comprise what has been termed the "16 °C compartment" (Morre *et al.*, 1989).

Variability in the time of migration of secretory and plasma membrane N-linked glycoproteins from the ER to the Golgi apparatus in human hepatoma HepG2 cells has prompted speculation that export from the ER is selective and possibly receptor-mediated (Lodish *et al.*, 1983; Ledford and Davis, 1983; Yeo *et al.*, 1985; Lodish, 1988). However, the existence of "transport receptors" remains to be demonstrated. In addition, evidence that an N-glycosylated tripeptide "bulk phase marker", which is too small to possess a positive transport signal, passes from the ER through the Golgi apparatus to the cell surface in CHO and HepG2 cells with a rapidity that is approximately equivalent to that of the most rapidly secreted proteins (Wieland *et al.*, 1987), has promoted the alternate theory that export from the ER is nonselective. Transport vesicles mediating ER to Golgi (and intra-Golgi) protein trafficking have been found to contain a coat protein that is not immunoreactive with polyclonal anti-clathrin antibodies and that possesses a distinctly different morphology than that exhibited by clathrin (Orci *et al.*, 1986; Malhotra *et al.*, 1989). Absence of clathrin from these transport vesicles infers that incorporation of protein into vesicles that bud from the smooth ER (and from Golgi cisternae) does not involve a preliminary receptor-mediated concentration step. Vesicle content therefore would be solely influenced by the local concentration of transportable protein present within the lumen of the parent compartment in the vicinity of the site of vesicle formation (Pfeffer and Rothman, 1987; Balch, 1989).

Differential transport rates of nascent proteins from the ER to the Golgi apparatus may be induced by the morphology of the ER itself. The ER possesses a very high membrane-surface to aqueous-volume ratio and this

may serve to retard protein trafficking in a nonspecific manner by enhancing the transient adsorption (through electrostatic and/or hydrophobic interactions) of newly synthesized soluble proteins to membrane-bound elements (Pfeffer and Rothman, 1987). In addition, time of export from the ER may also be reflective of the rapidity of attainment of correct tertiary and quaternary structure. The ER is the primary site of protein folding and oligomerization, and failure of polypeptides to fold or assemble correctly renders them "transport-incompetent" and precludes their efficient transfer to the Golgi apparatus (Carlin and Merlie, 1986; Rose and Doms, 1988; Hurtley and Helenius, 1989; Klausner, 1989). Specific retention within the ER of misfolded and misassembled proteins allows the cell to be protected against any deleterious effects that structurally defective proteins potentially may exert upon reaching their target compartments. Misfolded proteins, being of low solubility, have a tendency to aggregate (Rose and Doms, 1988), and frequently are found to be permanently, but noncovalently associated in the ER with a soluble protein known either as BiP (binding protein) or as the glucose-regulated protein grp78 (Hurtley *et al.*, 1989; Pelham, 1989a, b). BiP is a major luminal ER component in all cell types whose presence is apparently essential for viability (Pelham, 1989b), and whose synthesis is increased upon accumulation of abnormal proteins within the ER (Lee, 1987; Kozutsumi *et al.*, 1988). BiP has been shown to bind tightly to ATP, and ATP hydrolysis promotes the dissociation of BiP from its protein substrates (Munro and Pelham, 1986; Kassenbrock *et al.*, 1988). The noncovalent nature of the interaction between BiP and misfolded proteins allows the assumption that recognition of the latter is facilitated by the presence of abnormally exposed, inner core hydrophobic residues (Pelham, 1989b).

"Bulk-phase" nonselective transport of newly synthesized proteins from the ER to the Golgi apparatus necessarily implies that mechanisms exist for the selective retention of endogenous proteins. In theory, nascent proteins destined to become permanent residents of the ER either may not be transported to the Golgi apparatus at all, or alternatively may be transported to the Golgi apparatus and subsequently retrieved. Examination of the N-linked oligosaccharide side-chains of either the total population of ER glycoproteins or of specific resident ER glycoproteins has revealed the presence of predominantly high mannose $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ structures (Rosenfeld *et al.*, 1984; Lewis *et al.*, 1985; Brands *et al.*, 1985; Mutsaers *et al.*, 1985). Lack of evidence of Golgi-specific processing modifications on the covalently-bound carbohydrate of resident ER N-linked glycoproteins argues against retrograde trafficking of newly synthesized N-linked glycoproteins from the Golgi apparatus to the ER. Interestingly, presence of the sequence KDEL at the extreme COOH-terminus of the soluble ER proteins BiP, disulphide isomerase and grp94 has been shown to be an inter-species mechanism for retaining these proteins within the lumen of the ER (Munro and Pelham, 1987; Sorger and Pelham, 1987; Pelham, 1989b). Deletion of the KDEL sequence from the COOH-terminus of BiP resulted in its slow secretion from monkey COS cells, and addition of the SEKDEL sequence to the COOH-terminus of lysozyme caused this constitutively-secreted protein to be detained within the ER (Munro and Pelham, 1987).

Interaction between KDEL-containing ER proteins and putative KDEL-receptors appears to be very specific, given the demonstration that alteration of COOH-terminal sequences to KDAS, KDELGL or SEKDEV results in protein export from the ER (Pelham, 1989b; Zagouras and Rose, 1989). Although presence of the KDEL sequence confers ER residency, binding of

endogenous ER proteins to KDEL-receptors does not appear to take place within the ER. Evidence for this is predicated on the fact that KDEL-containing proteins are not membrane-associated within the ER, and that the rate of diffusion of BiP within the lumen of the ER of *Xenopus oocytes* has been shown to be independent of the presence or absence of the KDEL sequence (Ceriotti and Colman, 1988). Attachment of the KDEL sequence to the lysosomal enzyme cathepsin D was found to result in the accumulation of the phosphodiester-containing species, which implied that transport was arrested prior to arrival of cathepsin D in the Golgi compartment wherein the phosphodiesterase is located (Pelham, 1988). Accumulation of the phosphodiester-containing species of cathepsin D may be artificially induced by reduction of the incubation temperature, which blocks the fusion of ER transport vesicles with *cis* Golgi cisternal membranes and results in formation of the readily identifiable pre-Golgi "16 ^{o}C compartment" (Lazzarino and Gabel, 1988; Morre *et al.*, 1989). Consequently, it has been suggested that KDEL-containing endogenous ER proteins are transported, along with newly synthesized secretory and lysosomal proteins, from the ER to an intermediary pre-Golgi compartment which contains KDEL-receptors and phosphotransferase activity. Retention of soluble KDEL-containing proteins within the ER therefore is believed to be due to continuous receptor-mediated retrieval from a post-ER "salvage" compartment (Pelham, 1988, 1989a, b). Identification of a distinct post-ER compartment has recently been provided by the detection of 53 and 58 kDa proteins that localize predominantly within a tubulovesicular structure that lies proximal to the Golgi apparatus in human intestinal Caco-2, HepG2 and mouse myeloma cells (Saraste *et al.*, 1987; Schweizer *et al.*, 1988). Immunofluorescence studies using brefeldin A have provided evidence for the existence of a "recycling"

pathway from this 53 kDa protein-containing pre-Golgi compartment into the ER (Lippincott-Schwartz *et al.*, 1990). The antiviral antibiotic brefeldin A invokes the disruption of biosynthetic traffic along secretory and lysosomal pathways, and the rapid, but reversible dissolution of identifiable Golgi structure (Fujiwara *et al.*, 1988; Lippincott-Schwartz *et al.*, 1989; Doms *et al.*, 1989; Oda and Nishimura, 1989; Oda *et al.*, 1990). Brief exposure of rat kidney cells to brefeldin A was found to induce the retrograde movement of mannosidase II and galactosyltransferase along tubulovesicular processes that extended in a proximal direction from the Golgi apparatus and merged with the 53 kDa protein-containing compartment. Continued presence of brefeldin A resulted in the equivalent distribution of mannosidase II, galactosyltransferase and the 53 kDa protein between the intermediate "recycling" compartment and the ER. Addition of nocodazole, which inhibits polymerization of tubulin monomers (De Brabander *et al.*, 1976), caused an accumulation of these three proteins within the intermediate "recycling" compartment. This demonstrated that whereas anterograde movement out of the ER into the intermediate "salvage" or "recycling" compartment is microtubule-independent, retrograde movement into the ER is microtubule-dependent.

The Golgi apparatus is a topologically and functionally polarized, membranous organelle that is located distal to the ER in the pericentrosomal region of the cell. It typically consists of three to eight platelike saccules or cisternae, each of which exhibits a convex proximal face (directed towards the nucleus), a concave distal face, a fluid-filled lumen, and slightly dilated rims (Farquhar and Palade, 1981). Topological polarity of the Golgi apparatus is evident in the fact that nascent proteins enter at the proximal, immature or *"cis"* face and exit at the distal, mature or *"trans"* face. This has been clearly

demonstrated by immunocytochemical studies in CHO cells infected with vesicular stomatitis virus (VSV) in which alterations in incubation temperature generate synchronized waves of newly synthesized viral membrane N-linked glycoproteins that proceed vectorially from the *cis*, through the *medial*, to the *trans* cisternae of the Golgi apparatus (Bergmann *et al.*, 1981; Bergmann and Singer, 1983; Saraste and Hedman, 1983; Saraste and Kuismanen, 1984). Functional polarity of the Golgi apparatus is predicated on cisternal compositional heterogeneity, evidence for which has been obtained from numerous histochemical, immunocytochemical and Golgi subfractionation studies (Farquhar and Palade, 1981; Farquhar, 1985; Dunphy and Rothman, 1985; Kornfeld and Kornfeld, 1985; Roth, 1987; Rambourg and Clermont, 1990). Compartmentalization of N-linked glycoprotein processing enzymes within *cis*, *medial* and *trans* cisternae not only enhances fidelity of biosynthetic processing, but also allows extent of oligosaccharide modification to be used as an indication of passage through the Golgi apparatus. Immunocytochemical detection of secretory, plasma membrane and lysosomal N-linked glycoproteins in all Golgi cisternae, coupled with evidence that their oligosaccharide side-chains are similarly covalently modified by Golgi processing enzymes, has promoted the conclusion that proteins of all three classes traverse a common biosynthetic pathway that passes through *cis*, *medial* and *trans* cisternae of the Golgi apparatus (Kraehenbuhl *et al.*, 1977; Geuze *et al.*, 1979; Green *et al.*, 1981; Bergmann *et al.*, 1981; Brands *et al.*, 1982; Geuze *et al.*, 1984; Kornfeld and Kornfeld, 1985; Roth, 1987).

Intracellular location and architectural integrity of the Golgi apparatus are dependent upon an intact microtubule system. Anchoring of the Golgi complex to an underlying microtubule network appears to be mediated by 58

and 110 kDa proteins present at the cytoplasmic face of Golgi cisternal membranes (Allan and Kreis, 1986; Bloom and Brasher, 1989). However, maintenance of the stacked arrangement of Golgi cisternae in the pericentrosomal region of the cell is not a prerequisite for efficient biosynthetic processing and vectorial transport of newly synthesized N-linked glycoproteins through *cis, medial* and *trans* Golgi cisternae. Both the rate and extent of terminal sialylation of VSV G protein in virus-infected rat kidney cells was found to be unaltered by the presence of nocodazole (Rogalski *et al.*, 1984), and treatment of metabolically radiolabelled CHO cells with colcemid has been shown to affect neither rate nor fidelity of processing of plasma membrane N-linked glycoproteins (Stults *et al.*, 1989). Evidence that biosynthetic trafficking through the Golgi apparatus is a dissociative process involving repeated rounds of vesicle budding and fusion has been provided by Rothman and coworkers through a variety of cell-fusion studies (Rothman *et al.*, 1984a, b). Fusion of VSV-infected mutant CHO cells deficient in either GlcNAc-transferase I or galactosyltransferase with uninfected wild-type CHO cells was found to result in the efficient acquisition by VSV G protein of endoglycosidase H resistance and galactose, respectively, which demonstrated that intercisternal transport between different Golgi populations had occurred. Analogous studies using VSV-infected mutant CHO cells deficient in sialyltransferase showed that whereas transfer of VSV G protein between *medial* and *trans* Golgi cisternae of different Golgi populations was efficient, lateral transfer between *trans* Golgi cisternae was not.

Inter-Golgi compartmental transfer of VSV G protein has been successfully reconstituted in a cell-free system (Balch *et al.*, 1984a; Balch and Rothman, 1985), and incubation of isolated Golgi stacks obtained from VSV-infected CHO cells in the presence of cytosol and ATP precipitates the

formation from the dilated rims of Golgi cisternae of non-clathrin coated vesicles containing newly synthesized VSV G protein (Balch *et al.*, 1984b; Orci *et al.*, 1986). Results of cell-free morphological and biochemical studies of vesicular transport of VSV G protein between Golgi cisternae in CHO cells have demonstrated that a "coated bud (pit)-coated vesicle-uncoated vesicle" system is responsible for targeting and transport of biosynthetic traffic through the Golgi apparatus. Assembly and budding of coated pits requires the presence of cytosol and ATP, and the non-clathrin coat proteins are believed to be derived from the cytosol. Inhibition of long-chain acyl-CoA synthase prevents budding of coated pits from Golgi cisternae and it has been suggested that covalent modification of a soluble transport component(s) by fatty acid addition allows it to become anchored to the lipid bilayer. Targeting of coated transport vesicles to acceptor cisternae is followed by the GTP-dependent removal of the cytoplasmic coat. Fusion of uncoated vesicles with acceptor cisternal membranes has been demonstrated to be a complex process that requires the participation of NSF, ATP, acyl-CoA, a cytosolic Factor B, and three soluble NSF-attachment proteins (SNAP α, β and γ) (Pfanner *et al.*, 1989, 1990; Orci *et al.*, 1989; Clary *et al.*, 1990; Rothman and Orci, 1990).

Segregation of newly synthesized secretory, plasma membrane and lysosomal N-linked glycoproteins takes place within the TGN, which is a torturously extensive tubular reticulum that lies distal to, and is continuous with, *trans* Golgi cisternae (Saraste and Kuismanen, 1984; Roth *et al.*, 1985; Griffiths and Simons, 1986; Taatjes and Roth, 1986; Rambourg and Clermont, 1990). Immunocytochemical studies have shown that the TGN, *trans* Golgi cisternae and associated clathrin-coated vesicles preferentially accumulate 3-(2,4-dinitro-anilino)-3'-amino-N-methyldipropylamine (DAMP), which indicates that these structures are acidic compartments. Moreover,

comparative studies have revealed that acidification of *trans* Golgi cisternae and the TGN appears to be singularly characteristic of fibroblasts and hepatocytes (Anderson and Pathak, 1985; Schwartz *et al.*, 1985; Anderson and Orci, 1988). Detailed morphological examination of the TGN in VSV-infected BHK cells has revealed that a minor portion of the total surface area of this specialized organelle is attributable to a flattened cisternal compartment that lies immediately adjacent to the *trans* - most cisternae of the Golgi apparatus, and the bulk of the surface area is contained within a multiply evaginated tubulovesicular compartment (Griffiths *et al.*, 1989). Interestingly, three-dimensional electron microscopy of the Golgi apparatus and the TGN has provided evidence that there is a progressive perforation of Golgi cisternae in a *cis* - to - *trans* direction, and that formation of the TGN appears to arise from a "peeling-off" of highly fenestrated portions from non-compact regions of *trans* Golgi cisternae (Rambourg *et al.*, 1987; Rambourg and Clermont, 1990). Hypertrophy of the TGN in secretory cells may be induced by stimulation of secretion (Hand and Oliver, 1984; Griffiths and Simons, 1986), and an artificially-induced transient inhibition of VSV G protein transport in virus-infected BHK cells to the plasma membrane was found to result in a correspondingly rapid and transient enlargement of the TGN. Increase in surface area and volume of the TGN was found to occur concomitantly with both a large reduction in size, and a small increase in size, of the associated Golgi cisternal stack and ER, respectively, which revealed a vital cooperativity among these three structures in response to an increase in biosynthetic secretory traffic (Griffiths *et al.*, 1989).

Targeting of newly synthesized proteins to regulated and constitutive secretory pathways occurs concomitant with their exit from the TGN. Formation of regulated secretory granules is a sequential process that

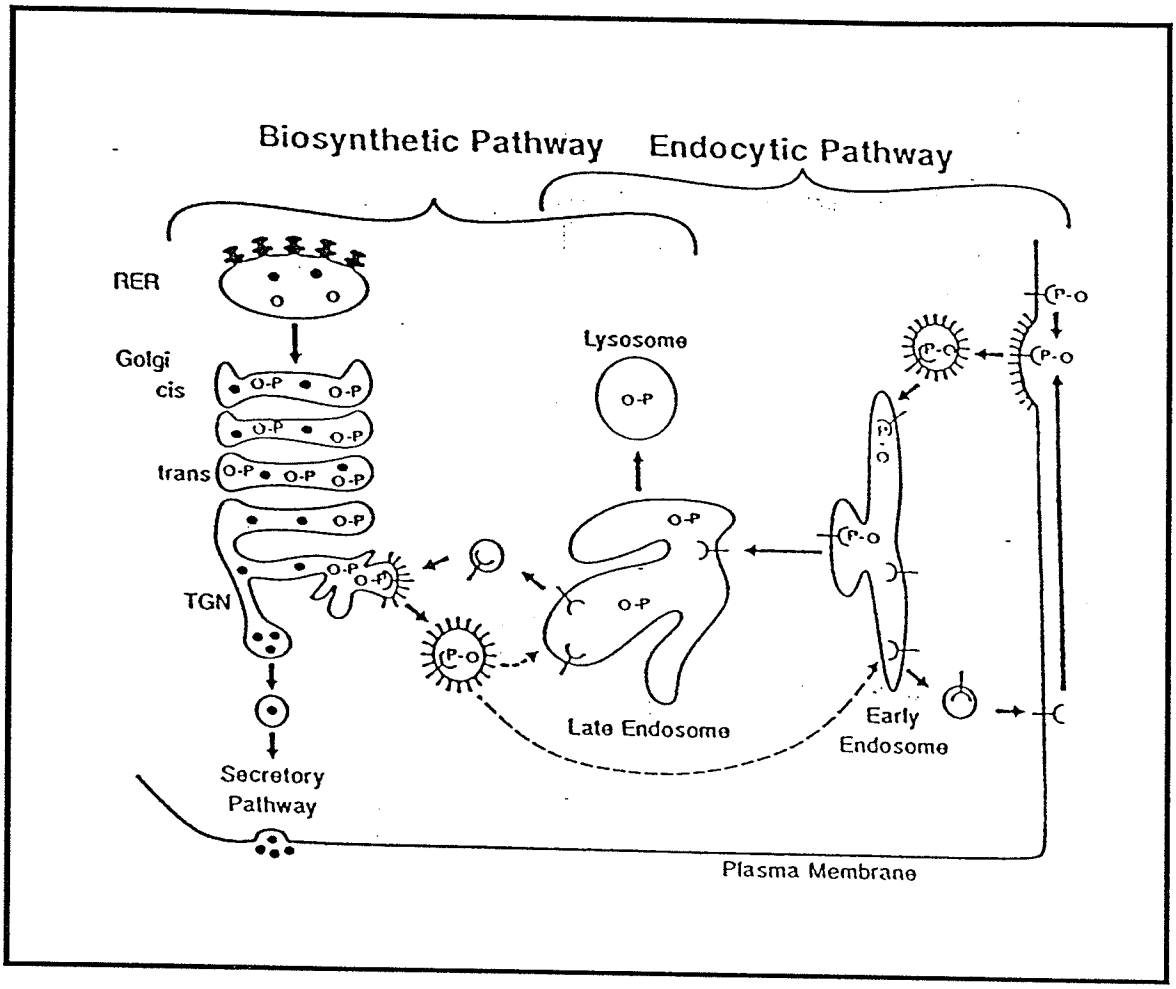
involves initially the selective aggregation of secretory protein within the TGN. Clathrin-associated, membrane-enveloped aggregates of secretory protein that bud from the TGN give rise to immature secretory granules, and these in turn mature into dense-core, secretory granules of uniform size upon removal of excess membrane and clathrin (Farquhar and Palade, 1981; Tooze and Tooze, 1986; Burgess and Kelly, 1987; Grossman, 1988). Electron microscopy studies using murine pituitary tumor AtT20 cells have demonstrated that influenza hemagglutinin (HA) and murine MHV-A59 progeny virions (markers for the constitutive secretory pathway), and proinsulin and ACTH (markers for the regulated secretory pathway), colocalize within individual Golgi cisternae. However, once within the post-Golgi area, HA and progeny virions are segregated into noncoated, constitutive secretory vesicles whereas proinsulin and ACTH are exclusively contained within dense-core, secretory granules. Segregation of constitutive and regulated secretory proteins has been found to precede the formation of clathrin-associated, immature secretory granules and is not concurrent with membrane-trimming during granule maturation (Orci *et al.*, 1987; Tooze *et al.*, 1987).

Targeting of lysosomal enzymes to lysosomes along biosynthetic or endocytic pathways within the central vacuolar system (Figure 10) is predicated on the recognition by specialized mannose 6-phosphate receptors (MPR) of exposed phosphomonoesters present within the N-linked carbohydrate moieties of lysosomal enzymes. Two distinct receptors have been identified and differentiated with respect to molecular size, ligand-binding characteristics and function (Sly and Fischer, 1982; von Figura and Hasilik, 1986; Kornfeld, 1986; Dahms *et al.*, 1989; Kornfeld and Mellman, 1989; Schwartz, 1990). The "cation-independent" mannose 6-phosphate receptor

FIGURE 10

Biosynthetic and Endocytic Lysosomal Pathways

Newly synthesized, phosphorylated lysosomal enzymes (O-P) are transported together with secretory proteins (●) along the main secretory pathway through the *cis*, *medial* and *trans* cisternae of the Golgi apparatus. Presence of exposed phosphomonoesters on nascent lysosomal enzymes allows them to be recognized by MPRs within the TGN, which results in their segregation from bulk secretory traffic, incorporation into clathrin-coated vesicles and subsequent transport along the biosynthetic lysosomal pathway to an acidified pre-lysosomal compartment. Similarly, transport of extracellular lysosomal enzymes along the endocytic lysosomal pathway to an intermediate endosomal compartment is also MPR-mediated. Dissociation of MPR-lysosomal enzyme complexes within the pre-lysosomal, endosomal compartment is promoted by a reduction in pH, following which the lysosomal enzymes are targeted to the lysosome and the MPRs return to either the TGN or the cell surface for additional rounds of enzyme transport. (Diagram taken from Dahms *et al.*, 1989).


Biosynthetic Pathway
Endocytic Pathway
RER
Golgi
cis
trans
TGN
Secretory
Pathway
Lysosome
Late Endosome
Early
Endosome
Plasma Membrane

(CI-MPR) exhibits an apparent molecular weight of 215 kDa and binds ligand within the pH range 6.0 - 7.4 in the absence of divalent cations. In contrast, the "cation-dependent" mannose 6-phosphate receptor (CD-MPR) has an apparent molecular weight of 46 kDa and displays optimal ligand binding within the pH range 6.0 - 6.3 in the presence of divalent cations. Significant ligand dissociation from both CI-MPR and CD-MPR is induced by reduction of pH to less than 5.5 (Sahagian *et al.*, 1981; Steiner and Rome, 1982; Hoflack and Kornfeld, 1985a, b; Hoflack *et al.*, 1987). Although selective binding of a particular lysosomal enzyme to either receptor has not been demonstrated, comparative binding studies have shown that the CI-MPR has a higher affinity for most mannose 6-phosphate-containing ligands than the CD-MPR (Tong and Kornfeld, 1989; Tong *et al.*, 1989). Both receptors are transmembrane N-linked glycoproteins, and cDNAs have been cloned from rat, human or bovine sources (Sahagian and Steer, 1985; von Figura *et al.*, 1985; Lobel *et al.*, 1987, 1988; Dahms *et al.*, 1987; Pohlmann *et al.*, 1987; Morgan *et al.*, 1987; Oshima *et al.*, 1988; MacDonald *et al.*, 1988). The CI-MPR exists either as a monomer or as an oligomer, and binds two moles of mannose 6-phosphate or one mole of a divalent phosphorylated oligosaccharide per monomer (Perdue *et al.*, 1983; Stein *et al.*, 1987a; Tong *et al.*, 1989). The CD-MPR, which exists as a dimer within the membrane and as either a dimer or a tetramer in solution, binds one mole of mannose 6-phosphate and 0.5 mole of divalent phosphorylated oligosaccharide per monomer (Stein *et al.*, 1987b; Dahms *et al.*, 1989; Tong and Kornfeld, 1989). Ligand binding studies using immobilized receptor and phosphorylated oligosaccharides have provided evidence that high affinity receptor-ligand interaction requires the presence within the ligand of at least two phosphomannosyl units in monoester linkage (Varki and Kornfeld, 1983; Fischer *et al.*, 1982; Hoflack *et al.*, 1987).

Therefore a "two-site" model for receptor-ligand binding has been proposed in which divalent interaction between CI-MPR monomer/CD-MPR dimer and ligand is mediated by two phosphomonoesters that are present on the lysosomal enzyme within either the same or different N-linked oligosaccharide side-chains.

Elucidation of the CI-MPR primary sequence allowed the surprising discovery that this mannose 6-phosphate receptor is identical to the insulin-like growth factor II (IGF-II) receptor (Morgan *et al.*, 1987). Biochemical studies undertaken to verify the bifunctional nature of this protein have shown that the CI-MPR possesses one high affinity binding site for the nonglycosylated polypeptide IGF-II per monomer. The CI-MPR also exhibits a low affinity for insulin-like growth factor I, however the CD-MPR does not bind to either growth factor (Roth *et al.*, 1987; Braulke *et al.*, 1988; Tong *et al.*, 1989; Kiess *et al.*, 1988; MacDonald *et al.*, 1988). Although distinct binding sites for phosphorylated ligand and IGF-II appear to be present within the CI-MPR, preliminary binding of either lysosomal enzyme or IGF-II exerts a negative influence on subsequent binding of the second substrate (Kiess *et al.*, 1988, 1989).

Insight into the itinerary of mannose 6-phosphate-dependent trafficking within the central vacuolar system has been provided by immunolocalization studies conducted with a variety of cell types, using antibodies prepared against the CI-MPR and the CD-MPR, which have demonstrated not only the varying presence of these receptors within the ER, the Golgi apparatus, the TGN, clathrin-coated vesicles, endosomes and clathrin-coated pits at the plasma membrane, but also their conspicuous absence within mature lysosomes (Fischer *et al.*, 1980; Willingham *et al.*, 1981, 1983; Campbell and Rome, 1983; Sahagian and Neufeld, 1983; Shepherd *et al.*,

1984; Brown and Farquhar, 1984; Sahagian and Steer, 1985; Geuze *et al.*, 1984, 1985; Braulke *et al.*, 1987; Griffiths *et al.*, 1988, 1989; Bleekemolen *et al.*, 1988; Brown, 1990). Demonstration of the accessibility of intracellular CI-MPR and CD-MPR to anti-MPR antibodies (Sahagian, 1984, Gartung *et al.*, 1985; Stein *et al.*, 1987c), coupled with results of immunolocalization studies which have shown that at steady state only a comparatively minor fraction is present within the plasma membrane as opposed to intracellular locations, have indicated that there are single pools of CI-MPR and CD-MPR which participate in mannose 6-phosphate-dependent trafficking. Interestingly, although both the CI-MPR and the CD-MPR are present within the plasma membrane, internalization and delivery of extracellular lysosomal enzymes to lysosomes along the endocytic pathway has been found to be mediated solely by the CI-MPR (Stein *et al.*, 1987c), which presumably is a reflection of the inability of the CD-MPR to bind ligand at neutral pH (Hoflack *et al.*, 1987).

Immunoperoxidase cytochemical detection of the CI-MPR in rat hepatic, exocrine pancreatic and epididymal cells, and in cultured Clone 9 rat hepatocytes, provided evidence that within the Golgi apparatus this receptor was exclusively concentrated in one or two *cis* cisternae (Brown and Farquhar, 1984; Brown *et al.*, 1984; Brown *et al.*, 1986). Apparent absence of the CI-MPR within *trans* Golgi cisternae and the TGN, coupled with previous biochemical evidence which showed that the enzymes involved in formation of the mannose 6-phosphate signal are associated with *cis* Golgi cisternal membranes (Pohlmann *et al.*, 1982), promoted speculation that segregation of newly synthesized lysosomal enzymes from bulk secretory and plasma membrane traffic occurred within an early, proximal Golgi compartment. However, immunogold labelling of ultrathin cryosections of rat liver and human hepatoma HepG2 cells revealed the colocalization of the CI-MPR,

albumin, and the lysosomal enzymes cathepsin D, β-hexosaminidase and α-glucosidase throughout the Golgi apparatus. Furthermore, tubules and clathrin-coated buds of the TGN appeared to be enriched in both the CI-MPR and lysosomal enzymes (Geuze *et al.*, 1984, 1985). Presence of the CI-MPR within *trans* Golgi cisternae and the TGN has also been reported in CHO cells (Willingham *et al.*, 1981, 1983), in NRK cells (Griffiths *et al.*, 1988), and in normal human and I-cell disease fibroblasts (Brown, 1990). In addition, both the CI-MPR and the CD-MPR have been found to be enriched within the TGN of U937 monocytes (Bleekemolen *et al.*, 1988). These data, combined with biochemical evidence that lysosomal enzymes may possess both phosphorylated high-mannose and terminally-processed complex N-linked oligosaccharides (Hasilik and von Figura, 1981; Giselmann *et al.*, 1983; Fedde and Sly, 1985; Kozutsumi *et al.*, 1986), have argued in favor of the hypothesis that the TGN is the site of diversion of the mainstream biosynthetic pathway into secretory and lysosomal routes.

Direct biochemical evidence that the CI-MPR and the CD-MPR constitutively recycle between the plasma membrane and sialyltransferase-containing compartments has been provided by Duncan and Kornfeld (1988) who demonstrated that limited exposure of CHO cells to neuraminidase was followed by the reappearance, at commensurable rates, of resialylated forms of both receptors at the cell surface. Significant delay in the α-mannosidase I processing of high-mannose N-linked oligosaccharides of cell surface receptors synthesized in the presence of dMM revealed the infrequent passage of recycling CI-MPR and CD-MPR through *cis* or *medial* Golgi cisternae. Identification of the TGN, as opposed to *trans* Golgi cisternae, as being the site of receptor resialylation was predicated on the lack of evidence of an increase

in galactosylation of recycling receptors within a mutant murine lymphoma cell line slightly impaired in UDP-Gal translocation.

MPR-lysosomal enzyme complexes that form within the TGN are subsequently enclosed within clathrin-coated vesicles (Campbell *et al.*, 1983; Geuze *et al.*, 1985; Lemansky *et al.*, 1987) and transported along the biosynthetic lysosomal pathway to a putative pre-lysosomal compartment (PLC). The acidic internal milieu of the PLC induces receptor-ligand dissociation, following which the MPR recycles back to the TGN for another round of enzyme transport by a transport mechanism that apparently does not involve clathrin (Draper *et al.*, 1990), and the dissociated lysosomal enzyme is delivered by an as yet unspecified means to the lysosome (Kornfeld and Mellman, 1989; Griffiths, 1989). Recently, an acidic, tubulovesicular structure believed to represent the putative PLC has been detected in normal and VSV-infected NRK cells in the vicinity of the TGN in the perinuclear region of the cell (Griffiths *et al.*, 1988). Differentiation of the PLC from the TGN has been predicated on both the conspicuous absence of the VSV G secretory glycoprotein, and the highly enriched presence of the CI-MPR, acid phosphatase and the heavily sialylated lysosomal membrane glycoprotein lgp120 (Griffiths *et al.*, 1988, 1989, 1990). Evidence that the PLC accumulates substantially greater amounts of the lysosomotropic agent DAMP than the TGN has revealed that it possesses a significantly lower luminal pH than the TGN, which has been postulated to have an internal pH of 6 - 6.5 (Griffiths and Simons, 1986). Significant dissociation of MPR-lysosomal enzyme complexes occurs at pH 5.5 (Sahagian *et al.*, 1981; Steiner and Rome, 1982; Hoflack and Kornfeld, 1985a, b; Hoflack *et al.*, 1987), and evidence that the luminal pH of the PLC is of sufficient low level to induce receptor-ligand dissociation has been inferred by the finding that endosomes and lysosomes,

which are believed to have an internal pH of 5.5 or below (Mellman *et al.*, 1986), incorporate DAMP to a similar extent as the PLC.

Although typically the bulk of newly-synthesized lysosomal enzymes are bound within the TGN to MPR and transported along the biosynthetic lysosomal pathway to the lysosome, a varying proportion of nascent lysosomal enzymes may be secreted from the cell (von Figura and Hasilik, 1986; Kornfeld, 1987). Extracellular lysosomal enzymes that bind to cell surface CI-MPR are internalized within clathrin-coated vesicles (Willingham *et al.*, 1981; Geuze *et al.*, 1985) and subsequently delivered to the lysosome along the endocytic pathway which involves sequential passage through "early" endosomal structures and the PLC. "Early" endosomes are defined as the population of small vesicles and tubulovesicular structures in the vicinity of the cell surface that are the first intracellular compartments to receive endocytosed material. Characterized by their ability to be selectively labelled with endocytic markers at 20 ^{o}C, "early" endosomes possess internal pH values within the range 5.5 to 6.3 and represent the site of acidification-dependent processes such as the release of iron from transferrin and the dissociation of insulin, low density lipoprotein and α_2-M from their respective receptors (Yamashiro and Maxfield, 1984; Schmid *et al.*, 1988; Kornfeld and Mellman, 1989; van Deurs *et al.*, 1989). Dissociation of ligand from the asialoglycoprotein receptor has been shown in rat liver and human hepatoma HepG2 cells to occur within a peripheral "early" endosomal, CI-MPR-enriched compartment, termed "CURL" for Compartment of Uncoupling of Receptor and Ligand (Geuze *et al.*, 1983; Stoorvogel *et al.*, 1989). Differentiation of CURL from the PLC has been predicated not only on location within the cell, relative time of arrival of endocytosed ligands and presence of acid phosphatase, but also on the finding that endocytic markers

are precluded from reaching the PLC at 20 °C. Demonstration that with prolonged chase at 37 °C, endocytic markers are transported from the PLC to mature lysosomes has revealed that the PLC represents not only the site of dissociation of MPR-lysosomal enzyme complexes, delivered from either the TGN or the plasma membrane, but also the "late" endosomal structure through which all endocytosed material passes enroute to the lysosome (Messner *et al.*, 1989; Griffiths, 1989; Griffiths *et al.*, 1988).

Insight into the molecular regulation of trafficking of the bovine CI-MPR between the plasma membrane, TGN and endosomal compartments has been provided by examination of the ability of mutant receptors containing truncated cytoplasmic tails to function in nascent lysosomal enzyme transport and endocytosis upon expression within receptor-deficient mouse L cells. Determinants for rapid endocytosis of radiolabelled β-galactosidase were found to reside within the inner 75 residues of the cytoplasmic tail, whereas the signal for efficient sorting of lysosomal cathepsin D was revealed to be present within the outer 40 residues (Lobel *et al.*, 1989). Receptor-mediated endocytosis was shown to be predicated on the presence of two tyrosine residues within the inner region of the cytoplasmic tail of the CI-MPR, which appear to be crucial participants in the binding of the cytoplasmic tail of the CI-MPR to a specific set of clathrin-binding 100, 50 and 16 kDa polypeptides that are present within coated pits in the plasma membrane (Pearse, 1988; Glickman *et al.*, 1989). Interestingly, a different set of clathrin-binding 100, 47 and 19 kDa polypeptides have been detected within Golgi membrane coated pits that are capable of interacting with mutant CI-MPR cytoplasmic tails that lack tyrosine residues (Brodsky, 1988; Pearse, 1988). These data reveal that selective routing of the CI-MPR appears to be regulated by interaction between the cytoplasmic tail of this receptor and specific sets of

clathrin-binding "adaptor" polypeptides located within coated pits at the plasma membrane and within the Golgi apparatus. However, evidence that a chimeric receptor, containing the extracellular and transmembrane domains of human EGF receptor joined to the cytoplasmic domain of the CI-MPR, remains localized at the surface of transfected mouse fibroblasts has revealed that presence of the intact cytoplasmic domain of the CI-MPR is not sufficient to direct receptor recycling between intracellular compartments (Dintzis and Pfeffer, 1990).

INTRODUCTION TO THE CURRENT WORK

Previous work in the Jamieson laboratory has been aimed at studying the effect of inflammation on glycoprotein biosynthesis in the liver, using α_1-AGP as a representative acute phase reactant. In the course of these studies it was discovered that turpentine-induced inflammation in the rat not only promotes an elevation in liver sialyltransferase activities (Turchen *et al.*, 1977; Kaplan *et al.*, 1983), but also invokes the release of hepatic sialyltransferase into the serum (Kaplan *et al.*, 1983). Use of rat and human asialo-α_1-AGP as high molecular weight acceptors, combined with use of lactose to confirm the nature of the linkage formed, led to the identification of the enzyme released during turpentine-induced inflammation in the rat to be the Galβ1,4GlcNAc α2,6-sialyltransferase (α2,6-ST, EC 2.4.99.1) responsible for the addition of N-acetylneuraminic acid to terminal positions of N-linked complex oligosaccharide chains (Kaplan *et al.*, 1983).

Elevated serum sialyltransferase levels have been reported in a variety of pathological and inflammatory conditions including rheumatoid arthritis, cystic fibrosis, partial hepatectomy, malignant melanoma, lymphoma, leukemia and cancer of the breast, ovary, lung and colon (Mookerjea *et al.*, 1972; Kessel and Allen, 1975; Kessel and Henderson, 1977; Kessel *et al.*, 1978; Kondo *et al.*, 1981; Bernacki and Kim, 1977; Dao and Ip, 1977; Ip and Dao, 1978; Ip, 1979; Silver *et al.*, 1978, 1979; Dwivedi *et al.*, 1984; Dall'Olio *et al.*, 1989). Increase in serum sialyltransferase activity in tumor-bearing animals has been shown to be related to the extent of neoplasia, and serum sialyltransferase levels have been found to return to normal during remission or following tumor removal (Kessel *et al.*, 1976; Kessel and

Henderson, 1977; Silver *et al.*, 1978; Dao *et al.*, 1980; Kondo *et al.*, 1981; Dwivedi *et al.*, 1984). Studies conducted in the Jamieson laboratory using lactose as the low molecular weight acceptor have shown that the enzyme responsible for the elevation of sialyltransferase activity in the sera of humans suffering from several different types of cancer was an α2,6-sialyltransferase (Kaplan, 1983).

Increased hepatic synthesis and secretion of serum N-linked glycoproteins during inflammation (Koj, 1974; Jamieson, 1983; Hatton *et al.*, 1983; Jamieson *et al.*, 1986) is accompanied by a proliferation of the liver Golgi complex and an accentuated presence of the glycosyltransferases involved in N-linked glycoprotein processing (Turchen *et al.*, 1977; Lombart *et al.*, 1980; Jamieson *et al.*, 1983; Kaplan *et al.*, 1983). However, although turpentine-induced inflammation in the rat has been shown to promote an increase in hepatic levels of the Galβ1,3(4)GlcNAc α2,3-sialyltransferase (EC 2.4.99.6) and the GlcNAc β1,4-galactosyltransferase (EC 2.4.1.38), these glycosyltransferases are not released into the serum (Kaplan *et al.*, 1983). Therefore the rat hepatic α2,6-ST represents a novel acute phase reactant. In contrast to all other known acute phase reactants which are plasma proteins that only transit through the organelles of the liver during their biosynthesis, the membrane-bound α2,6-ST is an integral component of the hepatic Golgi biosynthetic machinery involved in N-linked glycoprotein processing.

The goal of the work presented in this thesis was to provide an explanation for the preferential release of the hepatic α2,6-ST into the serum during inflammation. Theory preceded experimentation, and the model proposed to explain the acute phase behaviour of the hepatic α2,6-ST incorporated four major assumptions. The first assumption was that it was highly likely that release of the α2,6-ST from the Golgi membrane was due to

the action of a proteinase. The second assumption was that during turpentine-induced inflammation the observed increase in hepatic sialyltransferase activity, presumably due to an elevation in enzyme synthesis, was not accompanied by a change in intracellular location. This was a reasonable assumption given the demonstration that although malignant transformation can induce a reorganization of *trans* Golgi apparatus elements, the α2,6-ST and the GlcNAc β1,4-galactosyltransferase remain colocalized within rat hepatoma cells (Taatjes *et al.*, 1987).

The third assumption was that the α2,6-ST was physically divided into a globular catalytic domain and a flexible linker region that was contiguous with the sequence of hydrophobic amino acids which allowed insertion of the enzyme into the lipid bilayer of the Golgi membrane. Evidence of the membrane-bound nature of the rat hepatic α2,6-ST had been provided by the finding that it could be released from purified Golgi membranes with detergent (Weinstein *et al.*, 1982a, b). In addition, topological studies using "right-side-out" Golgi vesicles isolated from mouse and rat liver had demonstrated that sialyltransferases are integrally-bound to the inner face of the Golgi membrane such that the domain containing the active site is oriented towards the lumen (Carey and Hirschberg, 1981; Fleischer, 1981). Presence of a linker region between the catalytic domain and the lipid bilayer would infer that the latter is not necessary for the maintenance of enzymatic competence. The lack of participation of the Golgi membrane in stabilizing the catalytically-active tertiary conformation of the α2,6-ST had been implicated by the fact that a significant change in affinity towards either CMP-NeuAc or asialoglycoprotein acceptor was not exhibited by the rat enzyme following its secretion from the liver during inflammation (Kaplan *et al.*, 1983). Moreover, limited proteolytic cleavage of the extended linker region

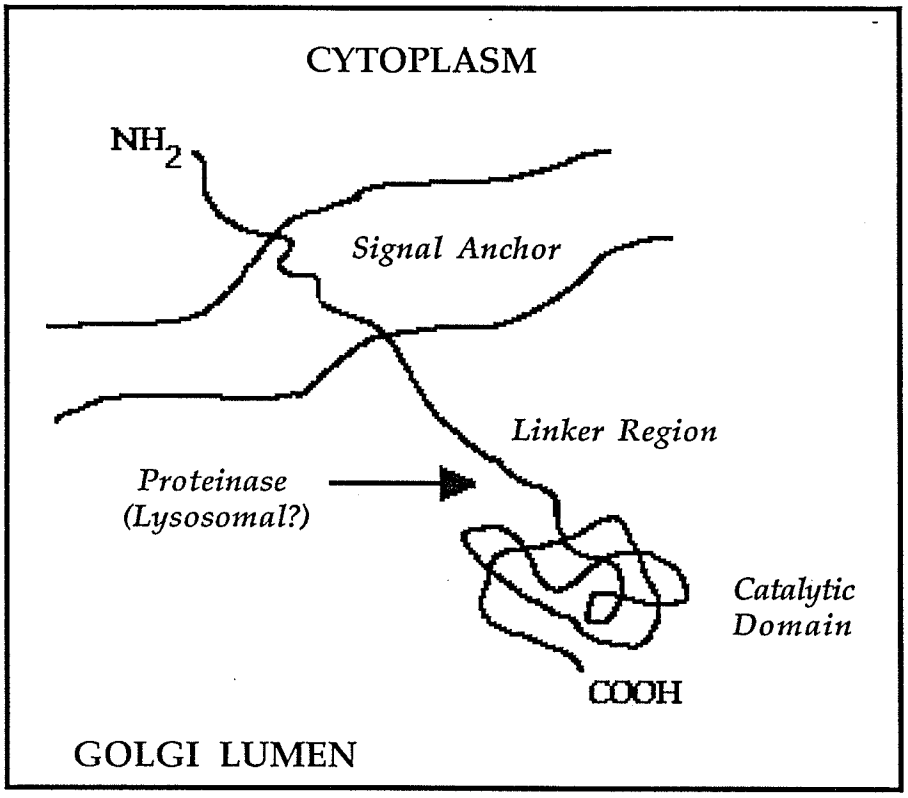
would allow release into the Golgi lumen of a lower molecular weight form of the α2,6-ST that possessed an undamaged catalytic domain. The presence of catalytically-active lower molecular weight forms of sialyltransferase have been detected during the purification of this glycosyltransferase from rat liver homogenate, which indicates that it is particularly sensitive to proteinase action (Weinstein *et al.*, 1982b).

The fourth assumption was that the proteinase involved in release of the α2,6-ST was a lysosomal enzyme that possessed a specificity for a uniquely exposed sequence of amino acids present within the linker region of the glycosyltransferase. It was hypothesized that an inflammation-induced alteration in lysosomal enzyme intracellular trafficking is invoked by some aspect of the acute phase response, (perhaps by the very increase in volume of secretory traffic itself), which results in the entrance of catalytically-active lysosomal proteinase into the Golgi compartment containing the α2,6-ST. Diversion of lysosomal enzymes from the biosynthetic lysosomal pathway into the mainstream constitutive secretory pathway had been implicated by the finding that during the acute phase response there is an elevation in the hepatic secretion of lysosomal enzymes (Jamieson *et al.*, 1983; Woloski *et al.*, 1983a, 1985a). In addition, evidence that regions of the *trans* Golgi apparatus and the TGN are acidic (Glickman *et al.*, 1983; Anderson and Pathak, 1985) provides a favourable environment for a misdirected lysosomal proteinase to be catalytically-active within the sialyltransferase-containing Golgi compartment.

The model proposed to explain the acute phase behaviour of the hepatic α2,6-ST (Figure 11) therefore was predicated on the hypothesis that the topology of the glycosyltransferase rendered it sensitive to proteolysis upon being exposed to a biologically-active lysosomal proteinase of the

FIGURE 11

Proposed Model for Release of Galβ1,4GlcNAc α2,6-Sialyltransferase During the Acute Phase Response



The model on which the experimental design was based incorporated a topological domain structure of liver Golgi Galβ1,4GlcNAc α2,6-sialyltransferase in which the catalytic domain was attached to a transmembrane signal anchor domain by an exposed linker region. The hypothesis was that during the acute phase response the linker region was subject to proteolytic cleavage, possibly by a mistargeted lysosomal enzyme, allowing the portion of the enzyme containing the catalytic site to be released into the extracellular space.

appropriate substrate specificity under conditions of reduced pH. Cleavage of the hypothetical linker region would result in release of a lower molecular weight form of sialyltransferase that was catalytically-active. Presence of inflammation-induced changes in hepatocyte biochemistry, such as increased mistargeting of lysosomal proteinases and reduction in pH of the sialyltransferase-containing compartment, would favour the likelihood of enzyme proteolysis and the net result would be an elevation in secretion into the serum of the hepatic α2,6-ST.

The experimental design employed in the attempt to verify this proposed model was first to determine the effect of pH on release of the hepatic α2,6-ST from purified rat liver Golgi membranes *in vitro*. The hypothesis that an improperly-targeted lysosomal enzyme was responsible for proteolysis of the membrane-bound glycosyltransferase resulted in the selection of the Leelavathi (1970) method of Golgi membrane purification, as modified by Moremen and Touster (1986), since microscopic and biochemical studies had demonstrated that Golgi membranes prepared in this manner are free of lysosomal contamination. Importance of pH in the release of sialyltransferase was further examined by investigating the effect of lysosomotropic agents on enzyme secretion from rat liver slices. In order to demonstrate that a proteolytic event was responsible for release of the hepatic α2,6-ST, molecular weights of soluble and membrane-bound forms of sialyltransferase were characterized by immunoblot analysis. In addition, kinetic properties of the soluble and membrane-bound enzyme forms were examined so as to provide evidence that proteolysis of the hypothesized linker region did not result in damage to the catalytic domain. The identity of the proteinase involved was probed with the use of proteinase inhibitors, exogenous proteinase and specific antibody. Moreover, experiments were

designed to establish the colocalization of proteinase and sialyltransferase within the same Golgi compartment. Finally, selected *in vitro* studies conducted with the rat were extended to include two additional species, the mouse and the guinea pig, so as to allow confirmation that the mechanism of release which promoted the acute phase behaviour of the hepatic α2,6-ST was an inter-species phenomenon.

EXPERIMENTAL

MATERIALS

Radioactive Compounds

CMP-[4,5,6,7,8,9,^{14}C] NeuAc (247mCi/mmol), UDP-[U-^{14}C] Gal (340 mCi/mmol) and neuramin-[1-^{3}H] lactitol (52 mCi/mmol) were obtained from New England Nuclear Corp., Lachine, Quebec. Affinity purified [^{125}I] protein A was obtained from Amersham Corp., Oakville, Ontario.

Sugars, Amino Acids, Peptides and Proteins

D-glucose, lactose, CMP-NeuAc and UDP-Gal were purchased from Sigma Chemical Co., St. Louis, Missouri.

Amino acids (L-configuration) and proteinase inhibitors aprotinin, bestatin, antipain, leupeptin, and soybean trypsin inhibitor were obtained from Sigma. Leupeptin was purchased from Boehringer Mannheim, Dorval, Quebec. Pepstatin A was obtained from either Sigma or Boehringer Mannheim. Bovine serum albumin, human α_1-AGP, Jack Bean Meal β-galactosidase and bovine spleen cathepsin D were obtained from Sigma.

Antiserum

Antiserum raised against rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase was kindly given by Dr. J. C. Paulson (University of California, Los Angeles, California). Antiserum containing antibodies to rat liver lysosomal cathepsin D was a gift from Dr. B. Wiederanders (Martin Luther University, German Democratic Republic).

Chromatographic, Electrophoretic and Electroblot Materials

Dowex 1X8 (Cl^- form, 100-200 mesh) was obtained from Sigma Chemical Co., St. Louis, Missouri. Nitrocellulose was obtained from Fisher Scientific Co., Toronto, Ontario. N,N'-methylene-bis-acrylamide (Bis), N,N,N',N'-tetramethylene-diamine (TEMED), ammonium persulfate, Tween 20, Coomassie Brilliant Blue R-250 and Low Molecular Weight Standards (10 - 10,000 Da) were purchased from Bio-Rad Laboratories, Richmond, California. Hyperfilm - βmax film was obtained from Amersham Corp., Oakville, Ontario.

Chemicals

Trizma Base (Tris), dimethylsulfoxide (DMSO), imidazole, Triton-X-100, chloroquine, methylamine, penicillin G, streptomycin sulfate were obtained from Sigma Chemical Co., St. Louis, Mo. Turpentine oil was purchased from Sargent-Welch Scientific Co., Skokie, Ill. Aqueous Counting Scintillant (ACS) was obtained from Amersham Corp., Oakville, Ont. All other chemicals were of analytical reagent grade and were of commercial origin.

METHODS

PHYSICAL METHODS

Determination of Radioactivity

Radioactivity was determined using a LKB-Wallac RackBeta II Model 1215 liquid scintillation counter that was programmed for calculation of disintegrations per minute (dpm) against external standards using the Hat

Trick Calibration Kit 1210-126 obtained from Fisher Scientific Co., Toronto, Ontario. Channel gate settings for ^{14}C and ^{3}H were 20-165 and 8-130, respectively. Radioactive samples obtained from sialyltransferase and galactosyltransferase assays were spotted on 2.5 cm-diameter circles of Whatman no. 1 filter paper and counted in 10 ml ACS (see below). Measurement of sialyllactose isomers separated by paper chromatography on Whatman 3 MM filter paper was performed by counting 0.5 X 8 cm strips cut from appropriate regions of chromatograms in 4 ml ACS using Omni-Vials obtained from Wheaton Scientific Co., Millville, New Jersey (see below). Sialyllactose isomer peaks were identified by scanning chromatograms with a Packard Model 7220 radiochromatogram scanner.

Polyacrylamide Gel Electrophoresis and Electroblotting

Discontinuous SDS/polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using a Bio-Rad Mini-Protean II Slab Cell. Acrylamide/bisacrylamide concentrations of stacking and separating gels were 4%/0.32% and 12%/0.08%, respectively. Samples of liver Golgi membrane and supernatant fractions were mixed with equal volumes of 0.125 M Tris/HCl, pH 6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 0.02% (w/v) Bromophenol Blue. Samples were heated in a boiling-water bath for 4 min, centrifuged for 10 min in a Fisher Model 235A Micro-Centrifuge, and applied to individual tracks of acrylamide gels (2-10 µl/track). Electrophoresis was for 1 hr at 100-150 V using a tank buffer of 0.025 M Tris/0.192 M glycine, pH 8.3, containing 0.1% SDS. Low molecular weight protein standards were soybean trypsin inhibitor (M_r 21,600), carbonic anhydrase (M_r 31,000), ovalbumin (M_r 42,699), bovine serum albumin (M_r 66,200) and phosphorylase *b* (M_r 97,400).

Electroblotting was performed using a Bio-Rad Mini Trans-Blot Cell. Electrophoresed gels were equilibrated for 1 hr in ice-cold 0.025 M Tris/0.192 M glycine/20% methanol blotting buffer, pH 8.3, and protein transfer to 0.45 µm-pore-size nitrocellulose was for 4 hr at 70 V. The ice-filled cooling unit was replaced every hr. Electroblotted nitrocellulose was washed three times for 20 min each with 200 ml deionized water, wrapped in aluminum foil and baked at 60 °C for 30 min. Molecular weight standards were visualized using the method of Hancock and Tsang (1983). Nitrocellulose was placed in a plastic watertight container and washed four times for 10 min each with 200 ml PBS-Tween (0.01 M Na_2HPO_4/NaH_2PO_4, pH 7.20, containing 0.15 M NaCl and 0.3% Tween 20) at 37 °C in a shaking water bath. After each PBS-Tween wash, nitrocellulose was rinsed with deionized water. Nitrocellulose was incubated overnight in 200 ml of PBS-Tween containing 200 µl Pelikan india drawing ink. Incubation was at room temperature in a plastic container that was placed on a rotary shaker. Stained nitrocellulose was then rinsed for 5 min with deionized water and air-dried.

Spectrophotometry , Measurement of pH and Other Methods

Measurements of absorbance in the visible and ultraviolet regions of the spectrum were made using a Philips Model PU8620 spectrophotometer. Measurements of pH were performed with a Fisher Accumet Model 915 pH Meter fitted with a temperature probe.

Ultrafiltration of protein solutions was performed with an Amicon Model 8400 Ultrafiltration Cell using YM10 Diaflo Ultrafiltration Membranes obtained from Amicon Canada Ltd., Oakville, Ontario. Dialysis of protein solutions against deionized water was routinely carried out at 4 °C using Spectrapor dialysis membranes purchased from Spectrum Medical Industries

Inc., Los Angeles, Ca. Deionized water was obtained from distilled water using a Barnstead NANOpure II purification system.

Dowex 1- phosphate resin was prepared from Dowex 1-chloride resin (100-200 mesh) according to the procedure of Paulson *et al.* (1977b). Dowex 1-chloride resin was initially washed three times with 20 volumes of 1 M hydrochloric acid, and then once with 60 volumes of deionized water. Dowex-1-chloride resin was then treated successively with 20 volumes of 1 M sodium hydroxide, 1 M phosphoric acid and 1 M sodium phosphate buffer, pH 6.8; after each treatment the resin was washed with 20 volumes of deionized water.

IMMUNOLOGICAL METHODS

Immunodetection of Sialyltransferase

Immunodetection of electroblotted sialyltransferase was by the method of Haas and Bright (1985). Nitrocellulose was blocked for 1 hr in Buffer A (25 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 25 mg/ml bovine serum albumin and 0.02 % NaN_3). Blocked nitrocellulose was then incubated for up to 12 hr in Buffer A containing rabbit antiserum raised against rat hepatic Galβ1,4GlcNAc α2,6 sialyltransferase (1 µl antiserum per 200 µl Buffer A). Incubation was carried out at room temperature in a heat-sealed plastic bag placed on a rotary shaker. Nitrocellulose was then washed for 5 min in 200 ml TBS (25 mM Tris/HCl, pH 7.5, containing 150 mM NaCl), twice for 5 min in 200 ml TTBS (TBS containing 0.05% (v/v) Triton X-100), and for 5 min in 200 ml TBS. Washed nitrocellulose was incubated at room temperature as described above for 75 min in 100 ml Buffer A containing 10 µCi affinity-purified ^{125}I-protein A. Nitrocellulose was then washed with TBS and TTBS

as described above, air-dried and autoradiographed on Hyperfilm-βmax film at -70 °C for 3 - 7 days.

Assay of α_1-AGP

Rat α_1-AGP was assayed immunologically using a modification of the methods of Simkin and Jamieson (1967) and Jamieson and Ashton (1973). Prior to precipitation with rabbit antiserum to rat α_1-AGP, precipitation with a heterologous immune system was performed in order to remove any nonspecific precipitating material. Samples of 3.0 ml of medium obtained from liver slice experiments (see below) were incubated with 500 μg human albumin, 0.15 M NaCl, 1 mM sodium azide, 4.7% Dextran T70 and 1.2 ml anti-human serum in a total volume of 4.5 ml. Solutions were incubated at 37 °C for 45 min in a shaking water bath and then allowed to stand for 24 hr at 4 °C. Precipitates were removed by centrifugation for 10 min at 4 °C at 2000 g_{av} in a Beckman Ti70 rotor. Samples of 0.5 ml of supernatant were incubated in the presence of 0.1 ml of rabbit antiserum raised against rat α_1-AGP, 0.15 M NaCl, 1 mM sodium azide and 4% Dextran T70 for 45 min at 37 °C in a shaking water bath. Incubation mixtures were then allowed to stand for 48 hr at 4 °C. Precipitates were collected by centrifugation for 10 min 4 °C at 2000 g_{av} in a Beckman Ti70 rotor, washed three times with 3 ml 0.15 M NaCl containing 4% Dextran T70 and then three times with 3 ml 0.15 M NaCl. Precipitates were dissolved in 1 ml 0.1 N NaOH and assayed for protein as described by Lowry *et al.* (1951) as modified by Miller (1959); α_1-AGP was determined by reference to a standard curve (Simkin and Jamieson, 1967; Jamieson and Ashton, 1973). Immune precipitation was not affected by the presence of ammonium chloride, methylamine or chloroquine.

PREPARATION OF SERUM AND LIVER SAMPLES

Treatment of Animals

Male Long-Evans hooded rats (150-200 g) and Swiss Webster mice (20-25 g) were purchased from the University of Manitoba Breeding Facility; male albino guinea pigs (100-150 g) were obtained from Charles River Inc., St. Constance, Quebec. Animals were maintained on a diet of Purina Laboratory Chow and tap water *ad libitum;* guinea pigs were also provided with a supply of fresh vegetables to ensure an adequate intake of vitamin C (Canadian Council on Animal Care, 1984).

Inflammation was induced according to Ashton *et al.* (1970). Animals were lightly anesthetized with ether, and then given a subcutaneous injection into the dorsolumbar region of 0.5 ml oil of turpentine per 100 g body weight; controls received injections of sterile 0.15 M NaCl.

Preparation of Serum and Liver Homogenate Samples

Serum was prepared as described by Jamieson *et al.* (1972). Animals were lightly anesthetized with ether and sacrificed by severing the jugular veins. Blood was collected for 2 min, allowed to clot for an additional 45 min on ice, and then the serum was obtained by centrifugation at $2500g_{av}$ for 10 min at 4 ^{0}C in a Sorvall RC2-B centrifuge. Livers were perfused *in situ* with ice-cold 0.15 M NaCl, and after excision were rinsed twice by gentle agitation in 200 ml of ice-cold 0.15 M NaCl. Liver homogenates were prepared according to Kaplan *et al.* (1983). Samples of 1 g of liver tissue were homogenized in 7.5 volumes of ice-cold 0.25 M sucrose using 10 up and down strokes of a Potter-Elvehjem homogenizer at 2000 rev/min. Serum and liver homogenate samples were either used immediately, or stored frozen at -20 ^{0}C until required.

Preparation of Liver Slices

Liver slices were prepared according to Jamieson *et al.* (1975). Liver tissue from livers that had been perfused with 0.15 M NaCl was placed on an ice-cold aluminum template containing a groove 7 mm wide and 0.36 mm deep (Hultin *et al.*, 1960; Simkin and Jamieson, 1967), and bathed in ice-cold 0.15 M NaCl. Slices were prepared manually using a blade supplied for the Stadie - Riggs tissue slicer, rinsed twice by gentle agitation in 200 ml of ice-cold 0.15 M NaCl, and maintained on ice in 0.15 M NaCl until use.

Preparation of Liver Slice Medium Samples

Incubation of liver slices and preparation of medium samples was according to the method of Jamieson *et al.* (1975) as modified by Kaplan *et al.* (1983). Freshly-prepared liver slices were transferred from 0.15 M NaCl and washed by gentle agitation in 200 ml of ice-cold incubation medium that had previously been purged with 95% O_2: 5% CO_2 for 30 min. Liver slices were blotted on filter paper, weighed, transferred to 25 ml incubation flasks that contained fresh medium and incubated at 37 ^{0}C in an atmosphere of 95% O_2: 5% CO_2 in a gently shaking water bath. Incubations were terminated by transferring the flasks to ice. Medium was aspirated by hand using a Pasteur pipet, centrifuged for 15 min in a Fisher Model 235A Micro-Centrifuge to remove particulate material, and supernatants were either used immediately or stored frozen at -20 ^{0}C until required. Incubation medium contained KCl (77 mM), NaCl (39 mM), $NaHCO_3$ (32.5 mM), KH_2PO_4 (0.6 mM), $MgSO_4$ (3.1 mM), $CaCl_2$ (1.3 mM), glucose (25 mM), penicillin G (25 mg/L) and streptomycin sulfate (25 mg/L) (Marsh and Drabkin, 1958). Each 5 ml of incubation medium was supplemented with 0.1 ml of a stock solution of L-amino acids that contained alanine (48 mM), arginine (22 mM), asparagine

(3.8 mM), aspartic acid (3.8 mM), cysteine (3.8 mM), glutamic acid (19 mM), glutamine (19 mM), glycine (43 mM), histidine (9 mM), isoleucine (9 mM), leucine (17 mM), lysine (48 mM), methionine (7 mM), phenylalanine (8 mM), proline (24 mM), serine (29 mM), threonine (29 mM), tryptophan (6.9 mM), tyrosine (9 mM) and valine (20 mM); this produced an amino acid concentration in the medium that was approximately twice that normally present in serum (Clemens and Korner, 1970; Woloski *et al.*, 1983a).

Experiments designed to test the effect of lysosomotropic agents on secretion of sialyltransferase and α_1-AGP from liver slices used 1 g of slices per 10 ml medium, and up to 50 mM NH_4Cl, 50 mM methylamine or 1 mM chloroquine as appropriate. Liver slices were preincubated for 30 min either alone or in presence of lysosomotropic agents, following which medium was removed and replaced with fresh medium of identical composition. Incubation continued for up to 4 hr, and at 1-hr intervals a 400 µl aliquot of medium was removed from each flask, centrifuged as described above, and the supernatant was assayed for sialyltransferase activities. Medium obtained from the 4-hr incubation of liver slices was assayed for the presence of α_1-AGP. Liver slice experiments designed to test the reversibility of inhibition of sialyltransferase release by lysosomotropic agents were carried out in a similar manner, except that preincubation was for 1 hr. Liver slices were preincubated either alone or in presence of 25 mM NH_4Cl, 25 mM methylamine or 0.5 mM chloroquine. After preincubation, medium was replaced with fresh medium that contained similar concentrations of lysosomotropic agents as appropriate, and incubation was allowed to proceed for an additional 70 min. Experiments designed to determine the effect of turpentine-induced inflammation on release of sialyltransferase used 1 g of slices per 5 ml medium (rat, guinea pig) or 0.5 g slices per 5 ml medium (mouse).

Experiments conducted to determine the effect of pepstatin A on release of sialyltransferase from liver slices used 250 mg slices per 2.0 ml medium. Liver slices were prepared under aseptic conditions, incubated at 37 ^{0}C in an atmosphere of 95% O_2: 5% CO_2 in sterile tissue-culture plates that had been placed on a rotary shaker inside a NAPCO Model 4200 Incubator.

Preparation of Liver Golgi Membranes

Golgi membranes free of lysosomal material were isolated according to the method of Leelavathi *et al.* (1970) as modified by Moremen and Touster (1986). All sucrose solutions were prepared using a 0.1 M potassium phosphate buffer, pH 6.5, containing 5 mM $MgCl_2$, and subcellular fractionation was carried out at 4 ^{0}C in a Beckman L5-70 ultracentrifuge. Excised livers were first minced by hand on an ice-cold aluminum chopping block, and then gently homogenized on ice for 30 s in 4 volumes of 0.5 M sucrose using a Potter-Elvehjem homogenizer at 1000 rev/min. Large granule material was removed by centrifugation for 10 min at 600 g_{av} in a Ti60 rotor. Supernatants were layered on top of 20 ml of 1.3 M sucrose solution and centrifuged for 90 min at 75,000 g_{av} in a Ti60 rotor. Clear supernatant solutions were aspirated by hand using a Pasteur pipet and discarded. The crude Golgi membrane fractions appearing immediately above the 1.3 M sucrose layer were aspirated, combined as appropriate, and adjusted by weight to 1.1 M sucrose with sucrose. Ten ml aliquots of this initial Golgi membrane fraction were then layered on top of 20 ml of 1.25 M sucrose, covered with 5 ml of 0.5 M sucrose and centrifuged for 90 min at 80,000 g_{av} in a SW28 rotor. The dense white Golgi bands appearing between the 0.5 M and the 1.1 M sucrose layers were carefully collected by aspiration, and after adjustment to 1.1 M sucrose were subjected to a second identical discontinuous sucrose

density gradient centrifugation. The resultant Golgi membrane bands were then carefully aspirated, pooled, diluted four-fold with 0.25 M sucrose, and then sedimented by centrifugation for 45 min at 175,000 g_{av} in a Ti60 rotor. Golgi membranes were gently resuspended by homogenization by hand in ice-cold 0.25 M sucrose, adjusted with 0.25 M sucrose to a protein concentration of 10 mg/ml, and stored refrigerated on ice until required for use. Protein content was assayed according to the method of Lowry *et al.* (1951), as modified by Miller (1959), using crystalline bovine serum albumin as the standard. The specific activity of sialyltransferase present in each purified Golgi membrane preparation was assayed immediately prior to use, and typically it was found to be at least 40-fold greater than that of the liver homogenate from which it was prepared.

Preparation of Liver Golgi Membrane and Supernatant Samples

Golgi membranes resuspended at a concentration of 10 mg Golgi membrane protein/ml in ice-cold 0.25 M sucrose, containing 0.1 M potassium phosphate buffer, pH 6.5/5 mM $MgCl_2$, were ultrasonicated in 2.5 ml portions at 50 W using an Artek dismembrator fitted with a 1 mm-diameter titanium probe. Samples of intact and permeabilized Golgi vesicle suspensions were adjusted to various pH values using equivalent amounts of 50 mM McIlvaine sodium phosphate - citric acid buffers (Elving *et al.*, 1956) and incubated in Beckman Ti70 rotor centrifuge tubes at 37 °C in a gently shaking water bath either alone, or in presence of proteinase inhibitors, bovine spleen cathepsin D, or antiserum raised against rat liver lysosomal cathepsin D. Protein concentration was typically kept at 1 mg of Golgi membrane protein/ml. At the end of incubation, tubes were transferred to ice, pH was adjusted to 7.0 with 0.1 M imidazole-HCl buffer, pH 9.0, and incubation

mixtures were adjusted to equal volumes with water. Incubation mixtures were centrifuged for 45 min at 175,000 g_{av} in the Ti70 rotor. Supernatants were carefully removed using a Pasteur pipet, and pellets were gently resuspended in ice-cold 0.25 M sucrose containing 0.1 M potassium phosphate buffer, pH 6.5/5 mM $MgCl_2$ using a 1 ml-capacity Potter-Elvehjem homogenizer at 2000 rev/min. Supernatant and pellet samples were assayed immediately for sialyltransferase, galactosyltransferase or cathepsin D activities, and then stored frozen at -20 °C until required for determination of protein content or for use in electrophoresis experiments.

Proteinase inhibitors leupeptin, antipain, aprotinin and bestatin were present in incubation mixtures at a concentration of 10^{-3} M, and soybean trypsin inhibitor was present at a concentration of 100 µg/ml. Stock solutions of pepstatin A dissolved in DMSO were added as appropriate to produce final concentrations of 10^{-3} - 10^{-11} M, and each incubation mixture contained equivalent amounts of DMSO; control incubation mixtures contained DMSO alone. Bovine spleen cathepsin D was present in incubation mixtures at a concentration of 1 µg per mg Golgi membrane protein. Incubations involving the use of rabbit antiserum raised against rat liver lysosomal cathepsin D contained 0.1 ml of permeabilized Golgi vesicle suspension (300 µg Golgi membrane protein) and up to 29 µl of antiserum. Preincubation with antiserum was at pH 7.0 at 37 °C for 45 min.

ENZYME ASSAYS

Preparation of Substrates

Isolation of α_1-AGP from Rat Serum

Rat α_1-AGP was isolated from rat serum according to the method of Simkin *et al.* (1964) as modified by Jamieson *et al.* (1972). Serum was diluted ten-fold with 0.15 M NaCl under conditions of constant stirring at room temperature, 1.8 M perchloric acid (400 ml per 80 ml serum) was slowly added, and then the mixture was allowed to stand for 10 min. Precipitated material was removed by centrifugation at 5000 g_{av} for 20 min at 4 ^{0}C in a Sorvall RC2-B centrifuge, and the supernatant which contained perchloric acid-soluble (PCA-soluble) material was brought to pH 6.5 with 2 M NaOH. Neutralized PCA-soluble fractions were dialyzed exhaustively against numerous changes of water, concentrated by ultrafiltration under N_2 using an Amicon Model 8400 Ultrafiltration Cell fitted with a YM10 Diaflo Ultrafiltration membrane, and lyophilized. Ion-exchange chromatography on a 2.5 x 45 cm column of CM-cellulose equilibrated in 0.05 M sodium acetate buffer, pH 4.9, was used to purify α_1-AGP from the PCA-soluble fraction. Samples of 250 mg of lyophilized PCA-soluble fraction dissolved in 10 ml of equilibrating buffer were applied to the column and eluted with the identical buffer at a flow rate of 20 ml/hr. The absorbance of the eluted fractions was monitored by a LKB Uvicord II detector and the α_1-AGP-containing peak which showed a maximum absorbance at 254 nm was dialyzed against deionized water, lyophilized and stored frozen at -20 ^{0}C until required for use.

Preparation of Asialo- and Asialoagalacto-α_1-AGP

Rat serum asialo-α_1-AGP was according to Jamieson *et al.* (1972) as modified by Kaplan (1983). Samples of 36 mg of rat serum α_1-AGP (prepared

as described above) were dissolved in 6.0 ml 0.025 M sulfuric acid and heated in a pyrex tube at 80 °C for 60 min. Hydrolysates were then dialyzed exhaustively against numerous changes of deionized water and lyophilized.

The agalacto derivative of asialo-α_1-AGP was prepared using Jack Bean Meal β-galactosidase by the method of Kaplan (1983). Samples of 20 mg of human asialo-α_1-AGP were incubated at 37 °C for 24 hr in 6.0 ml sodium citrate buffer, pH 3.5, containing 3 units of β-galactosidase activity. At the end of incubation, β-galactosidase was inactivated by heating for 10 min in a boiling water bath; hydrolysates were dialyzed for several days against numerous changes of deionized water and lyophilized.

Isolation of Hemoglobin from Bovine Serum

Bovine hemoglobin was prepared as described by Barrett (1977). Fresh beef blood containing 5 g trisodium citrate/L as anticoagulant was centrifuged for 10 min at 4 °C at 2000 g_{av} in a Beckman Ti19 Rotor. Sedimented erythrocytes were washed five times each in three volumes of 1.0% NaCl. Washed erythrocytes were then mixed well with an equal volume of deionized water and 0.5 volumes of CCl_4 and recentrifuged. Hemoglobin-containing supernatants were dialyzed for several days at 4 °C against numerous changes of deionized water, adjusted to a protein content of 8.0% with water, and stored at -20 °C until required.

Standard Enzyme Assay Procedures

Rat, mouse and guinea pig sialyltransferase, galactosyltransferase and cathepsin D activities present in each type of experimental sample (serum, liver homogenate, liver slice medium, Golgi membrane homogenate and supernatant) were assayed under conditions in which product formation was

linear with respect to both incubation time and enzyme concentration. Protein concentration was determined by the method of Lowry *et al.* (1951), as modified by Miller (1959) using crystalline bovine serum albumin as the standard. Incubations were carried out at 37 ^{0}C in covered test tubes (sialyltransferase and galactosyltransferase assays) or Eppendorf micro-centrifuge tubes (cathepsin D assays) in a gently shaking water bath.

Sialyltransferase

Rat, mouse and guinea pig sialyltransferase activities were routinely assayed according to the method of Baxter and Durham (1979) as modified by Kaplan *et al.* (1983). Each incubation mixture had a final volume of 150 µl and contained 10 µl of 0.75 M imidazole/HCl buffer (pH 7.0 at 37 ^{o}C), 10 µl of stock rat asialo-α_1-AGP (25 µg/µl), and up to 120 µl of experimental sample. Endogenous sialyltransferase activities were determined using controls in which the addition of asialo-α_1-AGP was omitted; values for these sialyltransferase activities were subtracted from those obtained using exogenous acceptor. Incubation mixtures containing either liver or Golgi membrane homogenate samples were supplemented with 10 µl of 15% Triton X-100. Additionally, 10 µl of 0.04 M CDP-choline were added to all incubation mixtures except those containing serum or liver slice medium samples to inhibit non-specific pyrophosphatase activity. A stock solution of the nucleotide sugar donor CMP-NeuAc was prepared by combining appropriate amounts of CMP-[^{14}C]NeuAc, unlabelled CMP-NeuAc and deionized water such that a 10 µl aliquot provided each incubation mixture with 20 nCi of radioactivity and 5 nmol of nucleotide sugar. Incubations were carried out for up to 90 min and were terminated by transferring tubes to ice. Samples of 60 µl were transferred to 2.5 cm-diameter circles of Whatman no.

1 filter paper; two circles were used per incubation mixture. Groups of 24 circles were washed once for ten min and twice for five min in 250 ml of ice-cold 10% and 5% (w/v) trichloroacetic acid, respectively. Trichloroacetic acid was removed by washing in 200 ml of ethanol : ether (2:1, v/v) for 10 min and then for 5 min in 30 ml of ether. Circles were air-dried and counted in 10 ml of ACS mixture. One unit of sialyltransferase activity was defined as equal to the transfer of 1 pmol of NeuAc from nucleotide donor to acceptor glycoprotein per unit time (min or hr).

Experiments designed to distinguish between α2,3 and α2,6 sialyltransferase activities used lactose as the low molecular weight acceptor and were carried out according to the methods of Hudgin and Schachter (1972) and Weinstein *et al.* (1982a) as modified by Kaplan *et al.* (1983). Each 150 µl incubation mixture contained 10 µl of 0.75 M imidazole/HCl buffer (pH 6.8 at 37 °C), 50 µl of 0.75 M lactose, 15 µl of CMP-[^{14}C]-NeuAc (300.9 nCi/nmole, 10 µCi/0.5 ml), and up to 75 µl of serum, liver homogenate or liver slice medium sample. Incubation was for 16 hr and was terminated by addition of 150 µl of ethanol. Precipitated protein was sedimented by centrifugation for 5 min in a Fisher Model 235A Micro-Centrifuge. Each pellet was washed with 1 ml of 50% aqueous ethanol, and the supernatant and wash solution were combined and dried under N_2 at 40 °C. Each sample was then dissolved in 1 ml of ice-cold 5 mM sodium phosphate buffer, pH 6.8, and applied to a 0.5 X 2 cm column of Dowex 1-phosphate (100-200 mesh). Sialyllactose isomers were eluted with 2 ml ice-cold 5 mM sodium phosphate buffer, pH 6.8, and column effluents were taken to dryness under N_2 at 40 °C. Each residue was dissolved in 30 µl of deionized water and the amount of radioactivity present in a 1 µl aliquot was determined by liquid scintillation counting in 10 ml ACS. Appropriate volumes of each sample containing an equivalent

amount of radioactivity were applied to Whatman 3MM paper (47 X 56 cm) along with a commercial mixture of 3′ and 6′ neuramin-[1-^{3}H]lactitol isomers. Sialyllactose isomers were separated by descending chromatography in ethyl acetate : pyridine : water (12:5:4, v/v/v) for up to 5 days. Eight cm wide longitudinal strips containing the separated 3′ and 6′ sialyllactose isomers of each sample were cut out of the dried chromatogram, and the radioactive regions were located using a radiochromatogram scanner. Semi-quantitative determination of sialyllactose isomers was performed by counting individual 0.5 X 8 cm wide strips cut from the radioactive region in 4 ml of ACS mixture.

Inhibition of Galβ1,4GlcNAc α2,6-sialyltransferase activity was achieved using rabbit antiserum containing antibodies to rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase. Sialyltransferase assays were identical to those described above except that the addition of the nucleotide sugar donor and either rat asialo-α_1-AGP or lactose was delayed until after the sample had been incubated in the presence of appropriate amounts of antiserum for 45 min.

Galactosyltransferase

Rat, mouse and guinea pig galactosyltransferase activities were assayed using human asialoagalacto-α_1-AGP as the acceptor according to the method of Fraser and Mookerjea (1976) as modified by Kaplan *et al.* (1983). Incubation mixtures were identical to those described for the sialyltransferase assay except for the addition of 10 µl of 0.2 M $MnCl_2$ and the substitution of UDP-Gal as the nucleotide sugar donor. Stock nucleotide sugar donor solutions were prepared as described for the sialyltransferase assay, except that appropriate amounts of UDP-[^{14}C]Gal, unlabelled UDP-Gal and deionized water were combined such that a 10 µl aliquot provided each incubation mixture with 20

nCi of radioactivity and a total of 5 nmol of nucleotide sugar. Incubations were carried out for up to 90 min, and incubation mixtures were treated as described above for the determination of radioactivity.

Cathepsin D

Cathepsin D activities were determined according to the procedures of Anson (1940) and Maguchi *et al.* (1988) as modified by Diment *et al.* (1988) to include the use of pepstatin A. Incubation mixtures had a final volume of 320 µl, and contained 100 µl of 0.5 M sodium formate buffer, pH 3.3, 150 µl of 3% bovine hemoglobin, up to 50 µl of experimental sample, 10 µl of either DMSO or 10^{-2} M pepstatin A dissolved in DMSO, and 10 µl of 3.2% Triton X-100. Triton-X-100 was omitted from assays of supernatant fractions containing solubilized enzyme. Incubation was for up to 60 min and was terminated by addition of 900 µl of 3% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation for 5 min in a Fisher Model 235A Micro-Centrifuge, and the absorbance of each resultant supernatant was measured at 280 nm. Proteolysis due to cathepsin D activity was calculated by subtracting the A_{280} of supernatants obtained from incubation mixtures that contained pepstatin A from that of supernatants of the corresponding incubation mixtures that had not been supplemented with proteinase inhibitor. A ΔA_{280} of 0.10 was taken to represent the activity of 0.10 unit of cathepsin D activity (Diment *et al.*, 1988).

RESULTS

INVESTIGATION INTO THE MECHANISM OF RELEASE OF HEPATIC Galβ1,4GlcNAc α2,6-SIALYLTRANSFERASE FROM RAT LIVER GOLGI MEMBRANES

Effect of pH on Release of Sialyltransferase from Rat Liver Golgi Membranes

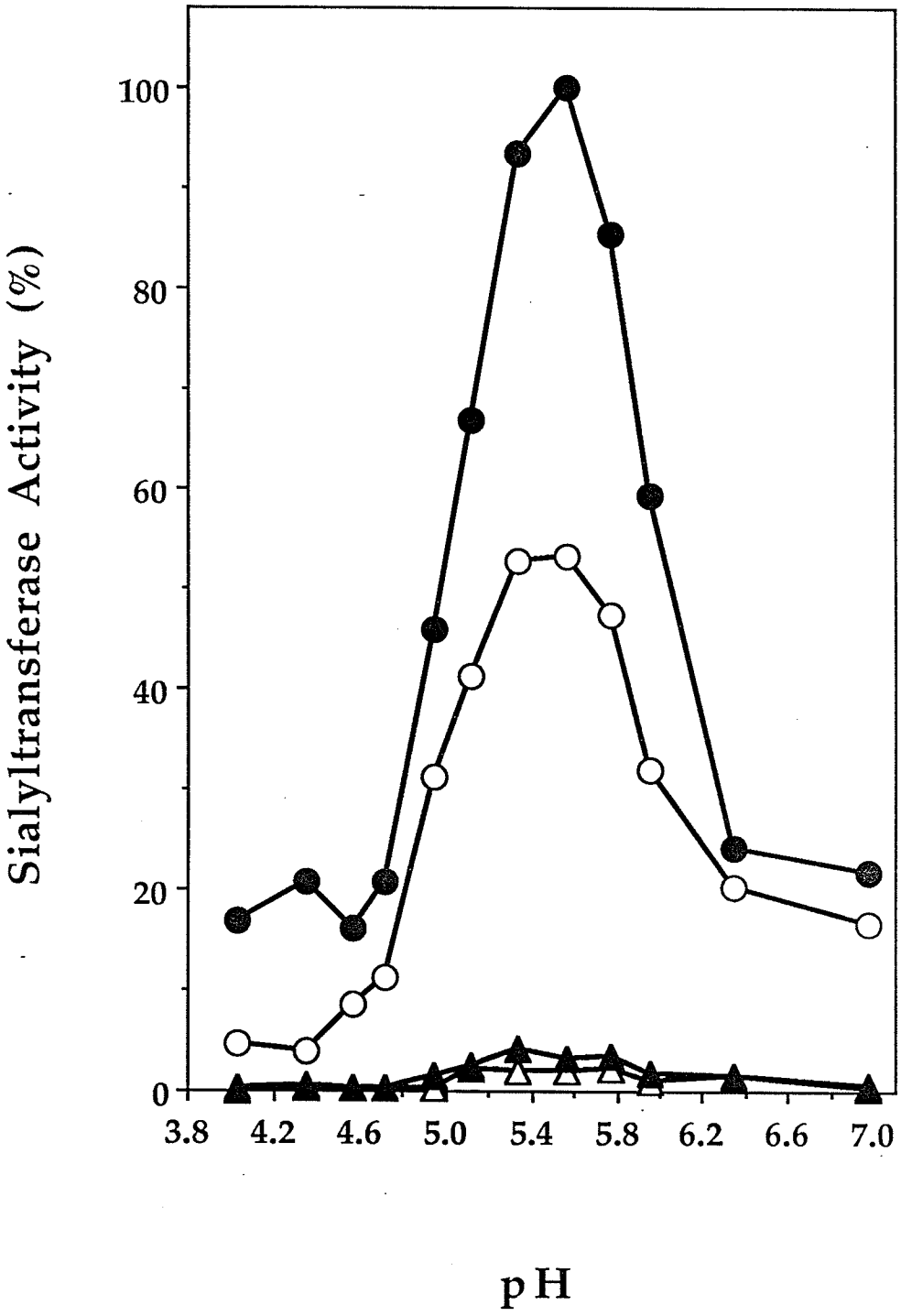
Golgi membranes prepared from rat livers according to the method of Leelavathi (1970) as modified by Moremen and Touster (1986) (see Methods) consist of sealed vesicles with the same membrane topography as Golgi membranes *in vivo*. In order to establish an *in vitro* system to investigate the effect of pH on release of sialyltransferase from the intraluminal face, it was necessary to devise a method to open up the intact Golgi vesicles to expose the sialyltransferase to the incubation medium. Ultrasonication was chosen since it opened up the Golgi vesicles but did not result in significant release of membrane-bound sialyltransferase. In order to demonstrate the specificity of the experimental conditions for the release of sialyltransferase, the release of galactosyltransferase, which is not an acute phase reactant, was used as an internal control. Galactosyltransferase codistributes with sialyltransferase within *trans* Golgi cisternae and the TGN in both the rat hepatocyte and the rat hepatoma (Taatjes *et al.*, 1987). Both glycosyltransferases share a common topology that includes the presence of an exposed luminal stem region which separates the catalytic domain from the transmembrane domain (Weinstein *et al*, 1987; D'Agostaro *et al.*, 1989). Experimental inflammation causes an elevation in liver galactosyltransferase activity, however large quantities of this enzyme are not secreted into the serum (Kaplan *et al.*, 1983).

Figure 12 shows the effect of pH on release of sialyltransferase and galactosyltransferase from Golgi vesicles that had been prepared from livers from controls, and from livers from rats which had been suffering from turpentine-induced inflammation for 36 hr and contained elevated levels of liver Golgi membrane-bound sialyltransferase and galactosyltransferase activities (Kaplan *et al.*, 1983). Golgi vesicles were first permeabilized by a 30 s exposure to ultrasonic vibrations and then incubated for 30 min in the presence of equimolar buffers of different pH (see Methods). Sialyltransferase was released from permeabilized Golgi vesicles in substantial amounts when the pH of the incubation medium was lowered. Golgi vesicles prepared from 36-hr inflamed-rat livers released a greater amount of sialyltransferase than those Golgi vesicles which had been isolated from control-rat livers, however the optimum pH for release of sialyltransferase was similar in both cases. Maximum release of sialyltransferase occurred at pH 5.6, and the amount of enzyme released at this pH from Golgi vesicles prepared from livers from controls and from inflamed rats was three and five times, respectively, the amount of enzyme released at pH 7.0. Examination of the sialyltransferase activities that remained associated with Golgi membranes following incubation revealed that as the pH of the incubation medium was lowered, increases in solubilized sialyltransferase activities were accompanied by corresponding decreases in membrane-bound enzyme activities. Although reduction of the pH of the incubation medium to below pH 5.6 did cause significant amounts of sialyltransferase to be released from Golgi membranes, an increasing amount of solubilized enzyme was apparently being degraded under these conditions. Lowering of the pH of the incubation medium was found to have a negligible effect on release of galactosyltransferase from Golgi vesicles that had been prepared from control-rat or inflamed-rat livers.

FIGURE 12

Effect of pH on Release of Sialyltransferase and Galactosyltransferase from Rat Liver Golgi Membranes

Golgi membranes were exposed to ultrasonic vibrations for 30 s and then incubated for 30 min in the presence of 50 mM McIlvaine buffers of different pH. At the end of incubation pH was adjusted to 7.0, particulate matter was removed by centrifugation, and supernatants were assayed for glycosyltransferase activities. Sialyltransferase (○,●) and galactosyltransferase (Δ,▲) activities released from Golgi vesicles prepared from livers from controls (open symbols) and 36-hr inflamed rats (closed symbols) are expressed relative to the amount of sialyltransferase activity present in the supernatant obtained from the incubation at pH 5.6 of Golgi membranes prepared from inflamed-rat livers. The 100% value was 3117 pmol NeuAc transferred to rat asialo-α_1-AGP per mg Golgi protein per hr. Results represent the means from four separate determinations; reproducibility was within $\pm$ 10%.


Sialyltransferase Activity (%)
100
80
60
40
20
0
3.8
4.2
4.6
5.0
5.4
5.8
6.2
6.6
7.0
pH

Galactosyltransferase activities were largely recovered in the pelleted Golgi membranes.

Sialyltransferase release from permeabilized Golgi vesicles at pH 5.6 increased with time of incubation up to 30 min, as shown in Figure 13. Golgi vesicles prepared from livers from 36-hr inflamed rats released a greater amount of sialyltransferase activity than did Golgi vesicles which had been isolated from livers from controls. Although there was variability from experiment to experiment in the amount of sialyltransferase activity recovered in the pelleted Golgi membranes, increases in solubilized sialyltransferase activities were typically accompanied by corresponding decreases in the membrane-bound enzyme activities during the 30-min incubation. Table 1 compares the amount of membrane-bound sialyltransferase and galactosyltransferase activity that remained associated with Golgi membranes following incubation at pH 5.6, at either 4 °C or 37 °C. Incubation for 30 min at pH 5.6 at 37 °C caused 78% and 88% of the total membrane-bound sialyltransferase activity to be released from control-rat and 36-hr inflamed-rat liver Golgi membranes, respectively. In contrast, marginal release of membrane-bound galactosyltransferase activity was observed under similar conditions.

In order to confirm that the sialyltransferase released from permeabilized Golgi vesicles at reduced pH had been attached to the luminal face of the Golgi membrane, sialyltransferase release from intact and permeabilized Golgi vesicles incubated under similar conditions of pH was investigated. Table 2 shows the combined effects of ultrasonication and pH on release of sialyltransferase from Golgi vesicles prepared from livers from controls and from 36-hr inflamed rats. Lowering of the pH of the incubation medium enhanced sialyltransferase release from both intact and

FIGURE 13

Effect of Incubation Time on Release of Sialyltransferase and Galactosyltransferase from Rat Liver Golgi Membranes Incubated at Reduced pH

Golgi membranes were exposed to ultrasonic vibrations for 30 s and then incubated at pH 5.6 for up to 30 min. At the end of incubation pH was adjusted to 7.0, particulate matter was removed by centrifugation, and supernatants were assayed for glycosyltransferase activities. Sialyltransferase (O,●) and galactosyltransferase (Δ,▲) activities released from Golgi vesicles prepared from livers from controls (open symbols) and 36-hr inflamed rats (closed symbols) are expressed relative to the amount of sialyltransferase activity present in the supernatant obtained from the 30-min incubation of Golgi membranes prepared from inflamed-rat livers. The 100% value was 2916 pmol NeuAc transferred to rat asialo-α_1-AGP per mg Golgi protein per hr. Results represent the means from four separate determinations; reproducibility was within $\pm$ 12%.

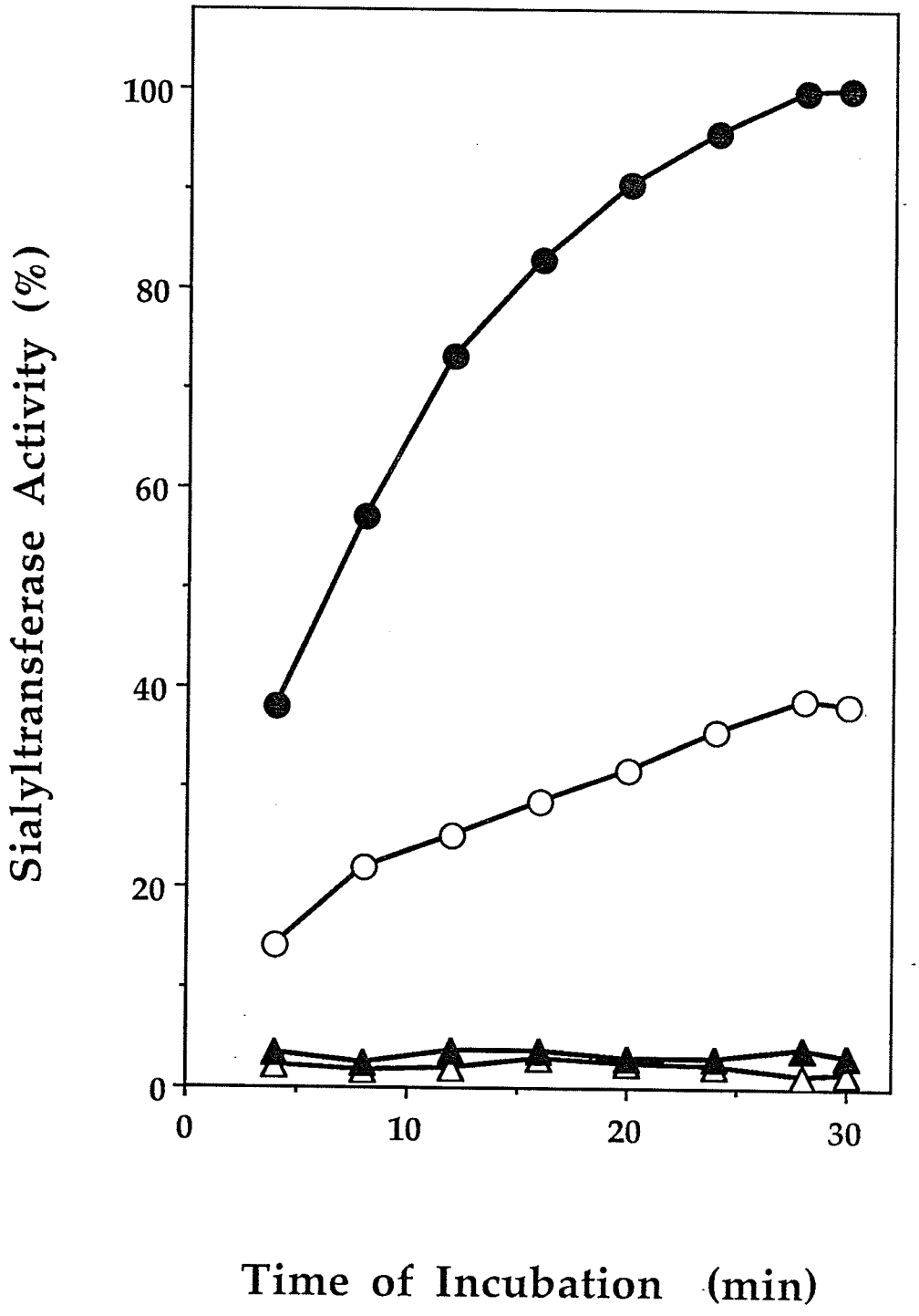

Sialyltransferase Activity (%)
100
80
60
40
20
0
0
10
20
30
Time of Incubation (min)

TABLE 1

EFFECT OF pH ON RAT LIVER GOLGI MEMBRANE-BOUND SIALYLTRANSFERASE AND GALACTOSYLTRANSFERASE

Conditions		GLYCOSYLTRANSFERASE ACTIVITY (pmol / mg Golgi protein / hr)			
		Sialyltransferase		Galactosyltransferase	
		Control	Inflamed	Control	Inflamed
pH 5.6	(4 °C)	4275 ± 513	7268 ± 681	2540 ± 203	5082 ± 503
pH 5.6	(37 °C)	938 ± 84	881 ± 72	2420 ± 210	4934 ± 543

Intact Golgi membranes prepared from livers from controls and from 36-hr inflamed rats were exposed to ultrasonic vibrations for 30 s, and then incubated for 30 min at pH 5.6 either on ice on a rotary shaker, or at 37 °C in a shaking water bath. At the end of incubation pH was adjusted to 7.0, and Golgi membranes were pelleted, resuspended in 0.25 M sucrose, and assayed for protein and for glycosyltransferase activities. Results represent the means from three separate determinations ± SD.

TABLE 2

EFFECT OF ULTRASONICATION AND pH ON RELEASE OF SIALYLTRANSFERASE FROM RAT LIVER GOLGI MEMBRANES

	SIALYLTRANSFERASE ACTIVITY RELEASED (units/ mg Golgi protein / hr)			
pH.....	7.0		5.6	
Condition.....	Control	Inflamed	Control	Inflamed
Sample				
Intact Golgi	36 ± 3	111 ± 10	238 ± 25	646 ± 55
Ultrasonicated Golgi	48 ± 4	234 ± 22	895 ± 70	3087 ± 250

Intact Golgi membranes prepared from livers from controls and from 36-hr inflamed rats were incubated for 30 min in the presence of 50 mM McIlvaine buffer (pH 7.0 or 5.6), either directly or after treatment with ultrasonic vibrations for 30 s. At the end of incubation pH was adjusted to 7.0, particulate material was removed by centrifugation and supernatants were assayed for sialyltransferase activities. Results represent the means from four separate determinations ± SD.

permeabilized Golgi vesicles, with a greater amount of enzyme being released from Golgi vesicles that had been prepared from livers from inflamed rats. Sialyltransferase release from Golgi vesicles incubated at pH 5.6 increased five-fold when the Golgi vesicles were exposed to ultrasonic vibrations for 30 s prior to incubation. Permeabilization of Golgi membranes caused a much smaller increase in the amount of sialyltransferase released at pH 7.0. These data confirmed that the procedure used in this study to isolate Golgi membranes resulted largely in the isolation of sealed Golgi vesicles and that ultrasonication was necessary to permit entrance into the incubation medium of sialyltransferase that had been released from the intraluminal face of the membrane.

Control experiments were conducted in order to determine the effect of ultrasonication on release of sialyltransferase and galactosyltransferase from Golgi membranes. Intact Golgi vesicles were exposed to ultrasonic vibrations for up to 60 s, following which particulate material was sedimented and supernatants were assayed for glycosyltransferase activities. Release of sialyltransferase and galactosyltransferase by ultrasonication alone was compared to the maximum amount of sialyltransferase and galactosyltransferase released from intact Golgi vesicles that had been exposed to 60 s of ultrasonic vibrations and then incubated for 30 min at pH 5.6. Table 3 shows that increase in the time of ultrasonication did result in an increase in solubilized sialyltransferase activity. However, the amount of sialyltransferase activity released by ultrasonication alone was insignificant when compared to that which was released by ultrasonication and incubation at reduced pH. Intact Golgi vesicles prepared from 36-hr inflamed-rat livers that had been treated with ultrasonic vibrations for 60 s and then incubated at pH 5.6 for 30 min released a total of 3088 units of sialyltransferase activity. A

TABLE 3

EFFECT OF ULTRASONICATION ON RELEASE OF SIALYLTRANSFERASE AND GALACTOSYLTRANSFERASE FROM RAT LIVER GOLGI MEMBRANES

Time of Ultrasonication (s)	GLYCOSYLTRANSFERASE ACTIVITY RELEASED (%)			
	Sialyltransferase		Galactosyltransferase	
	Control	Inflamed	Control	Inflamed
0	3.2	3.8	0.6	2.2
15	5.6	6.2	3.3	5.5
30	6.8	9.3	3.4	11.8
45	8.2	12.4	6.1	11.5
60	9.3	14.8	10.9	15.0

Intact Golgi membranes prepared from livers from controls and from 36-hr inflamed rats were exposed for increasing periods of time to ultrasonic vibrations, after which particulate matter was removed by centrifugation and supernatants were assayed for glycosyltransferase activities. Results are expressed relative to the amount of glycosyltransferase activity released from Golgi membranes prepared from livers from 36-hr inflamed rats that had been exposed to ultrasonic vibrations for 60 s prior to incubation for 30 min at pH 5.0. The 100% values for sialyltransferase and galactosyltransferase were 3088 and 240 units, respectively, of glycosyltransferase activity (pmol of sugar transferred to acceptor per mg Golgi protein per hr). Results represent the means from three separate determinations; reproducibility was within $\pm$ 10%.

60-s exposure to ultrasonic vibrations caused the release from Golgi vesicles prepared from control-rat and inflamed-rat livers of only 9.3% and 14.8%, respectively, of the total sialyltransferase activity released by similarly-treated Golgi vesicles following incubation at pH 5.6. Ultrasonication for 30 s, the time used routinely in the *in vitro* Golgi membrane experiments, contributed to less than 10% of the total sialyltransferase activity detected in the medium following incubation of ultrasonicated Golgi vesicles at reduced pH. Similar values were found for galactosyltransferase. Intact Golgi vesicles prepared from inflamed-rat livers that had been exposed to ultrasonic vibrations for 30 s and then incubated at pH 5.6 for 30 min released a total of 240 units of galactosyltransferase activity. Less than 12% of the total galactosyltransferase activity was found to be released by ultrasonication alone. Control experiments were also conducted in order to determine the effect of ultrasonication on released sialyltransferase and galactosyltransferase activities, and it was found that a 60-s exposure to ultrasonic vibrations did not significantly affect solubilized glycosyltransferase activities.

Sialyltransferase activity released from permeabilized Golgi vesicles upon incubation at either neutral or reduced pH was assayed using rat asialo-α_1-AGP, which is a suitable substrate for both the Galβ1,(3)4GlcNAc α2,3-sialyltransferase and the Galβ1,4GlcNAc α2,6-sialyltransferase (Yoshima *et al.*, 1981). In order to demonstrate that reduced pH promoted specific release of the α2,6-ST, sialyltransferase activity released from Golgi vesicles was assayed before and after treatment with antiserum containing antibodies to rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase. Table 4 shows the effect of preincubation with antiserum on the sialyltransferase activity released at neutral or reduced pH from permeabilized Golgi vesicles prepared from livers from controls and from 36-hr inflamed rats. Incubation of

TABLE 4

EFFECT OF PREINCUBATION WITH ANTISERUM CONTAINING ANTIBODIES AGAINST RAT HEPATIC Galβ1,4GlcNAc α2,6-SIALYLTRANSFERASE ON SIALYLTRANSFERASE ACTIVITIES RELEASED FROM RAT LIVER GOLGI MEMBRANES

	SIALYLTRANSFERASE ACTIVITY (pmol/ mg Golgi protein / hr)			
pH.....	7.0		5.6	
Condition.....	Control	Inflamed	Control	Inflamed
Sample				
Ultrasonicated Golgi				
- Antiserum	102 ± 9	319 ± 16	945 ± 70	3347 ± 298
+Antiserum	70 ± 6	164 ± 10	138 ± 12	590 ± 39

Golgi membranes prepared from livers from controls and from 36-hr inflamed rats were exposured to ultrasonic vibrations for 30 s and then incubated for 30 min in the presence of 50 mM McIlvaine buffer (pH 7.0 or 5.6). At the end of incubation pH was adjusted to 7.0, particulate material was removed by centrifugation, and supernatants were assayed for sialyltransferase activities, either directly, or after a 45 min preincubation with antiserum containing antibodies against the rat hepatic Galβ1,4GlcNAc α2,6- sialyltransferase. Results represent the means from three separate determinations ± SD.

permeabilized Golgi vesicles at reduced pH was found to promote the preferential release of the α2,6-ST. Pretreatment with antiserum caused a greater reduction in total sialyltransferase activity in medium obtained from the incubation of Golgi vesicles at pH 5.6 than in medium obtained from the incubation of Golgi vesicles at pH 7.0. Total sialyltransferase activity in medium obtained from the incubation at pH 5.6 of permeabilized Golgi vesicles prepared from livers from controls and from inflamed rats was reduced to less than 18% following inhibition of α2,6-ST activity.

Effect of Lysosomotropic Agents on Secretion of Sialyltransferase and α_1-AGP from Rat Liver Slices

The importance of reduced pH on release of the α2,6-ST *in vitro* from rat liver Golgi membranes was further investigated by examining the effect of lysosomotropic agents on secretion of sialyltransferase from liver slices. Lysosomotropic agents are weakly basic amines such as ammonium chloride, methylamine and chloroquine, and in their unprotonated forms they readily penetrate lipid bilayers. In their protonated forms they accumulate within acidic compartments of the cell such as lysosomes and endosomes and cause increases in pH of the local environment (Poole and Ohkuma, 1981; Maxfield, 1982). Lysosomotropic agents have been shown by immunocytochemical methods to concentrate within cisternae and vesicles associated with the *trans* face of the Golgi apparatus in human fibroblast and HepG2 hepatoma cells (Anderson and Pathak, 1985; Schwartz *et al.*, 1985; Strous *et al.*, 1985). The acidic nature of regions of the *trans* Golgi apparatus has been attributed to the presence of ATP-dependent H^+ pumps (Glickman *et al.*, 1983; Zhang and Schneider, 1983) and an H^+ - translocating ATPase has recently been purified from rat liver Golgi membranes (Moriyama and Nelson, 1989). Use of the

whole-cell liver slice system, as opposed to isolated Golgi vesicles, to study the effect of lysosomotropic agents provided greater assurance that at the start of experimentation both the physical integrity and the intraluminal pH of the sialyltransferase-containing *trans* Golgi compartments were more representative of the situation *in vivo*. In addition, it allowed the release of sialyltransferase to be monitored over longer periods of time. Figure 14 shows the effect of ammonium chloride on sialyltransferase release from liver slices prepared from livers from rats suffering from turpentine-induced inflammation for 36 hr. Liver slices were incubated either alone, or in the presence of 10, 25 or 50 mM ammonium chloride, and sialyltransferase release into the medium was monitored as a function of time for up to 4 hr (see Methods). Dramatic decreases in sialyltransferase release were exhibited by liver slices exposed to ammonium chloride. Inhibition of enzyme release was concentration-dependent, with lesser amounts of sialyltransferase release occurring in the presence of higher concentrations of lysosomotropic agent. After 4 hr of incubation, only 15% of the total sialyltransferase activity had been released in the presence of 50 mM ammonium chloride. The effect of ammonium chloride on sialyltransferase release from liver slices prepared from controls was also investigated. As shown in Figure 15, increases in the concentration of this lysosomotropic agent in the incubation medium promoted significant decreases in the amount of sialyltransferase secreted. However, the inhibitory effect of ammonium chloride on secretion of sialyltransferase from control-rat liver slices was not as dramatic as that demonstrated with inflamed-rat liver slices due to their decreased capacity to release sialyltransferase into the medium.

Methylamine and chloroquine were also found to be potent inhibitors of secretion of sialyltransferase from rat liver slices. This provided additional

FIGURE 14

Effect of Ammonium Chloride on Release of Sialyltransferase from Liver Slices Prepared from Livers from Inflamed Rats

Liver slices (1 g) were preincubated with 10 ml of medium either alone (○), or in the presence of 10 (▲), 25 (●), or 50 (■) mM ammonium chloride for 30 min. The medium was then replaced with fresh medium of identical composition, and sialyltransferase in the medium was monitored as a function of time for up to 4 hr. Results are expressed relative to the amount of sialyltransferase activity present in medium obtained from the 4-hr incubation, in the absence of ammonium chloride, of liver slices prepared from 36-hr inflamed-rat livers. The 100% value was 400 pmol NeuAc transferred to rat asialo-α_1-AGP per ml medium per hr. Each point represents the means from three separate determinations; reproducibility was within $\pm$ 15%.

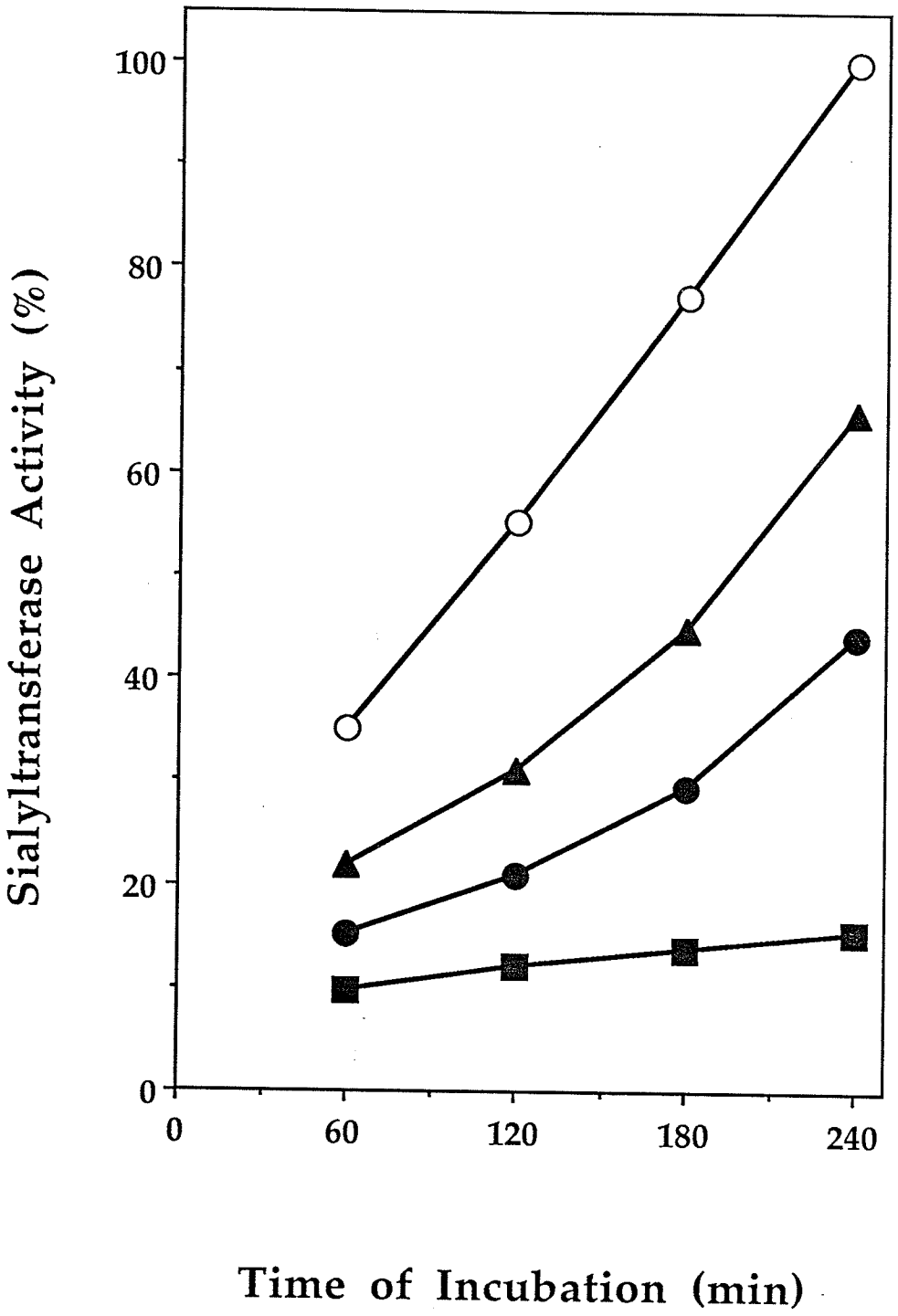
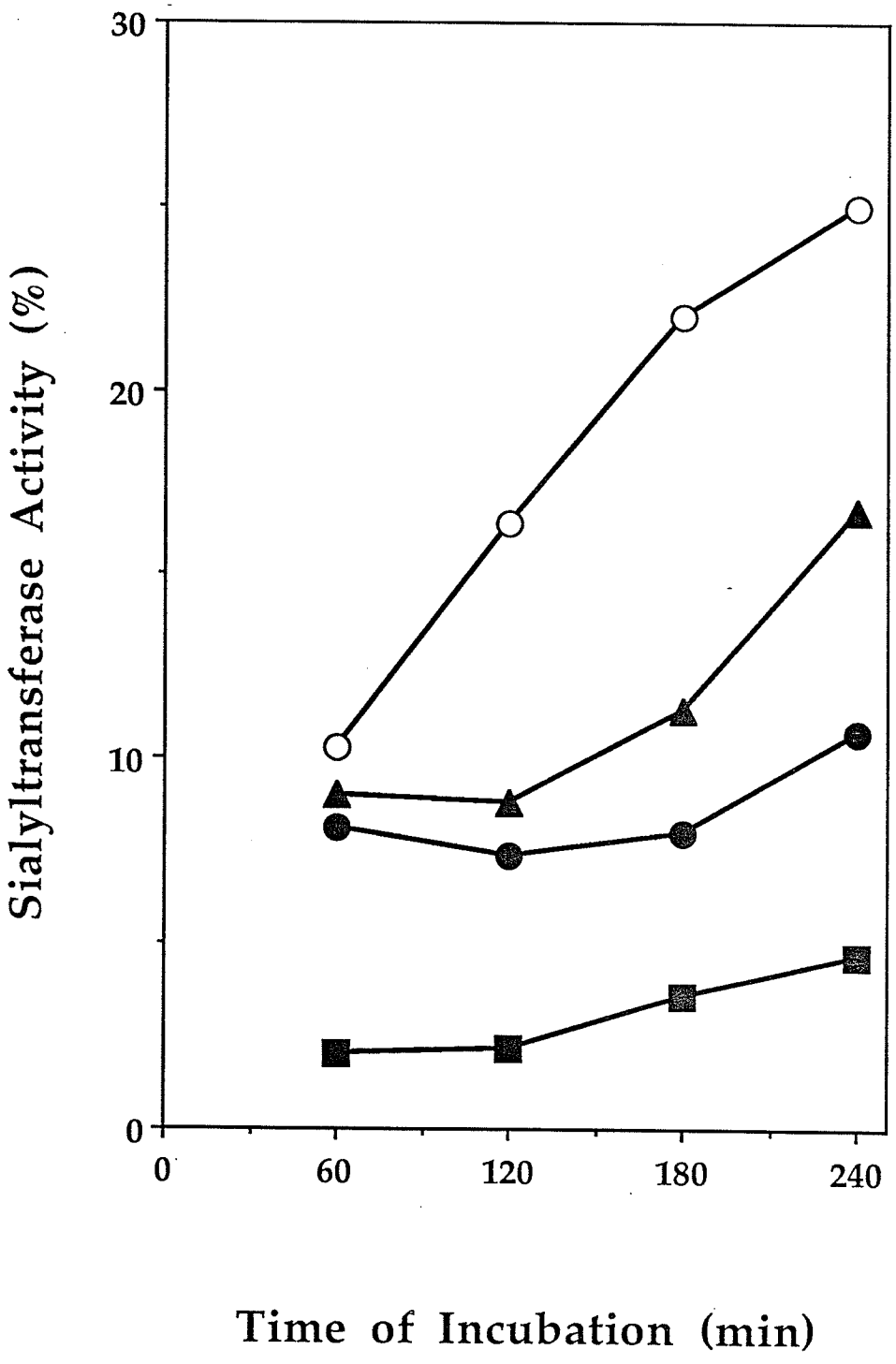

Sialyltransferase Activity (%)
100
80
60
40
20
0
0
60
120
180
240
Time of Incubation (min)

FIGURE 15

Effect of Ammonium Chloride on Release of Sialyltransferase from Liver Slices Prepared from Livers from Control Rats

Liver slices (1 g) were incubated with 10 ml of medium either alone (○), or in the presence of 10 (▲), 25 (●), or 50 (■) mM ammonium chloride. Treatment of liver slices and expression of results were as described in the legend for Figure 14. The 100% value was 400 pmol NeuAc transferred to rat asialo-α_1-AGP per ml medium per hr. Each point represents the means from three separate determinations; reproducibility was within $\pm$ 15%.


30
20
10
0
0
60
120
180
240
Sialyltransferase Activity (%)
Time of Incubation (min)

confirmation that reduced intraluminal pH was a prerequisite for sialyltransferase release from Golgi membranes. Exposure of liver slices to increasing amounts of methylamine (from 10 to 50 mM) produced corresponding decreases in the amount of sialyltransferase secreted from slices prepared from livers from 36-hr inflamed rats (Figure 16) and from controls (Figure 17). Although at a concentration of 10 mM, methylamine was less inhibitory than ammonium chloride, at higher concentrations both lysosomotropic agents produced an equivalent reduction in sialyltransferase release. Chloroquine inhibited secretion of sialyltransferase from liver slices at a much lower concentration than ammonium chloride or methylamine. Significant inhibition of sialyltransferase release occurred in the presence of 0.2 mM chloroquine, and the presence of 1.0 mM chloroquine was sufficient to produce a 65% inhibition in the amount of sialyltransferase secreted from inflamed-rat liver slices after 4 hr of incubation (Figure 18). As in the case of ammonium chloride and methylamine, the inhibitory effect of chloroquine on sialyltransferase release from control-rat liver slices (Figure 19), though significant, was less pronounced than that exerted on sialyltransferase release from inflamed-rat liver slices. Control experiments were conducted to determine the effect of lysosomotropic agents on both the pH of the incubation medium and the activity of solubilized sialyltransferase. The presence of 50 mM ammonium chloride, 50 mM methylamine or 1.0 mM chloroquine did not alter the pH of the incubation medium, nor did the addition of lysosomotropic agents interfere with the assay of sialyltransferase activity.

Although greatly diminished amounts of sialyltransferase were secreted from rat liver slices exposed to ammonium chloride, methylamine and chloroquine over the entire 4-hr incubation period, there was evidence

FIGURE 16

Effect of Methylamine on Release of Sialyltransferase from Liver Slices Prepared from Livers from Inflamed Rats

Liver slices (1 g) were preincubated with 10 ml of medium either alone (○), or in the presence of 10 (▲), 25 (●), or 50 (■) mM methylamine for 30 min. The medium was then replaced with fresh medium of identical composition, and sialyltransferase in the medium was monitored as a function of time for up to 4 hr. Results are expressed relative to the amount of sialyltransferase activity present in medium obtained from the 4-hr incubation, in the absence of methylamine, of liver slices prepared from 36-hr inflamed-rat livers. The 100% value was 308 pmol NeuAc transferred to rat asialo-α_1-AGP per ml medium per hr. Each point represents the means from three separate determinations; reproducibility was within $\pm$ 15%.

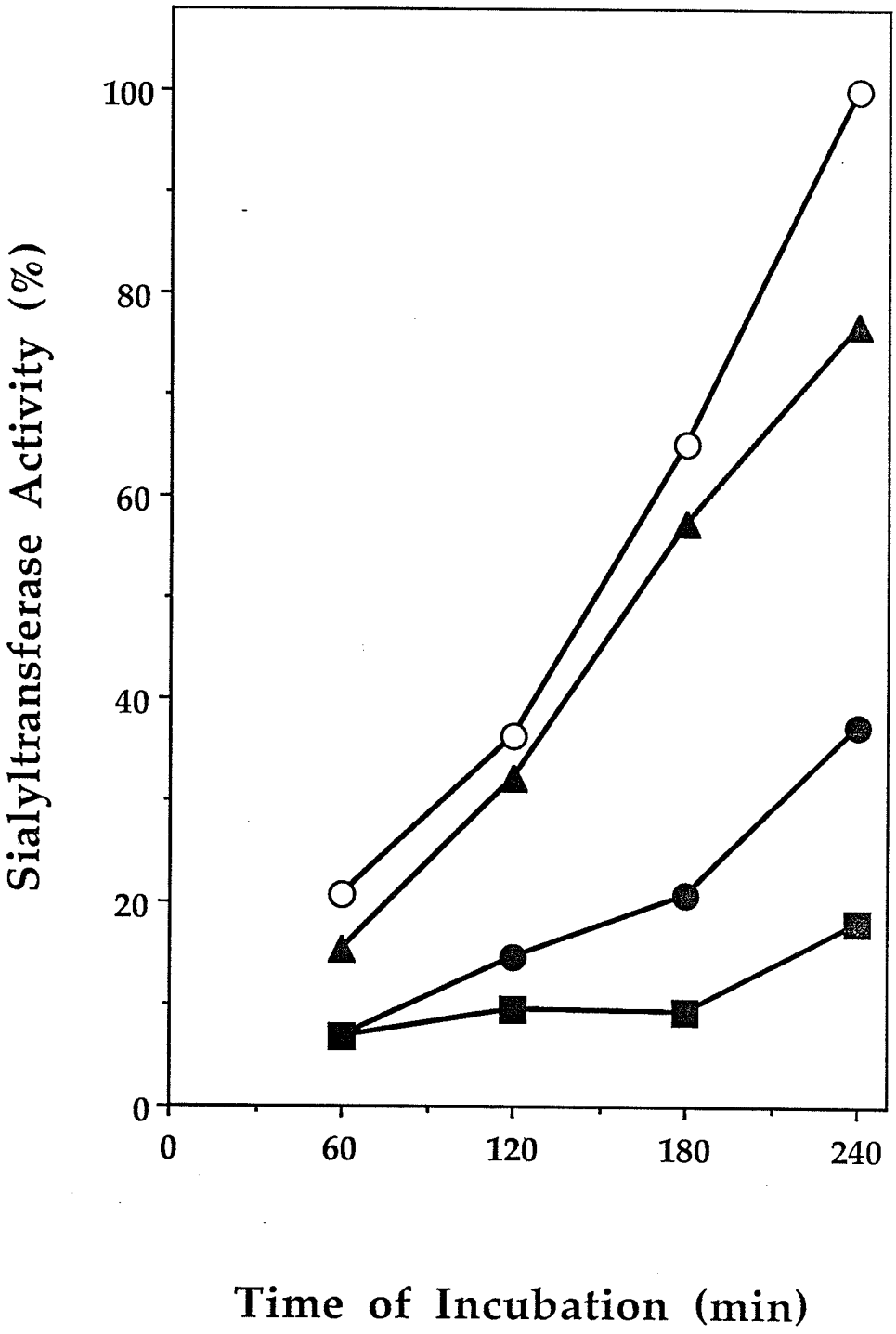

Sialyltransferase Activity (%)
100
80
60
40
20
0
0
60
120
180
240
Time of Incubation (min)

FIGURE 17

Effect of Methylamine on Release of Sialyltransferase from Liver Slices Prepared from Livers from Control Rats

Liver slices (1 g) were incubated with 10 ml of medium either alone (○), or in the presence of 10 (▲), 25 (●), or 50 (■) mM methylamine. Treatment of liver slices and expression of results were as described in the legend for Figure 16. The 100% value was 308 pmol NeuAc transferred to rat asialo-α_1-AGP per ml medium per hr. Each point represents the means from three separate determinations; reproducibility was within $\pm$ 15%.

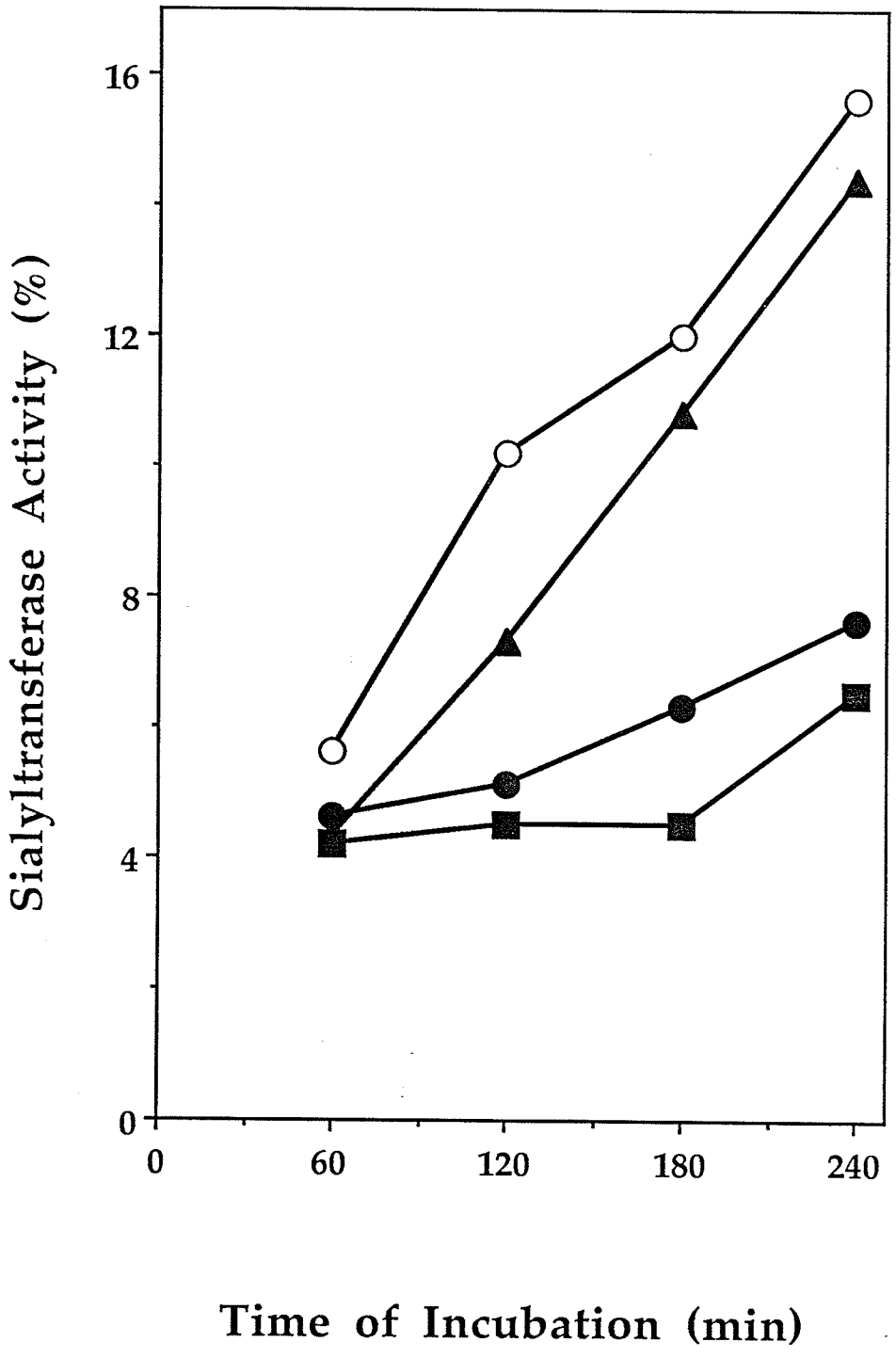

Sialyltransferase Activity (%)
16
12
8
4
0
0
60
120
180
240
Time of Incubation (min)

FIGURE 18

Effect of Chloroquine on Release of Sialyltransferase from Liver Slices Prepared from Livers from Inflamed Rats

Liver slices (1 g) were incubated with 10 ml of medium either alone (○), or in the presence of 0.2 (▲), 0.5 (●), or 1.0 (■) mM chloroquine for 30 min. The medium was then replaced with fresh medium of identical composition, and sialyltransferase in the medium was monitored as a function of time for up to 4 hr. Results are expressed relative to the amount of sialyltransferase activity present in medium obtained from the 4-hr incubation, in the absence of chloroquine, of liver slices prepared from 36-hr inflamed-rat livers. The 100% value was 255 pmol NeuAc transferred to rat asialo-α_1-AGP per ml medium per hr. Each point represents the means from three separate determinations; reproducibility was within $\pm$ 15%.

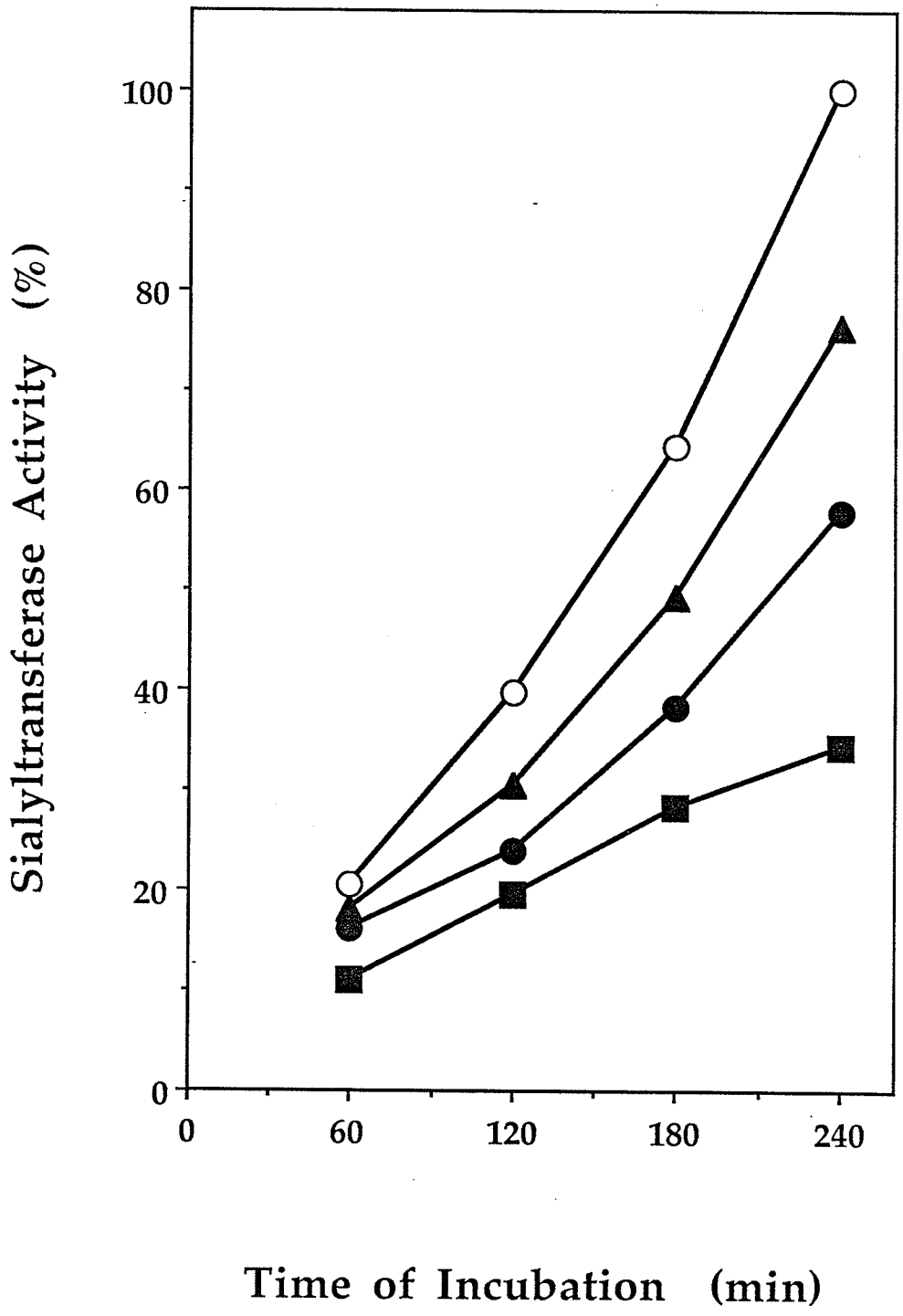
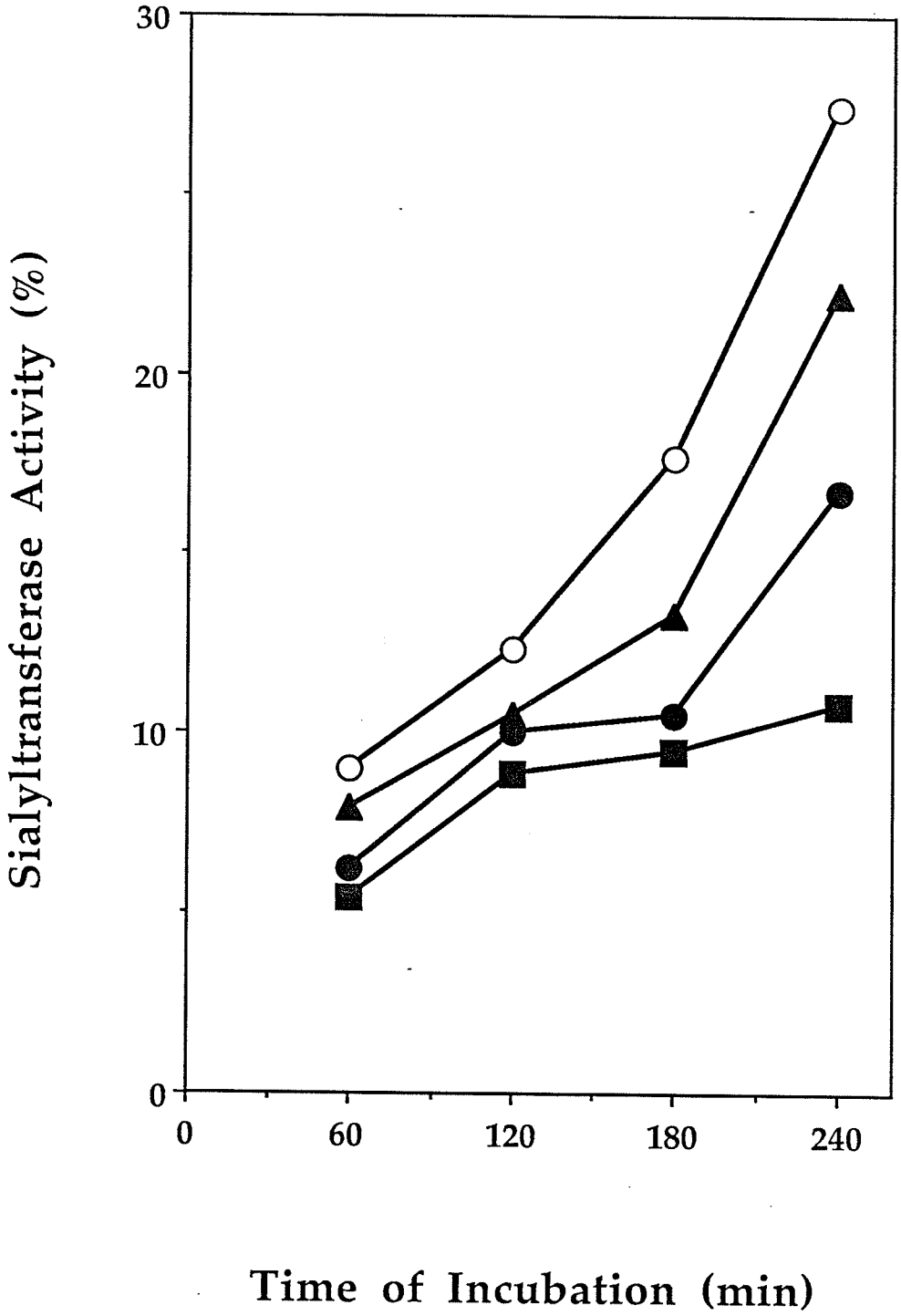

Sialyltransferase Activity (%)
100
80
60
40
20
0
0
60
120
180
240
Time of Incubation (min)

FIGURE 19

Effect of Chloroquine on Release of Sialyltransferase from Liver Slices Prepared from Livers from Control Rats

Liver slices (1 g) were incubated with 10 ml of medium either alone (○), or in the presence of 0.2 (▲), 0.5 (●), or 1.0 (■) mM chloroquine. Treatment of liver slices and expression of results were as described in the legend for Figure 18. The 100% value was 255 pmol NeuAc transferred to rat asialo-α_1-AGP per ml medium per hr. Each point represents the means from three separate determinations; reproducibility was within $\pm$ 15%.

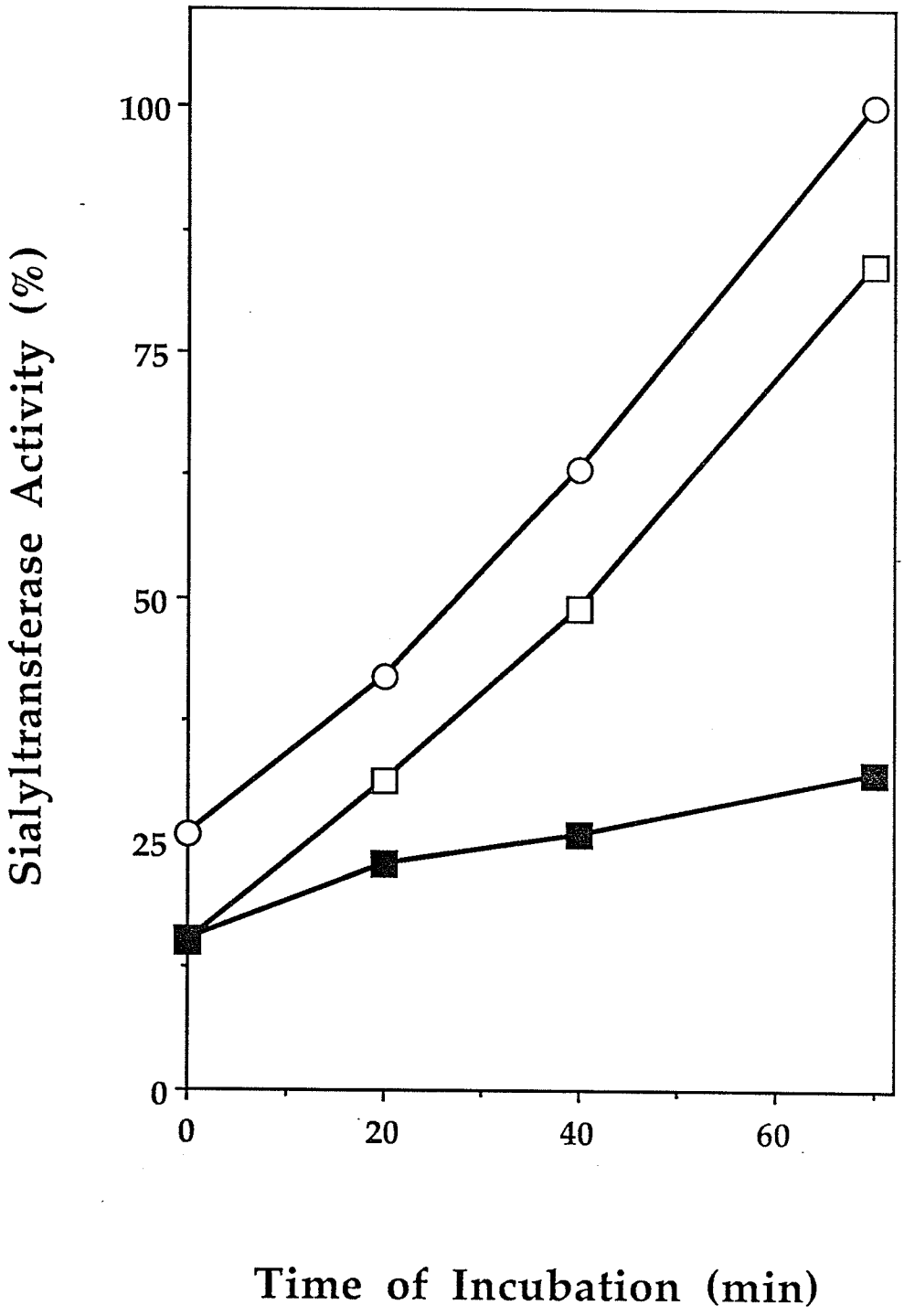

30
20
10
0
0
60
120
180
240
Sialyltransferase Activity (%)
Time of Incubation (min)

that the rate of enzyme release was slightly increasing with time of incubation. This suggested that the pH of the sialyltransferase-containing *trans* Golgi compartments were slowly being restored to homeostatic values. In order to directly test the reversibility of the inhibitory effects of ammonium chloride, methylamine and chloroquine on secretion of sialyltransferase from liver slices, reversal experiments were conducted in which the effect of removal of these lysosomotropic agents from the incubation medium was investigated. Liver slices prepared from livers from 36-hr inflamed rats were preincubated for 1 hr in the presence of either 25 mM ammonium chloride, 25 mM methylamine or 0.5 mM chloroquine, following which the medium was replaced with fresh medium that did not contain any lysosomotropic agents. Control flasks were also prepared in which inflamed-rat liver slices were preincubated either alone, or in the presence of either 25 mM ammonium chloride, 25 mM methylamine or 0.5 mM chloroquine, and then the medium was replaced with fresh medium of identical composition. Incubation was then allowed to continue for an additional 70 min, and sialyltransferase activity in the medium was monitored as a function of time. The inhibition of sialyltransferase release from inflamed-rat liver slices produced by the presence of lysosomotropic agents was found to be rapidly reversible. Figure 20 shows the results of experiments carried out with ammonium chloride. Within 20 min of the removal of ammonium chloride from the incubation medium, liver slices actively resumed the release of elevated amounts of sialyltransferase activity. Secretion of sialyltransferase increased linearly as a function of time and at the end of the 70-min incubation period, sialyltransferase release had returned to 80% of the uninhibited value. In contrast, only 25% of the sialyltransferase activity secreted by liver slices that had not been exposed to

FIGURE 20

Effect of Removal of Ammonium Chloride on Release of Sialyltransferase from Liver Slices Prepared from Livers from Inflamed Rats

Liver slices (1 g) were preincubated with 10 ml of medium either alone (○), or in the presence of 25 mM ammonium chloride (□, ■) for 1 hr. After preincubation the medium was replaced, and liver slices were incubated either alone (○, □), or in the presence of 25 mM ammonium chloride (■) for up to 70 min. Sialyltransferase in the medium was monitored as a function of time. Results are expressed relative to amount of sialyltransferase activity present in medium obtained from the 70-min incubation, in the absence of ammonium chloride, of liver slices prepared from 36-hr inflamed-rat livers (○). The 100% value was 144 pmol NeuAc transferred to rat asialo-α_1-AGP per ml medium per hr. Each point represents the means from three separate determinations; reproducibility was within $\pm$ 15%.


Sialyltransferase Activity (%)
100
75
50
25
0
0
20
40
60
Time of Incubation (min)

ammonium chloride was found to be secreted by liver slices that had been constantly exposed to this lysosomotropic agent. Similar results were obtained from experiments conducted with methylamine and chloroquine, as shown in Figures 21 and 22, respectively. Removal of either methylamine or chloroquine promoted the rapid appearance of elevated sialyltransferase activities in the incubation medium. At the end of the 70-min incubation period, liver slices that had been exposed to methylamine or chloroquine only during the preincubation period had recovered sufficiently to secrete 60% and 90%, respectively, of the uninhibited value of sialyltransferase activity. Comparison of the extent of recovery of liver slices from the inhibitory effects of lysosomotropic agents revealed that methylamine exerted a slightly longer-lasting inhibition than did either ammonium chloride or chloroquine. However, the rapidity with which liver slices resumed the secretion of elevated amounts of sialyltransferase following the removal of any of these three lysosomotropic agents suggested that their presence was not cytotoxic under the experimental conditions used.

The reversible inhibition of sialyltransferase secretion caused by transient rises in intraluminal pH of *trans* Golgi compartments due to the presence of lysosomotropic agents suggested that a proteolytic event was involved in release of this glycosyltransferase from Golgi membranes, and that proteolysis was most effective at reduced pH. In order to determine if lysosomotropic agents interfered with the secretion of a protein which is not subject to proteolytic processing in the Golgi apparatus, control experiments were conducted in which the effect of weakly basic amines on the secretion of α_1-AGP from liver slices was investigated. Rat α_1-AGP is a major acute phase reactant that traverses the entire Golgi apparatus during its biosynthesis and therefore possesses sialylated complex carbohydrate chains in its mature,

FIGURE 21

Effect of Removal of Methylamine on Release of Sialyltransferase from Liver Slices Prepared from Livers from Inflamed Rats

Liver slices (1 g) were preincubated with 10 ml of medium either alone (○), or in the presence of 25 mM methylamine (□, ■) for 1 hr. After preincubation the medium was replaced, and liver slices were incubated either alone (○, □), or in the presence of 25 mM methylamine (■) for up to 70 min. Sialyltransferase in the medium was monitored as a function of time. Results are expressed as described in the legend for Figure 20. The 100% value was 144 pmol NeuAc transferred to rat asialo-α_1-AGP per ml medium per hr. Each point represents the means from three separate determinations; reproducibility was within $\pm$ 15%.

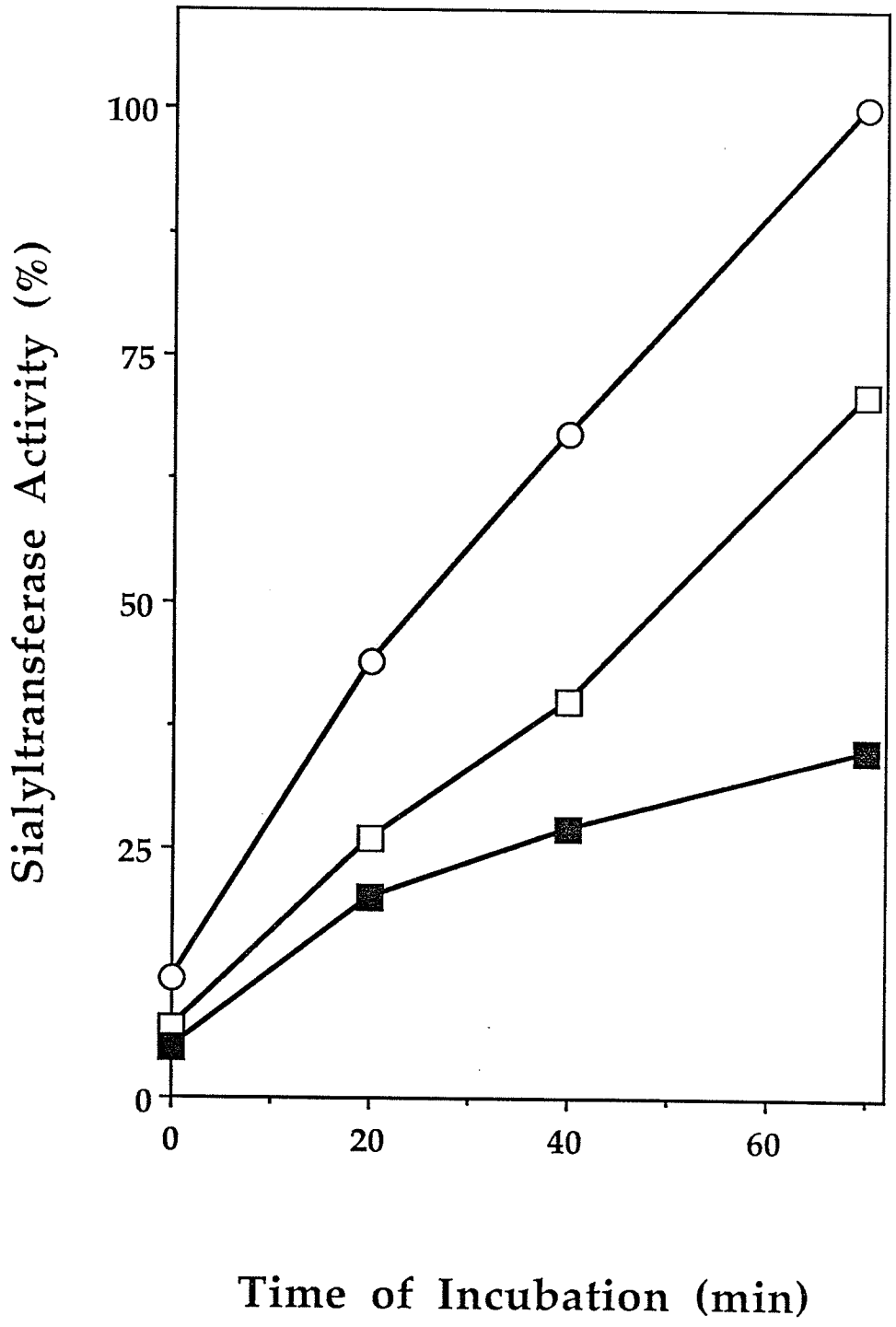
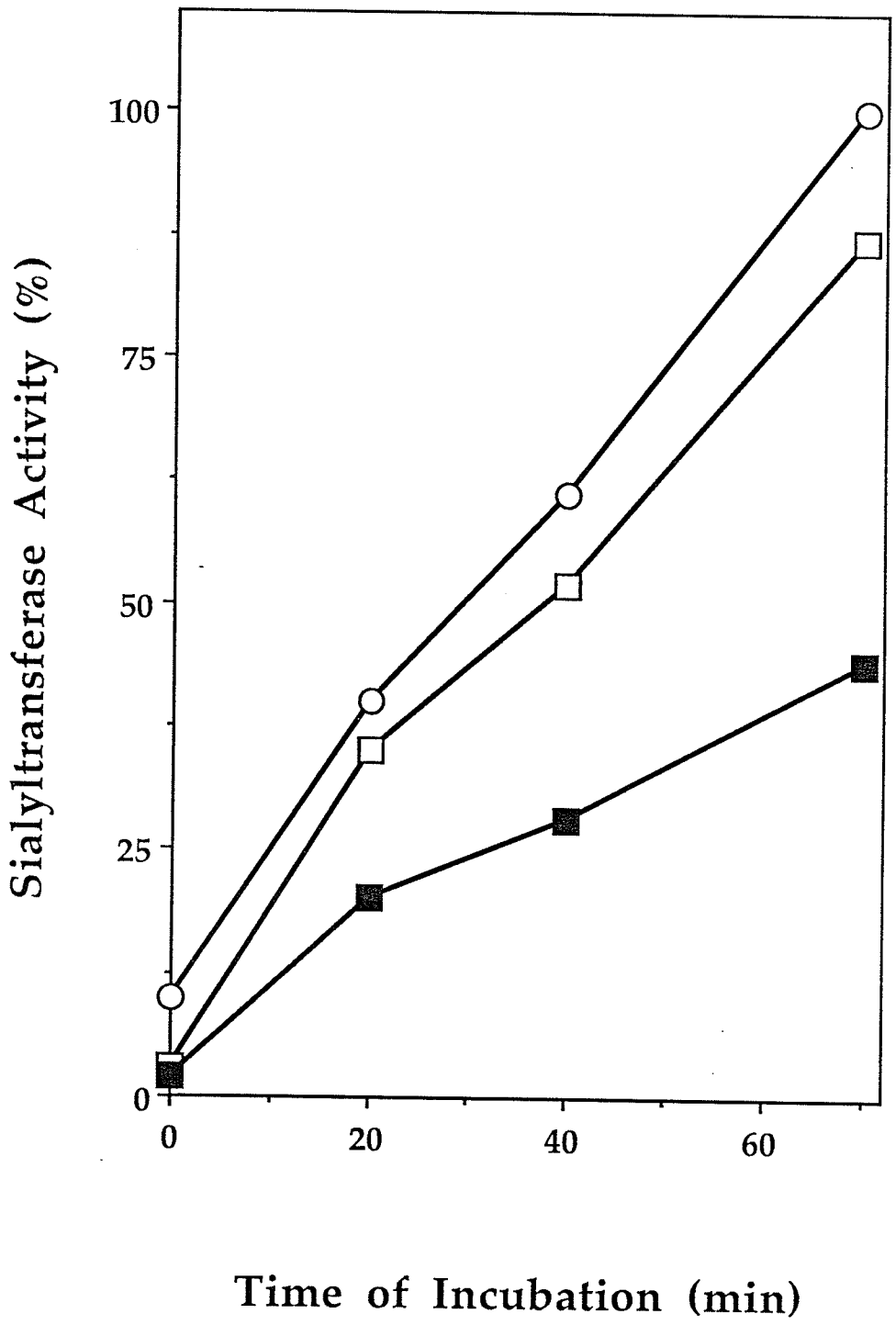

Sialyltransferase Activity (%)
100
75
50
25
0
0
20
40
60
Time of Incubation (min)

FIGURE 22

Effect of Removal of Chloroquine on Release of Sialyltransferase from Liver Slices Prepared from Livers from Inflamed Rats

Liver slices (1 g) were preincubated with 10 ml of medium either alone (○), or in the presence of 0.5 mM chloroquine (□, ■) for 1 hr. After preincubation the medium was replaced, and liver slices were incubated either alone (○,□), or in the presence of 25 mM chloroquine (■) for up to 70 min. Sialyltransferase in the medium was monitored as a function of time. Results are expressed as described in the legend for Figure 20. The 100% value was 144 pmol NeuAc transferred to rat asialo-α_1-AGP per ml medium per hr. Each point represents the means from three separate determinations; reproducibility was within $\pm$ 15%.


Sialyltransferase Activity (%)
100
75
50
25
0
0
20
40
60
Time of Incubation (min)

secreted form (Jamieson *et al.*, 1972; Jamieson and Ashton, 1973). However, it is not subject to proteolytic processing beyond the stage of removal of the signal sequence in the rough endoplasmic reticulum (Ricca and Taylor, 1981; Reinke and Fiegelson, 1985). Table 5 shows the effect of ammonium chloride, methylamine and chloroquine on secretion of α_1-AGP from liver slices prepared from livers from controls and from rats suffering from turpentine-induced inflammation for 36 hr. Slices were incubated for 4 hr in the presence of different concentrations of lysosomotropic agents, and the amount of α_1-AGP in the medium was assayed immunologically (see Methods). The presence of ammonium chloride had little effect on the secretion of α_1-AGP into the medium, whereas the presence of higher concentrations of methylamine and chloroquine was slightly inhibitory. However, the inhibition of α_1-AGP secretion in the presence of even high concentrations of lysosomotropic agents was much less than the inhibition of sialyltransferase release under similar experimental conditions. Inflamed-rat liver slices exposed to 50 mM methylamine or 1 mM chloroquine were found to have secreted at the end of the 4-hr incubation period only 18% and 35% of the total uninhibited value of sialyltransferase activity (Figures 16, 18). In contrast, inflamed-rat liver slices were able to secrete 77% and 90%, respectively, of the total uninhibited value of α_1-AGP under similar experimental conditions. Therefore the pH-sensitive event that was instrumental in release of sialyltransferase from liver slices was not associated with the secondary secretory machinery of the Golgi apparatus.

TABLE 5

EFFECT OF LYSOSOMOTROPIC AGENTS ON SECRETION OF α_1-AGP FROM RAT LIVER SLICES

Additions	α_1-AGP IN MEDIUM (μg/g wet weight of liver)	
	Control-Rat Liver Slices	Inflamed-Rat Liver Slices
None	69 ± 6	162 ± 12
NH_4Cl		
10 mM	73 ± 9	180 ± 15
25 mM	69 ± 7	179 ± 12
50 mM	77 ± 7	170 ± 12
Chloroquine		
250 μM	67 ± 6	150 ± 10
500 μM	65 ± 7	149 ± 9
1 mM	71 ± 6	145 ± 10
Methylamine		
10 mM	64 ± 6	142 ± 10
25 mM	60 ± 6	129 ± 8
50 mM	58 ± 5	125 ± 9

Liver slices (1 g) prepared from livers from controls and from 36-hr inflamed rats were incubated with 10 ml of medium for 4 hr either alone, or in the presence of NH_4Cl, chloroquine or methylamine at the concentrations indicated. After incubation particulate material in the medium was removed by centrifugation and rat α_1-AGP present in supernatants was assayed immunologically using rabbit antiserum. Results represent the means from four separate determinations ± SD.

CHARACTERIZATION OF HEPATIC Galβ1,4GlcNAc α2,6-SIALYLTRANSFERASE RELEASED FROM RAT LIVER GOLGI MEMBRANES AT REDUCED pH

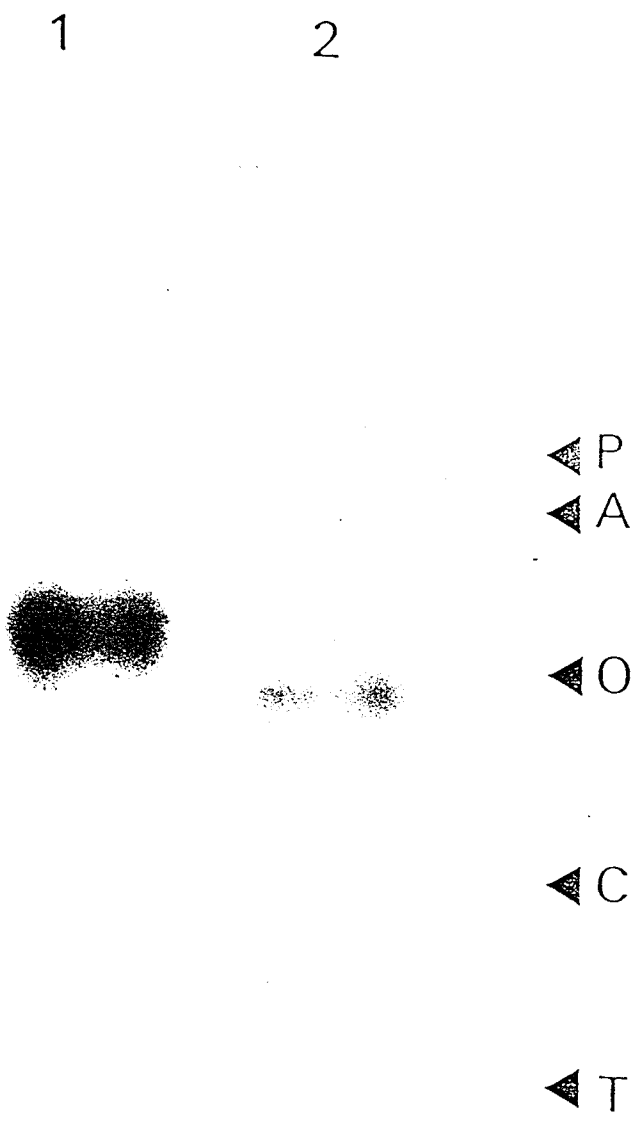
Immunoblot Analysis of Sialyltransferase Released at Reduced pH from Rat Liver Golgi Membranes

Purified, catalytically-active forms of rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase have been shown by immunoblot analysis of SDS gels to have M_r of 37,000 - 43,000 Da (Weinstein *et al.*, 1982b; Miagi and Tsuiki, 1982). A higher molecular weight of 47,000 Da was reported for the Golgi membrane-bound form of the enzyme (Roth *et al.*, 1985; Weinstein *et al.*, 1987). This suggested that limited proteolysis could be occurring during the purification procedure which resulted in the formation of truncated, biologically-active forms of soluble sialyltransferase. Analysis of the primary structure of rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase deduced from the cDNA sequence has revealed the presence of a short 9-amino acid NH_2-terminal cytoplasmic domain, a 17-residue hydrophobic membrane-anchor domain and a large 377-residue luminal domain. The difference in molecular weights between the membrane-bound and soluble forms of the α2,6-sialyltransferase was reported to be the result of cleavage by endogenous Golgi proteinases of a 63-amino acid peptide from the NH_2-terminus (Paulson *et al.*, 1987; Weinstein *et al.*, 1987). In order to determine if proteolysis was involved in release of sialyltransferase from rat liver Golgi membranes incubated at reduced pH, samples containing membrane-bound and solubilized forms of sialyltransferase were subjected to SDS-gel electrophoresis followed by immunoblotting using rabbit antiserum containing antibodies to the rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase. Figure 23 shows that the intact, membrane-bound form of α2,6-ST present within Golgi membrane

FIGURE 23

Immunoblot of Membrane-Bound and Solubilized Rat Liver Golgi Sialyltransferase

Intact Golgi membranes prepared from livers from 36-hr inflamed rats were extracted with Triton X-100 to release the membrane-bound sialyltransferase, which was compared with solubilized sialyltransferase released from Golgi membranes by sonication followed by incubation at pH 5.6 for 30 min. Samples containing membrane-bound and solubilized sialyltransferases were subjected to SDS-gel electrophoresis for 1 hr using a Bio-Rad Mini-Protean II Slab Cell system, and immunoblot analysis was performed using antiserum to rat liver Golgi Galβ1,4GlcNAc α2,6-sialyltransferase (see Methods). Track 1, Golgi membrane-bound sialyltransferase; Track 2, sialyltransferase released from Golgi membranes by sonication and incubation at reduced pH. Molecular weight markers, indicated by arrows, were: soybean trypsin inhibitor (T, M_r 21,600), carbonic anhydrase (C, M_r 31,000), ovalbumin (O, M_r 42,699), bovine serum albumin (A, M_r 66,200), and phosphorylase B (P, M_r 97,400).


1
2
P
A
O
C
T

preparations obtained from livers from 36 hr-inflamed rats has a molecular weight of approximately 49,000 Da. In contrast, the major enzyme form present within supernatants obtained following centrifugation of permeabilized Golgi membranes that had been incubated for 30 min at pH 5.6 was found to exhibit a molecular weight of approximately 42,000 Da.

Kinetic Properties of Rat Liver Golgi Membrane-Bound and Released Sialyltransferase

Secretion of sialyltransferase from rat livers *in vivo,* or from rat liver slices *in vitro,* was reported to not be accompanied by significant changes in the catalytic activities of sialyltransferase towards CMP-NeuAc and either rat or human asialo-α_1-AGP (Kaplan *et al.*, 1983). This implied that attachment of sialyltransferase to the lipid bilayer was not necessary for maintenance of enzymatic competence. In addition, it suggested that the mechanism of release of sialyltransferase from intact Golgi membranes did not produce significant alterations in the three-dimensional conformations of substrate binding and catalytic sites. An *in vivo* topological orientation of rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase in which the membrane-anchor domain is separated from the catalytic, luminal domain by an intervening linker domain would allow truncated forms of sialyltransferase possessing intact catalytic domains to be released from Golgi membranes at reduced pH if the site of proteolysis was within the linker domain. In order to provide evidence that proteolytic cleavage of sialyltransferase from rat Golgi membranes during incubation at reduced pH did not cause significant damage to the catalytic domain, the kinetic properties of membrane-bound and solubilized sialyltransferase activities were investigated. Table 6 shows the apparent K_m values towards CMP-NeuAc and rat asialo-α_1-AGP determined for Golgi

TABLE 6

APPARENT K_m VALUES FOR RAT LIVER GOLGI MEMBRANE-BOUND AND SOLUBILIZED SIALYLTRANSFERASE ACTIVITIES

Enzyme Source	CMP-NeuAc K_m (μM)	Asialo-α_1-AGP K_m (μM)
Control Rat		
Membrane-Bound	155 ± 14	21 ± 3
Solubilized	143 ± 10	16 ± 2
Inflamed Rat		
Membrane-Bound	153 ± 11	15 ± 2
Solubilized	143 ± 9	19 ± 4

Golgi membranes prepared from livers from controls and from 36-hr inflamed rats were exposed to ultrasonic vibrations for 30 s and then incubated for 30 min either at pH 7.0 or 5.6. After incubation pH was adjusted to 7.0, and incubation mixtures were centrifuged to obtain pellet and supernatant samples. Activities towards CMP-NeuAc and rat asialo-α_1-AGP were determined for sialyltransferase associated with pelleted Golgi membranes or present in supernatants obtained from centrifugation of Golgi membrane samples incubated at pH 7.0 and pH 5.6, respectively. K_m values for rat asialo-α_1-AGP were calculated using a molecular weight of 34,600 Da (Jamieson *et al.*, 1972; Kaplan *et al.*, 1983). Results represent the means from three separate determinations ± SD.

membrane-bound sialyltransferase activities and for sialyltransferase activities released from permeabilized Golgi vesicles during incubation for 30 min at pH 5.6. Apparent K_m values were determined by varying the concentration of one substrate against a constant concentration of the other (see Methods). Significant impairment of catalytic efficiency was not found to occur following sialyltransferase release at reduced pH. In addition, there was little difference in apparent K_m values towards either substrate demonstrated by sialyltransferase activities released from Golgi membranes prepared from control-rat and 36-hr inflamed rat livers. Values of apparent K_m for rat asialo-α_1-AGP were 16-19 μM and 15-21 μM for solubilized and membrane-bound sialyltransferase activities, respectively. The apparent K_m values reported for rat serum and liver sialyltransferase activities towards rat asialo-α_1-AGP were 7.6-8.3 μM and 7.1-10 μM, respectively (Kaplan *et al.*, 1983), which compared favourably to those found for rat liver Golgi solubilized and membrane-bound sialyltransferase activities. An apparent K_m value of 143 μM for CMP-NeuAc was found for solubilized sialyltransferase activities and this was very similar to the apparent K_m values of 153-155 μM exhibited by Golgi membrane-bound sialyltransferase activities. However, values for apparent K_m for rat Golgi solubilized and membrane-bound sialyltransferase activities determined with respect to CMP-NeuAc were higher than those reported for rat serum and liver sialyltransferase activities, which were 16-29 μM and 25-32 μM, respectively (Kaplan *et al.*, 1983).

CHARACTERIZATION OF THE PROTEINASE INVOLVED IN RELEASE OF HEPATIC Galβ1,4GlcNAc α2,6-SIALYLTRANSFERASE FROM RAT LIVER GOLGI MEMBRANES

Effect of Proteinase Inhibitors on Release of Sialyltransferase from Rat Liver Golgi Membranes

In order to determine the identity of the endogenous Golgi proteinase responsible for cleavage of rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase from Golgi membranes incubated at reduced pH, a variety of proteinase inhibitors were individually tested for their ability to inhibit sialyltransferase release. Permeabilized Golgi vesicles were incubated at pH 5.6 for 30 min in the presence of 10^{-3} M concentrations of leupeptin, antipain, aprotinin, bestatin and pepstatin A, or 100 μg/ml soybean trypsin inhibitor (see Methods). The concentrations of proteinase inhibitors chosen were those which had been used by others to characterize lysosomal and non-lysosomal proteinases in rat hepatocytes (Grinde and Seglen, 1980). Table 7 shows the effect of proteinase inhibitors on release of sialyltransferase from Golgi membranes prepared from livers from control rats and from 36-hr inflamed rats. Sialyltransferase release was dramatically inhibited in the presence of pepstatin A. Significant inhibition of sialyltransferase release did not occur in the presence of any of the other proteinase inhibitors tested. All of the proteinase inhibitors were dissolved in water except pepstatin A, which was dissolved in DMSO. Control experiments were conducted to determine the effect of DMSO on sialyltransferase release and it was found that this solvent was slightly inhibitory. However the amount of inhibition produced by DMSO alone was much less than that produced by pepstatin A. In addition, control experiments were carried out to determine the effect of pepstatin A or

TABLE 7

EFFECT OF PROTEINASE INHIBITORS ON RELEASE OF SIALYLTRANSFERASE FROM RAT LIVER GOLGI MEMBRANES

Additions		SIALYLTRANSFERASE ACTIVITY (pmol / mg Golgi protein / hr) Control-Rat Golgi Membranes	Inflamed-Rat Golgi Membranes
None		1280 ± 100	2830 ± 250
Leupeptin	(10^{-3} M)	1220 ± 118	2895 ± 242
Antipain	(10^{-3} M)	1264 ± 123	2928 ± 234
Aprotinin	(10^{-3} M)	1195 ± 72	2870 ± 144
Bestatin	(10^{-3} M)	1222 ± 115	3000 ± 207
Soybean Trypsin Inhibitor	(100 µg/ml)	1346 ± 95	3376 ± 271
Pepstatin A	(10^{-3} M)	230 ± 45	250 ± 50
DMSO		938 ± 40	2195 ± 165

Golgi membranes prepared from livers from controls and from 36-hr inflamed rats were exposed to ultrasonic vibrations for 30 s and then incubated for 30 min at pH 5.6 in the presence of proteinase inhibitors at the concentrations indicated. At the end of incubation pH was adjusted to 7.0, particulate material was removed by centrifugation and supernatants were assayed for sialyltransferase activities. Results represent the means from three separate determinations ± SD.

DMSO on the activity of solubilized sialyltransferase and it was found that the presence of 10^{-3} M pepstatin A in DMSO, or DMSO alone, had little or no effect on enzyme activity.

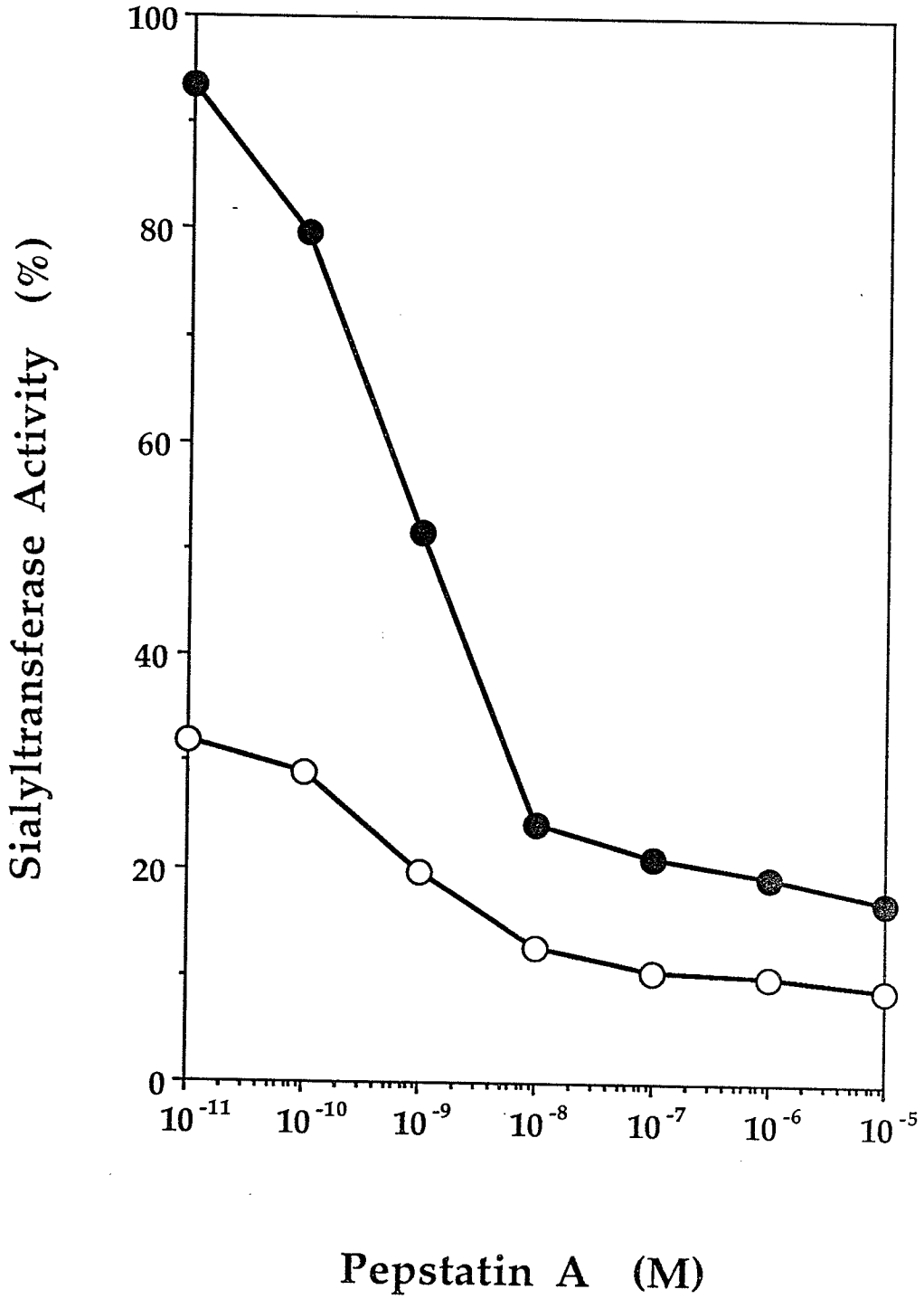
Effect of Inhibition of Cathepsin D on Release of Sialyltransferase from Rat Liver Golgi Membranes and Rat Liver Slices

Pepstatin A is a potent, reversible inhibitor of cathepsin D. Inhibition is equimolar and is most effective under conditions of acidic pH (Barrett, 1977). The equimolar nature of pepstatin A inhibition of cathepsin D provided a convenient means of estimating the endogenous concentration of the cathepsin D-like proteinase responsible for cleavage of the rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase from Golgi membranes incubated at reduced pH. Figure 24 shows the effect of up to 10^{-5} M pepstatin A on release of sialyltransferase from Golgi membranes prepared from livers from controls and from 36-hr inflamed rats. Golgi vesicles were permeabilized by ultrasonication and then incubated at pH 5.6 for 30 min in the presence of different amounts of pepstatin A dissolved in a constant volume of DMSO. Inhibition of sialyltransferase release from Golgi membranes increased substantially as the concentration of pepstatin A was increased from 10^{-11} M to 10^{-8} M, following which only minor increases in inhibition of enzyme release were observed. Sialyltransferase activities that remained associated with Golgi membranes following incubation at reduced pH increased with increases in concentration of pepstatin A in the incubation medium. A concentration of 10^{-8} M pepstatin A was sufficient to cause a 76% inhibition in the total amount of sialyltransferase activity released from Golgi membranes prepared from inflamed-rat livers in the presence of DMSO alone. Sialyltransferase release from Golgi membranes obtained from inflamed-rat

FIGURE 24

Effect of Pepstatin A on Release of Sialyltransferase from Rat Liver Golgi Membranes

Golgi membranes were exposed to ultrasonic vibrations for 30 s and then incubated for 30 min at pH 5.6 in the presence of different concentrations of pepstatin A dissolved in DMSO. At the end of incubation pH was adjusted to 7.0, particulate matter was removed by centrifugation and supernatants were assayed for sialyltransferase activities. Sialyltransferase activities released from Golgi membranes prepared from livers from control rats (O) and from 36-hr inflamed rats (●) are expressed relative to the amount of sialyltransferase activity present in the supernatant obtained from the incubation in the presence of DMSO alone of Golgi membranes prepared from livers from 36-hr inflamed rats. The 100% value was 3126 pmol NeuAc transferred to rat asialo-α_1-AGP per mg Golgi protein per hr. Results represent the means from six separate determinations; reproducibility was within $\pm$ 10%.


Sialyltransferase Activity (%)
100
80
60
40
20
0
10 -11
10 -10
10 -9
10 -8
10 -7
10 -6
10 -5
Pepstatin A (M)

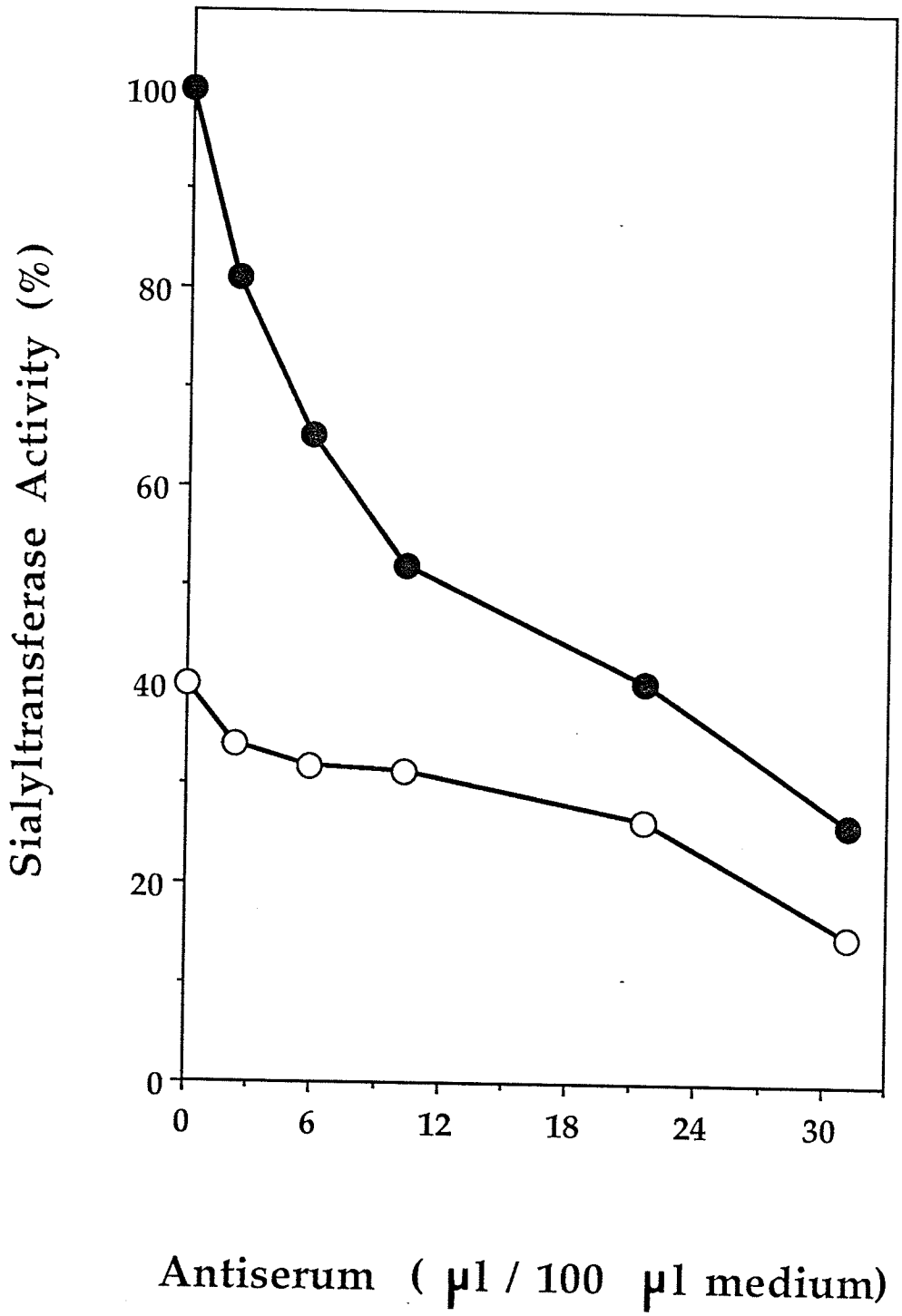
livers was inhibited by pepstatin A to a greater extent than sialyltransferase release under similar conditions from Golgi membranes prepared from control-rat livers.

In order to confirm the cathepsin D-like identity of the proteinase involved in release of rat Golgi membrane-bound sialyltransferase, the effect of rabbit antiserum raised against rat liver lysosomal cathepsin D on sialyltransferase release from Golgi membranes was investigated. Permeabilized Golgi vesicles prepared from livers from controls and from 36-hr inflamed rats were preincubated for 45 min at pH 7.0 in the presence of increasing amounts of antiserum; the pH was then reduced to pH 5.6 and incubation was allowed to continue for an additional 30 min. As shown in Figure 25, significant reduction in sialyltransferase release was observed with Golgi membranes that had been pretreated with antiserum prior to incubation at reduced pH. Exposure of Golgi membranes to increasing amounts of antiserum resulted in corresponding decreases in the amount of sialyltransferase released when the pH of the incubation medium was lowered to pH 5.6, and this effect was more pronounced with Golgi membranes obtained from inflamed-rat livers. Sialyltransferase release from Golgi membranes prepared from inflamed-rat livers was reduced by 74% upon pretreatment of Golgi membranes with 30 µl antiserum / 100 µl incubation medium. Examination of the sialyltransferase activities that remained associated with Golgi membranes following incubation at reduced pH revealed that pretreatment of Golgi membranes with increasing amounts of antiserum resulted in corresponding increases in membrane-bound enzyme activities. Control experiments were conducted to determine the effect of non-immune rabbit serum on solubilized sialyltransferase activities and on sialyltransferase release from Golgi membranes. Normal rabbit serum

FIGURE 25

Effect of Antiserum Containing Antibodies to Cathepsin D on Release of Sialyltransferase from Rat Liver Golgi Membranes

Golgi membranes were exposed to ultrasonic vibrations for 30 s and then incubated for 45 min at pH 7.0 in the presence of increasing volumes of rabbit antiserum raised against rat liver lysosomal cathepsin D. The pH was then lowered to 5.6 and incubation was allowed to continue for an additional 30 min. The pH was then adjusted to 7.0, particulate matter was removed by centrifugation and supernatants were assayed for sialyltransferase activities. Sialyltransferase activities released from Golgi membranes prepared from livers from control rats (○) and from 36-hr inflamed rats (●) are expressed relative to the amount of sialyltransferase activity present in the supernatant obtained from the incubation, in the absence of antiserum, of Golgi membranes prepared from livers from 36-hr inflamed rats. The 100% value was 3126 pmol NeuAc transferred to rat asialo-α_1-AGP per mg Golgi protein per hr. Results represent the means from four separate determinations; reproducibility was within $\pm$ 15%.


Sialyltransferase Activity (%)
100
80
60
40
20
0
0
6
12
18
24
30
Antiserum (µl / 100 µl medium)

had no detectable effect on released sialyltransferase activities, and Golgi membranes incubated at pH 7.0 in the presence of normal rabbit serum secreted significant amounts of sialyltransferase activity when the pH of the incubation medium was reduced to pH 5.6.

The ability of pepstatin A to inhibit sialyltransferase release in a whole-cell system was investigated by examining the effect of this inhibitor on secretion of sialyltransferase from rat liver slices. Figure 26 shows the effect of 10^{-4} M pepstatin A on sialyltransferase release from liver slices prepared from livers from controls and from rats suffering from turpentine-induced inflammation for 30 hr. Pepstatin A has been reported to penetrate lipid bilayers very slowly (Dean, 1977), therefore liver slices were prepared under aseptic conditions and incubated for up to 18 hr in the presence of pepstatin A that had been previously dissolved in 50% ethanol and then added to the incubation medium to provide a final concentration of 10^{-4} M (see Methods). Secretion of sialyltransferase from liver slices was significantly inhibited in the presence of pepstatin A, with greater inhibition occurring with liver slices obtained from 30-hr inflamed-rat livers. At the end of the 18-hr incubation, the total amount of sialyltransferase released from inflamed-rat liver slices incubated in the absence of pepstatin A was reduced by 50% in the presence of this inhibitor. This data supported the conclusion that release of sialyltransferase from Golgi membranes under conditions of reduced pH was due to the action of endogenous Golgi cathepsin D-like activity.

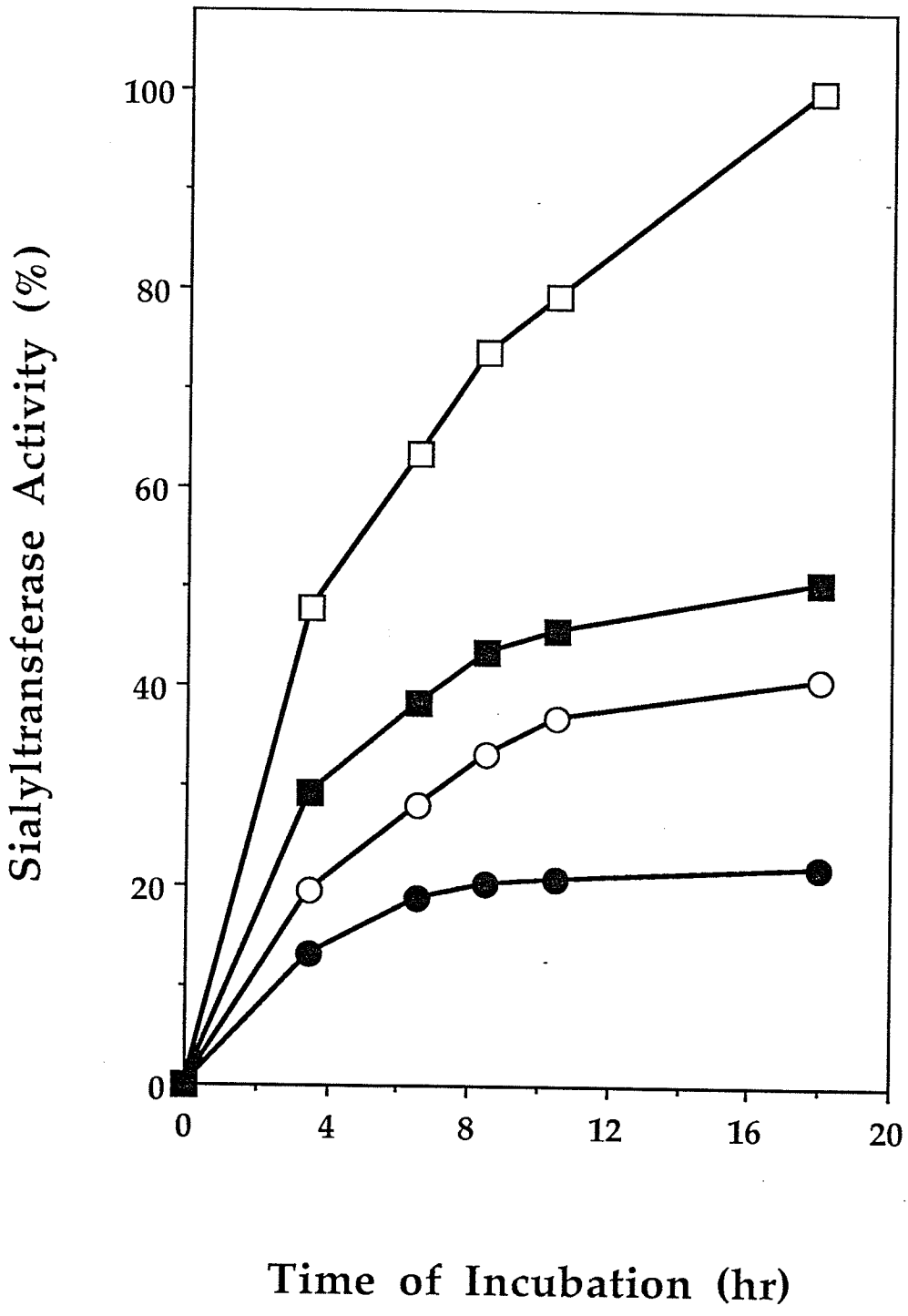
Effect of Exogenous Cathepsin D on Release of Sialyltransferase from Rat Liver Golgi Membranes

The observed inhibition of sialyltransferase release at reduced pH from rat liver Golgi membranes exposed to pepstatin A (Table 7, Figure 24) or

FIGURE 26

Effect of Pepstatin A on Release of Sialyltransferase from Rat Liver Slices

Liver slices (250 mg) prepared from livers from controls (○, ●) and from 30-hr inflamed rats (□,■) under aseptic conditions were incubated with 2.0 ml of medium either alone (open symbols) or in the presence of pepstatin A (closed symbols). Pepstatin A was dissolved in 50% ethanol and then added to the incubation medium to provide a final concentration of 10^{-4} M. Equivalent amounts of ethanol were present in each incubation mixture. Sialyltransferase present in the medium was monitored as a function of time for up to 18 hr. Results are expressed relative to the amount of sialyltransferase activity present in medium obtained from the 18-hr incubation, in the absence of pepstatin A, of liver slices prepared from inflamed rats. The 100% value was 327 pmol NeuAc transferred to rat asialo-α_1-AGP per ml medium per hr. Each point represents the means from three separate determinations; reproducibility was within $\pm$ 15%.


Sialyltransferase Activity (%)
100
80
60
40
20
0
0
4
8
12
16
20
Time of Incubation (hr)

pretreated with antiserum raised against rat liver lysosomal cathepsin D (Figure 25) confirmed that a cathepsin D-like proteinase was responsible for cleavage of Golgi membrane-bound sialyltransferase and that this proteinase was present in Golgi membrane suspensions at very low concentrations. In order to determine if sialyltransferase release from Golgi membranes could be enhanced by the increased presence of proteinase, the effect of exogenous cathepsin D on sialyltransferase release was investigated. Permeabilized Golgi vesicles were incubated at pH 5.6 either alone, or in the presence of 1 μg bovine spleen cathepsin D per mg Golgi membrane protein. Table 8 shows the effect of exposure to exogenous cathepsin D for up to 12 min on release of sialyltransferase from Golgi membranes prepared from livers from controls and from 36-hr inflamed rats. Exogenous cathepsin D caused significant increases in sialyltransferase release from Golgi membranes. Incubation periods longer than 12 min, or the addition of higher concentrations of exogenous cathepsin D, did result in greater release of sialyltransferase from Golgi membranes; however, under these conditions solubilized sialyltransferase was also degraded to a greater extent. Control experiments were conducted to determine the effect of exogenous cathepsin D on release of galactosyltransferase from Golgi membranes. Galactosyltransferase was found to be resistant to proteolysis by cathepsin D under these conditions since most or all of the galactosyltransferase activities were recovered in the pelleted Golgi membranes following incubation at reduced pH.

Evidence that Sialyltransferase and Cathepsin D Are Localized in the Same Compartments of the Rat Liver Golgi Apparatus

Experiments conducted to determine the identity of the proteinase involved in cleavage of the rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase

TABLE 8

EFFECT OF EXOGENOUS CATHEPSIN D ON RELEASE OF SIALYLTRANSFERASE FROM RAT LIVER GOLGI MEMBRANES

Incubation time		SIALYLTRANSFERASE ACTIVITY (pmol / mg Golgi protein / hr)			
		Control Golgi		Inflamed Golgi	
(min)	Cathepsin D.....	-	+	-	+
2		47 ± 3	80 ± 5	135 ± 12	166 ± 10
6		141 ± 8	222 ± 16	406 ± 30	621 ± 20
12		281 ± 10	466 ± 25	812 ± 55	1144 ± 82

Golgi membranes prepared from livers from controls and from 36-hr inflamed rats were exposed to ultrasonic vibrations for 30 s and then incubated at pH 5.6 for the times indicated alone (-), or in the presence of 1 μg bovine spleen cathepsin D per mg Golgi membrane protein (+). At the end of incubation pH was adjusted to 7.0, particulate material was removed by centrifugation and supernatants were assayed for sialyltransferase activities. Results represent the means from four separate determinations ± SD.

from Golgi membranes at reduced pH utilized Golgi vesicles that had been permeabilized by ultrasonication. Consequently they did not provide unequivocal evidence that the cathepsin D-like activity was present at the luminal side of the Golgi membrane. Therefore experiments were designed to show that release of sialyltransferase was due to an intraluminal proteolytic event carried out by endogenous Golgi cathepsin D. Intact Golgi vesicles were incubated for up to 16 min at neutral or reduced pH either alone, or in the presence of 10^{-6} M pepstatin A. At the end of incubation the pH was adjusted to 7.0, and portions of the incubation medium were removed and assayed for sialyltransferase activity. The remainder of the incubation medium was then exposed to ultrasonic vibrations for 30 s and then assayed for sialyltransferase activity (see Methods). The presence of pepstatin A in the incubation medium would inhibit mainly extraluminal cathepsin D activities. Table 9 shows the effect of pepstatin A on intraluminal release of sialyltransferase from intact Golgi vesicles prepared from livers from controls and from 36-hr inflamed rats. Increases in sialyltransferase activities were detected in the medium following ultrasonication of Golgi vesicles that had been incubated at either pH 7.0 or 5.6. Incubation of Golgi vesicles prepared from control-rat and inflamed-rat livers at reduced pH followed by ultrasonication caused a significantly greater amount of sialyltransferase to be released, and this effect was more pronounced with Golgi vesicles obtained from inflamed-rat livers. In addition, increase in incubation time from 4 to 16 min significantly enhanced proteolysis of sialyltransferase from Golgi membranes incubated at reduced pH. Substantial amounts of sialyltransferase were also released into the medium following ultrasonication of intact Golgi vesicles that had been incubated at pH 5.6 in the presence of pepstatin A. These values were only slightly lower than those

TABLE 9

EFFECT OF INCUBATION OF INTACT RAT LIVER GOLGI MEMBRANES WITH PEPSTATIN A FOLLOWED BY ULTRASONICATION TO RELEASE INTRALUMINAL SIALYLTRANSFERASE

		SIALYLTRANSFERASE ACTIVITY (pmol / mg Golgi protein / hr)			
		Control Golgi		Inflamed Golgi	
Conditions	Incubation time	4 min	16 min	4 min	16 min
pH 7.0					
Before Sonication		22 ± 2	41 ± 3	62 ± 5	73 ± 7
After Sonication		58 ± 5	74 ± 6	159 ± 12	194 ± 15
pH 5.6					
Before Sonication		123 ± 10	198 ± 18	271 ± 20	565 ± 55
After Sonication		327 ± 25	406 ± 35	1270 ± 80	1749 ± 90
pH 5.6 + Pepstatin A					
Before Sonication		82 ± 7	107 ± 10	204 ± 21	431 ± 53
After Sonication		261 ± 20	321 ± 30	1077 ± 101	1570 ± 125

Golgi membranes prepared from livers from controls and from 36-hr inflamed rats were incubated for 4 min and 16 min at pH 7.0, or at pH 5.6 in either the presence or absence of 10^{-6} M pepstatin A. At the end of incubation pH was adjusted to 7.0, 400 µl portions were removed from each sample, centrifuged to remove particulate matter and supernatants were assayed for sialyltransferase activities. The remaining material was then ultrasonicated for 30 s to release any sialyltransferase that was no longer membrane-bound, centrifuged and supernatants were assayed for sialyltransferase activities. Results represent the means from six separate determinations ± SD.

obtained under similar conditions from intact Golgi vesicles that had not been exposed to this inhibitor. Medium obtained from the 16-min incubation at pH 5.6 in the absence of pepstatin A of intact Golgi vesicles prepared from control-rat and 36-hr inflamed-rat livers contained 406 and 1749 units of sialyltransferase activity, respectively; the presence of pepstatin A in the incubation medium only caused these amounts of solubilized sialyltransferase activity detected in the medium following ultrasonication to be reduced to 321 and 1570 units, respectively. These data strongly suggested that both membrane-bound sialyltransferase and cathepsin D were present at the luminal side of Golgi membranes.

Characterization of Rat Liver Golgi Membrane Cathepsin D Activities

Inability of pepstatin A in the medium to inhibit proteolysis of rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase within intact Golgi vesicles (Table 9) confirmed the intraluminal location of cathepsin D activity. However it did not provide evidence that cathepsin D was membrane-bound. Therefore experiments were conducted to characterize endogenous cathepsin D activities present in Golgi membrane suspensions. Table 10 shows the effect of treatment with ultrasonic vibrations, Triton-X-100 and mannose 6-phosphate on release of cathepsin D from Golgi membranes prepared from livers from controls and from 36-hr inflamed rats. Cathepsin D activities were assayed using bovine hemoglobin as substrate (see Methods). Golgi membranes obtained from livers from inflamed rats contained approximately twice the amount of proteinase activity than Golgi membranes prepared from controls. Exposure of intact Golgi vesicles to ultrasonic vibrations caused only marginal release of cathepsin D activities. However,

TABLE 10

CATHEPSIN D ACTIVITIES IN INTACT RAT LIVER GOLGI MEMBRANES AND SUPERNATANT FRACTIONS PREPARED BY ULTRASONICATION, EXTRACTION WITH TRITON X-100 AND INCUBATION WITH MANNOSE 6-PHOSPHATE

Sample	CATHEPSIN D ACTIVITY (ΔA_{280} / ml / min) Control Golgi	Inflamed Golgi
Intact Golgi	2.50 ± 0.30	4.04 ± 0.35
Supernatant Fractions		
Ultrasonicated Golgi	0.10 ± 0.01	0.61 ± 0.07
Triton X-100	2.42 ± 0.21	3.57 ± 0.35
Mannose 6-phosphate	1.90 ± 0.11	3.17 ± 0.25

Golgi membranes prepared from livers from controls and from 36-hr inflamed rats were assayed directly for cathepsin D activity ("intact Golgi" samples). Golgi membranes were then either exposed to ultrasonication for 30 s ("ultrasonicated Golgi" samples) or extracted with 0.1% Triton X-100 ("Triton X-100" samples), following which particulate material was removed by centrifugation and supernatants were assayed for cathepsin D activities. Golgi membranes were also ultrasonicated for 30 s and then incubated for 30 min in the presence of 5 mM mannose 6-phosphate, following which particulate material was removed by centrifugation and supernatants were assayed for cathepsin D activities ("mannose 6-phosphate" samples). Supernatants obtained from centrifugation of intact Golgi membranes, and centrifugation of intact Golgi membranes that had been incubated with mannose 6-phosphate contained low levels of cathepsin D activities, and these values were subtracted from those present in the "ultrasonicated Golgi" and "mannose 6-phosphate" samples, respectively. Results represent the means of three separate determinations ± SD.

treatment with Triton-X-100 caused substantial amounts of cathepsin D to be released. This suggested that cathepsin D within intact Golgi vesicles was membrane-bound. The possibility that endogenous Golgi cathepsin D activities were due to transitory cathepsin D proteinase bound to mannose 6-phosphate receptors that were enroute to lysosomes was investigated by examining the effect of pretreatment with mannose 6-phosphate on release of cathepsin D. Incubation of permeabilized Golgi vesicles for 30 min at neutral pH in the presence of 5 mM mannose 6-phosphate resulted in significant release of cathepsin D. Exposure to mannose 6-phosphate caused 76% and 78% of the total cathepsin D activity to be released from Golgi membranes prepared from livers from controls and from inflamed rats, respectively. This amount of proteinase release was only slightly lower than that achieved by treatment of Golgi membranes with the detergent Triton X-100. These data strongly suggested that endogenous Golgi cathepsin D-like activities responsible for cleavage of rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase were associated with membrane-bound mannose 6-phosphate receptors.

ACUTE PHASE RESPONSE OF MOUSE AND GUINEA PIG HEPATIC Galβ1,4GlcNAc α2,6-SIALYLTRANSFERASE

Effect of Inflammation on Mouse and Guinea Pig Serum, Liver and Liver Slice Medium Sialyltransferase Activities

Studies on the effect of inflammation on sialyltransferase activities in the rat were repeated in the mouse and the guinea pig in order to determine if the acute phase response of hepatic Galβ1,4GlcNAc α2,6-sialyltransferase was an inter-species phenomenon. Injection of turpentine was reported to cause rat serum and liver sialyltransferase activities towards rat asialo-α_1-

AGP to rapidly increase to a maximum of five times and three times, respectively, over control values after 48 hr (Kaplan *et al.*, 1983). Figures 27 and 28 show the effect of turpentine-induced inflammation on serum and liver sialyltransferase activities towards rat asialo-α_1-AGP in the mouse and the guinea pig, respectively. Inflammation caused significant elevations in sialyltransferase activities in the mouse and the guinea pig, however a species variation was observed in both the timing and the magnitude of the response of sialyltransferase to inflammation. Sialyltransferase activities in mouse serum and liver were found to rise rapidly following the onset of inflammation, reaching maximum values of 24.7 units/ml and 14 units/mg, respectively, after 72 hr. These maximum values for mouse serum and liver sialyltransferase activities represented a five-fold and a three-fold increase, respectively, over control values. In contrast to both the rat and the mouse, guinea pig serum and liver sialyltransferase activities showed a delayed response to inflammation. No significant change in guinea pig serum sialyltransferase activities could be detected within 48 hr after the onset of turpentine-induced inflammation. However, after 48 hr guinea pig serum sialyltransferase activities sharply increased, reaching a maximum of 147 units/ml at 96 hr. Detectable increases in guinea pig liver sialyltransferase activities occurred 24 hr after the onset of inflammation, and a maximum of 23 units/mg was also found at 96 hr. These maximum values for sialyltransferase activities in guinea pig serum and liver represented a 50% increase in activity over control values. Control values for guinea pig serum and liver sialyltransferase activities were 100 units/ml and 15 units/mg, respectively. These control values were much higher than the control values of 5.4 units/ml and 4.5 units/mg found for mouse serum and liver sialyltransferase activities; consequently, the magnitude of response of

FIGURE 27

Effect of Inflammation on Mouse Liver and Serum Sialyltransferase Activities

Liver (■) and serum (▲) sialyltransferase activities are expressed as pmol of NeuAc transferred to rat asialo-α1-AGP per mg liver protein or per ml serum per min. Results represent means from 5-8 animals for each value; replicates were within ± 8%.

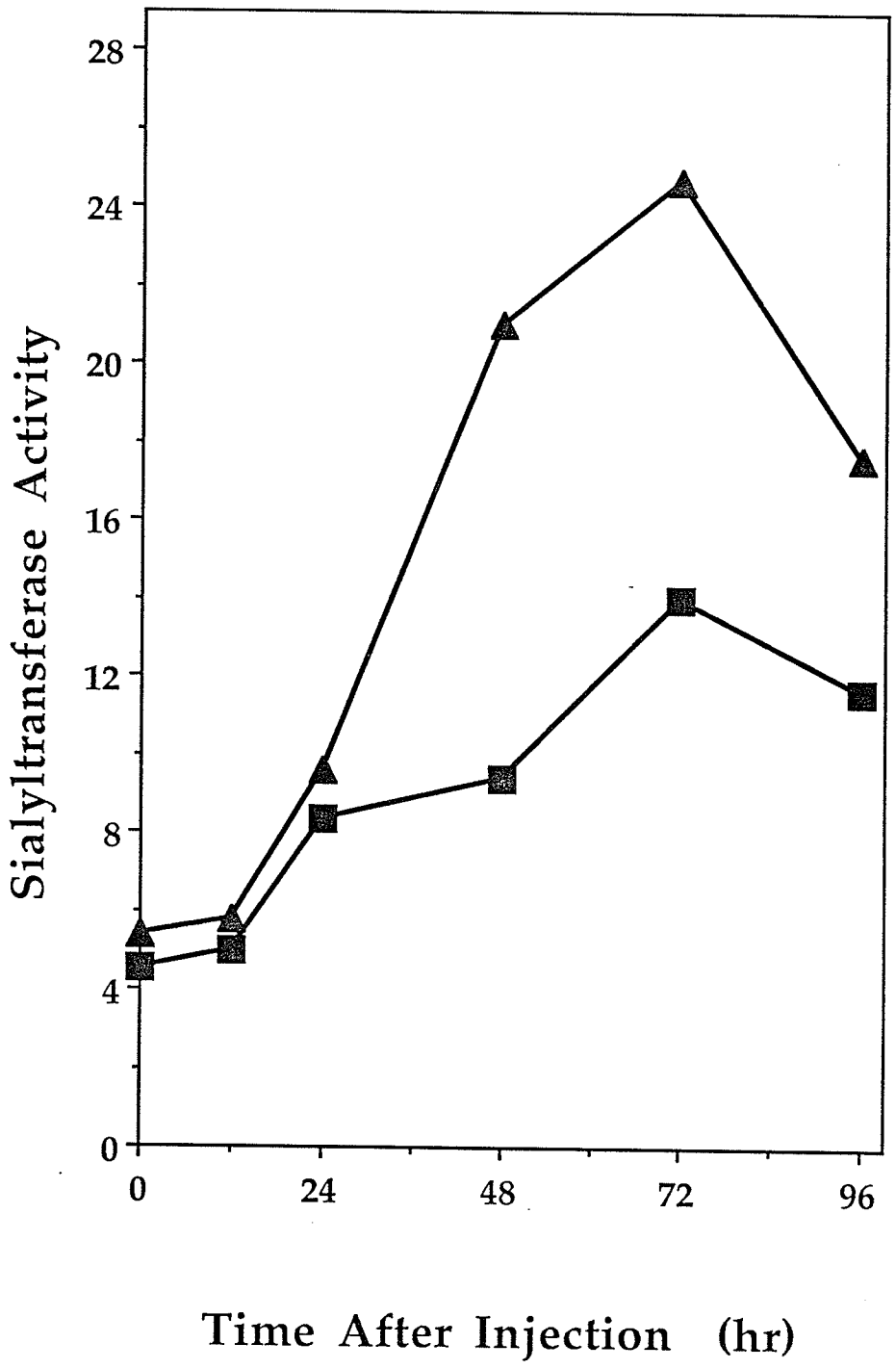
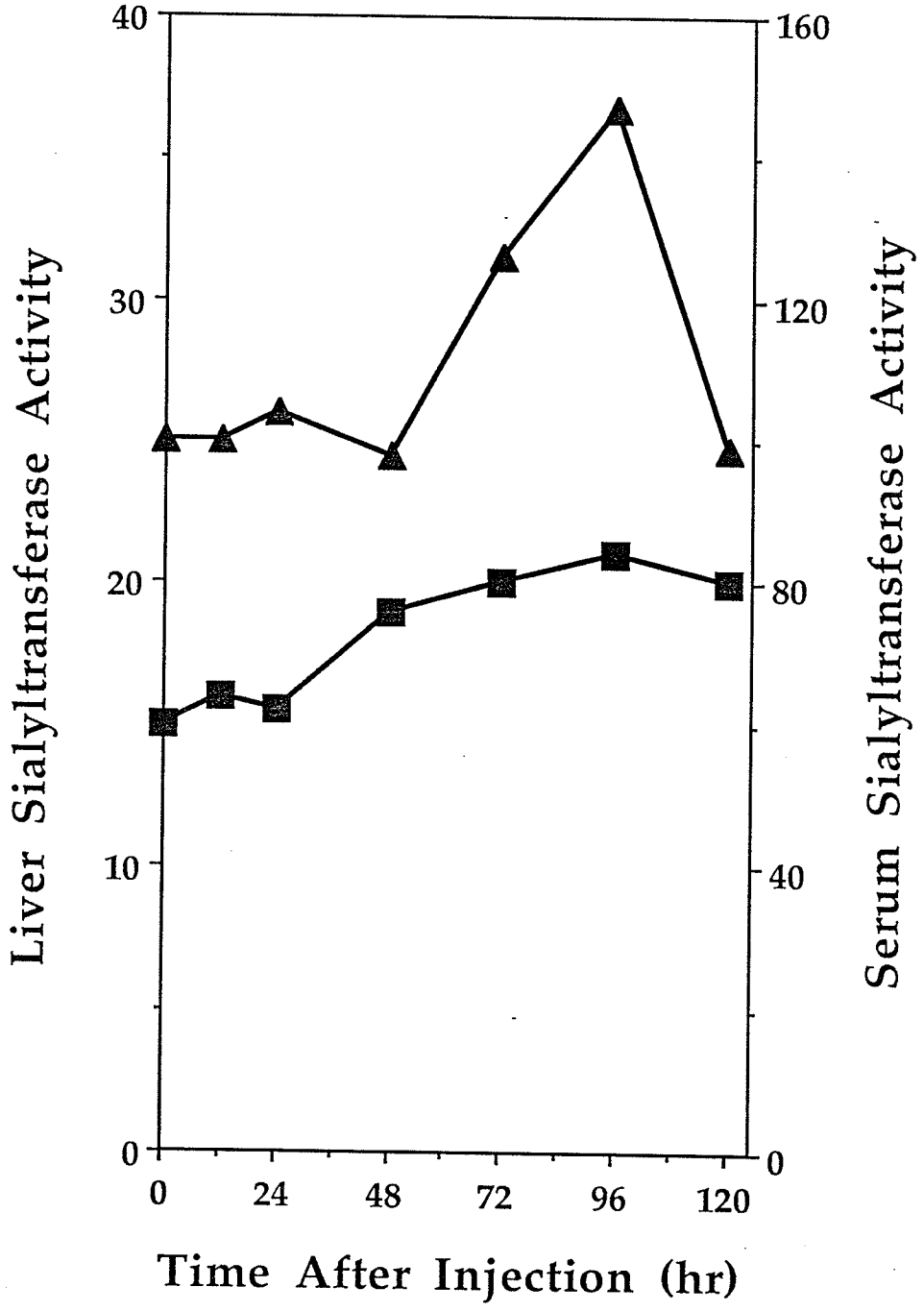

Sialyltransferase Activity
28
24
20
16
12
8
4
0
0
24
48
72
96
Time After Injection (hr)

FIGURE 28

Effect of Inflammation on Guinea Pig Liver and Serum Sialyltransferase Activities

Liver (■) and serum (▲) sialyltransferase activities are expressed as pmol of NeuAc transferred to rat asialo-α_1-AGP per mg liver protein or per ml serum per min. Results represent means from 5-8 animals for each value; replicates were within $\pm$ 8%.


Liver Sialyltransferase Activity
Serum Sialyltransferase Activity
Time After Injection (hr)
0
10
20
30
40
0
40
80
120
160
0
24
48
72
96
120

sialyltransferase to inflammation was smaller in the guinea pig than in the mouse.

In order to confirm the hepatic origin of the elevated serum sialyltransferase activities detected in the mouse and the guinea pig during turpentine-induced inflammation, secretion of sialyltransferase from liver slices was investigated. Inflammation was allowed to proceed for 72 hr in mice and for 96 hr in guinea pigs before sacrifice, and release of sialyltransferase from liver slices prepared from livers obtained from controls and from experimentally-inflamed animals was monitored as a function of time for up to 6 hr. Figures 29 and 30 show the secretion of sialyltransferase activities from liver slices prepared from mouse and guinea pig livers, respectively. Sialyltransferase secretion from liver slices increased fairly linearly with time of incubation. Liver slices prepared from 72-hr inflamed-mouse and 96-hr inflamed-guinea pig livers secreted equivalent amounts of sialyltransferase activity. However, liver slices prepared from control-mouse livers released lesser amounts of sialyltransferase than did slices obtained from control-guinea pig livers, and therefore turpentine-induced inflammation caused a three-fold and a two-fold increase in sialyltransferase secretion from mouse and guinea pig liver slices, respectively. Elevated secretion of sialyltransferase from liver slices prepared from livers obtained from inflamed mice and guinea pigs showed that sialyltransferase was behaving as an acute phase reactant in the mouse and the guinea pig.

Effect of pH on Mouse and Guinea Pig Liver and Serum Sialyltransferase Activities

There is little published information on the effect of pH on mouse and guinea pig sialyltransferase activities. Therefore experiments were

FIGURE 29

Effect of Incubation Time on Secretion of Sialyltransferase from Mouse Liver Slices

Sialyltransferase activities released from liver slices prepared from livers from controls (○) and from 72-hr inflamed mice (●) are expressed as pmol of NeuAc transferred to rat asialo-α_1-AGP per ml medium per min. Results represent means from 5-8 animals for each value; replicates were within $\pm$ 8%.

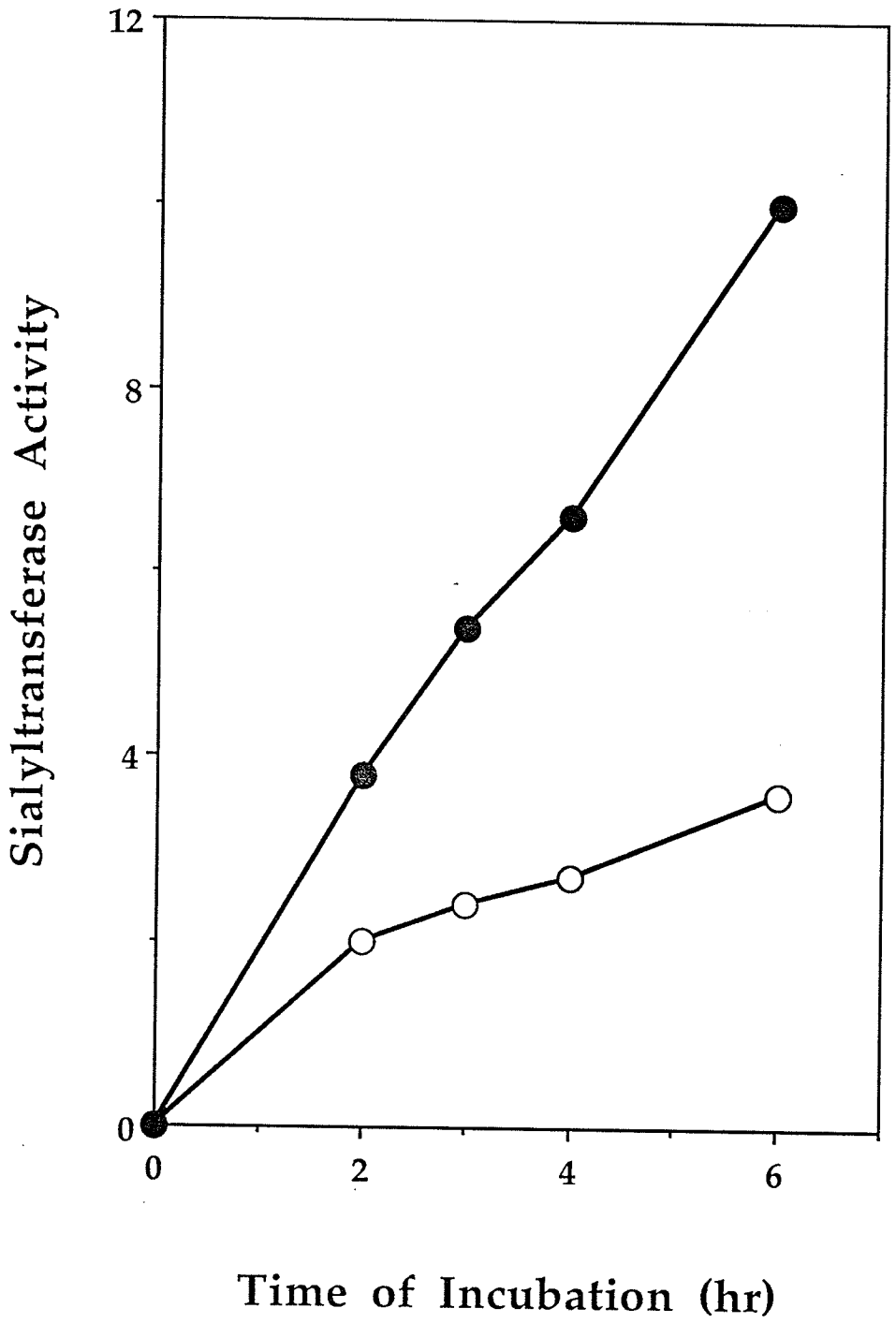
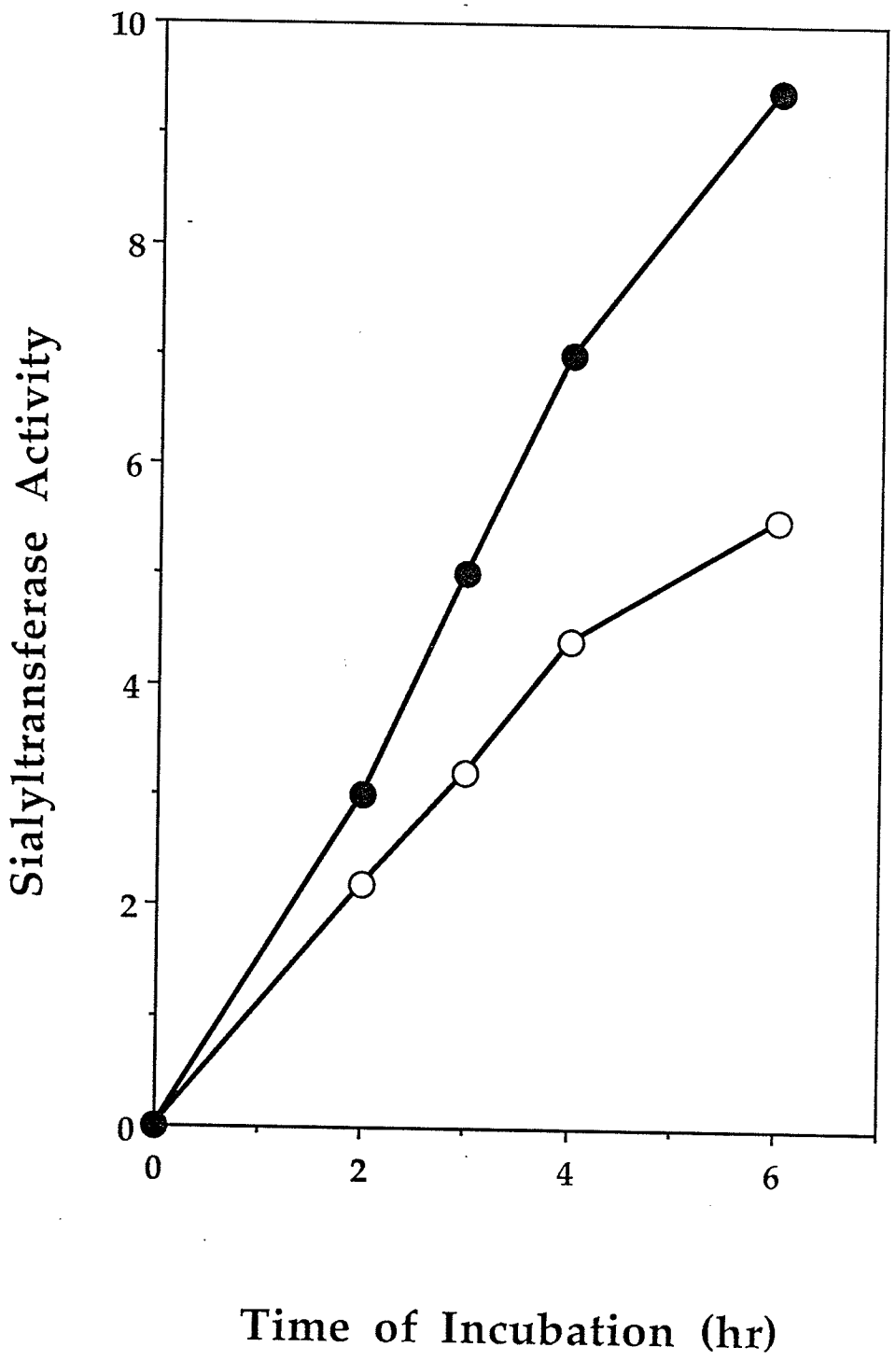

12
8
4
0
0
2
4
6
Sialyltransferase Activity
Time of Incubation (hr)

FIGURE 30

Effect of Incubation Time on Secretion of Sialyltransferase from Guinea Pig Liver Slices

Sialyltransferase activities released from liver slices prepared from livers from controls (○) and from 96-hr inflamed guinea pigs (●) are expressed as pmol of NeuAc transferred to rat asialo-α_1-AGP per ml medium per min. Results represent means from 5-8 animals for each value; replicates were within $\pm$ 8%.


Sialyltransferase Activity
10
8
6
4
2
0
0
2
4
6
Time of Incubation (hr)

conducted to determine the pH optima of sialyltransferase activities in mouse and guinea pig liver and serum with respect to the acceptor rat asialo-α_1-AGP. Sialyltransferase activities in serum and liver samples prepared from controls, and from either 72-hr inflamed mice or 96-hr inflamed guinea pigs, were assayed in the presence of equimolar McIlvaine sodium phosphate - citric acid buffers of different pH values (see Methods). Figures 31 and 32 show the effect of pH on mouse liver and serum sialyltransferase activities, respectively. Control and 72-hr inflamed-mouse liver sialyltransferase activities exhibited a similar response to pH, producing smooth, bell-shaped pH profiles that shared a common region of near-maximum enzyme activity between pH 6.10 and 7.00, and a pH optimum of 6.54. Mouse serum sialyltransferase activities showed a greater tolerance to pH and significant enzyme activity was found to occur within the pH region 5.72 - 8.06. The pH optima of control and 72-hr inflamed-mouse serum sialyltransferase activities were pH 7.19 and 7.00, respectively. The effect of pH on guinea pig liver and serum sialyltransferase activities is shown in Figures 33 and 34, respectively. Sialyltransferase activities in control and 96-hr inflamed-guinea pig liver produced very broad, bell-shaped pH profiles that exhibited significant activity within the pH region 6.24 - 7.24 and a coincident pH optima at pH 6.71. Control and 96-hr inflamed-guinea pig serum sialyltransferase activities were found to have a pH optimum of 6.87, and significant enzyme activity was found to occur within the pH region 5.93 - 8.15. These data demonstrated that turpentine-induced inflammation did not significantly alter the pH optima of mouse and guinea pig liver and serum sialyltransferase activities. The range of pH optima found for mouse and guinea pig liver and serum sialyltransferase activities was between pH 6.54 and 7.19. Sialyltransferase activities towards rat asialo-α_1-AGP in liver

FIGURE 31

Effect of pH on Mouse Liver Sialyltransferase Activities

Sialyltransferase activities present in livers from controls (□) and 72-hr inflamed mice (■) were assayed in presence of 50 mM McIlvaine buffers of different pH, the values of which were measured at 37 °C. Results are expressed relative to the maximum amount of sialyltransferase activity present in livers obtained from inflamed animals. The 100% value was 14.0 pmol NeuAc transferred to rat asialo-α_1-AGP per mg liver protein per min. Each point represents the means from four to six separate determinations; reproducibility was within $\pm$ 10%.

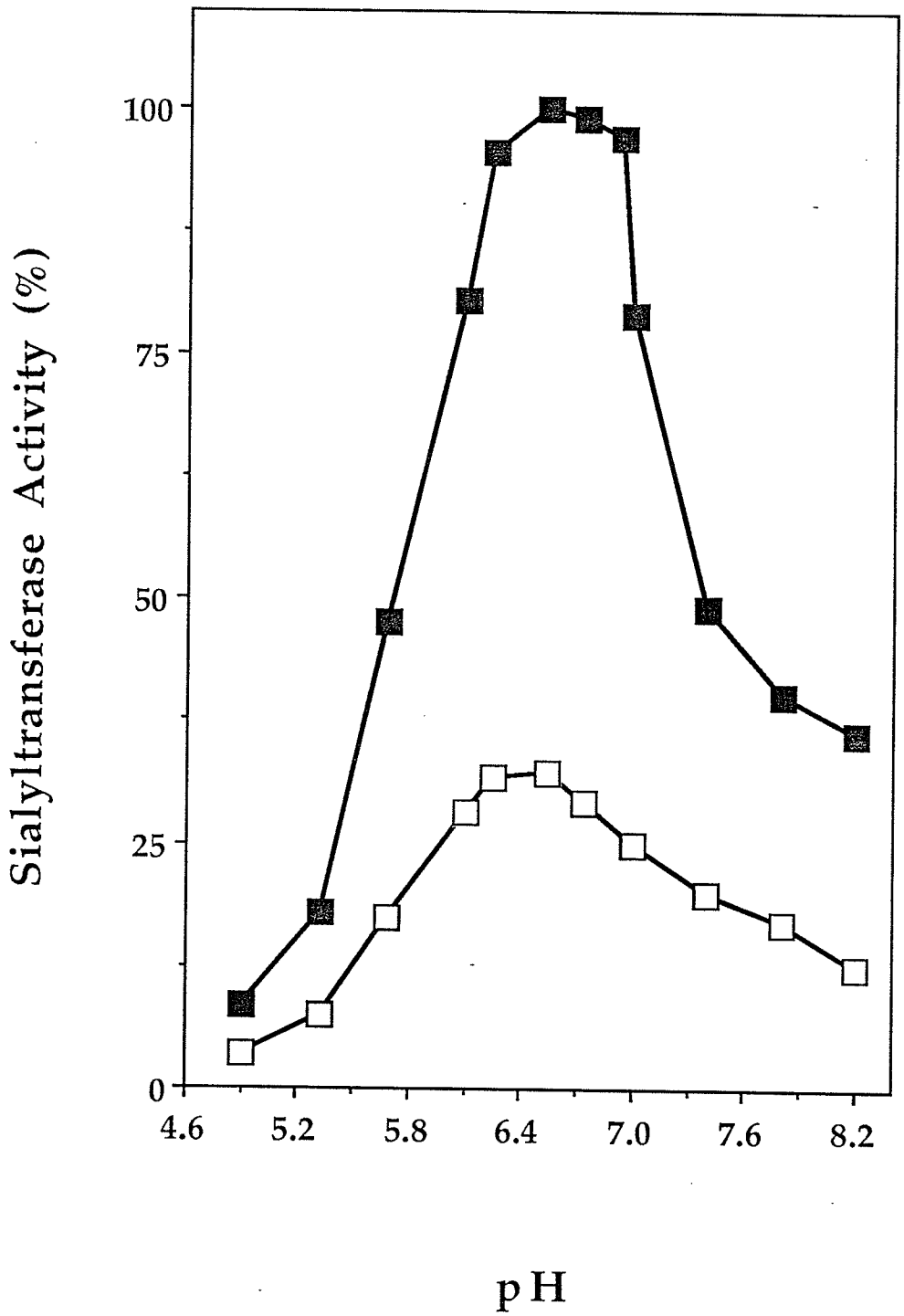

Sialyltransferase Activity (%)
100
75
50
25
0
4.6
5.2
5.8
6.4
7.0
7.6
8.2
pH

FIGURE 32

Effect of pH on Mouse Serum Sialyltransferase Activities

Sialyltransferase activities present in serum from controls (Δ) and 72-hr inflamed mice (▲) were assayed in presence of 50 mM McIlvaine buffers of different pH, the values of which were measured at 37 ^{o}C. Results are expressed relative to the maximum amount of sialyltransferase activity present in serum obtained from inflamed animals. The 100% value was 26.8 pmol NeuAc transferred to rat asialo-α1-AGP per ml serum per min. Each point represents the means from four to six separate determinations; reproducibility was within ± 10%.

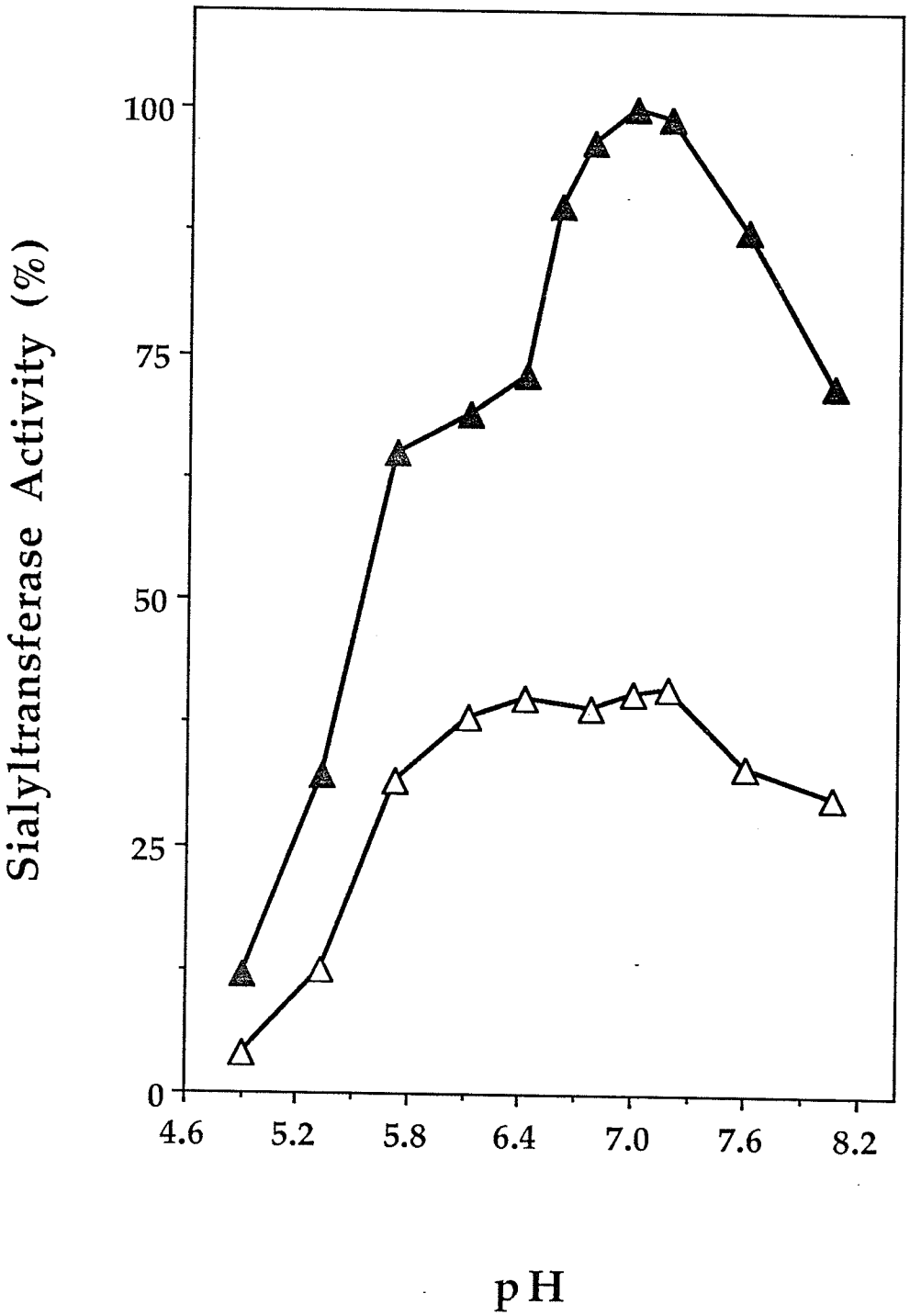

Sialyltransferase Activity (%)
100
75
50
25
0
4.6
5.2
5.8
6.4
7.0
7.6
8.2
pH

FIGURE 33

Effect of pH on Guinea Pig Liver Sialyltransferase Activities

Sialyltransferase activities present in livers from controls (❑) and 96-hr inflamed guinea pigs (■) were assayed in presence of 50 mM McIlvaine buffers of different pH, the values of which were measured at 37 ^{o}C. Results are expressed relative to the maximum amount of sialyltransferase activity present in livers obtained from inflamed animals. The 100% value was 22.5 pmol NeuAc transferred to rat asialo-α_1-AGP per mg liver protein per min. Each point represents the means from four to six separate determinations; reproducibility was within $\pm$ 10%.

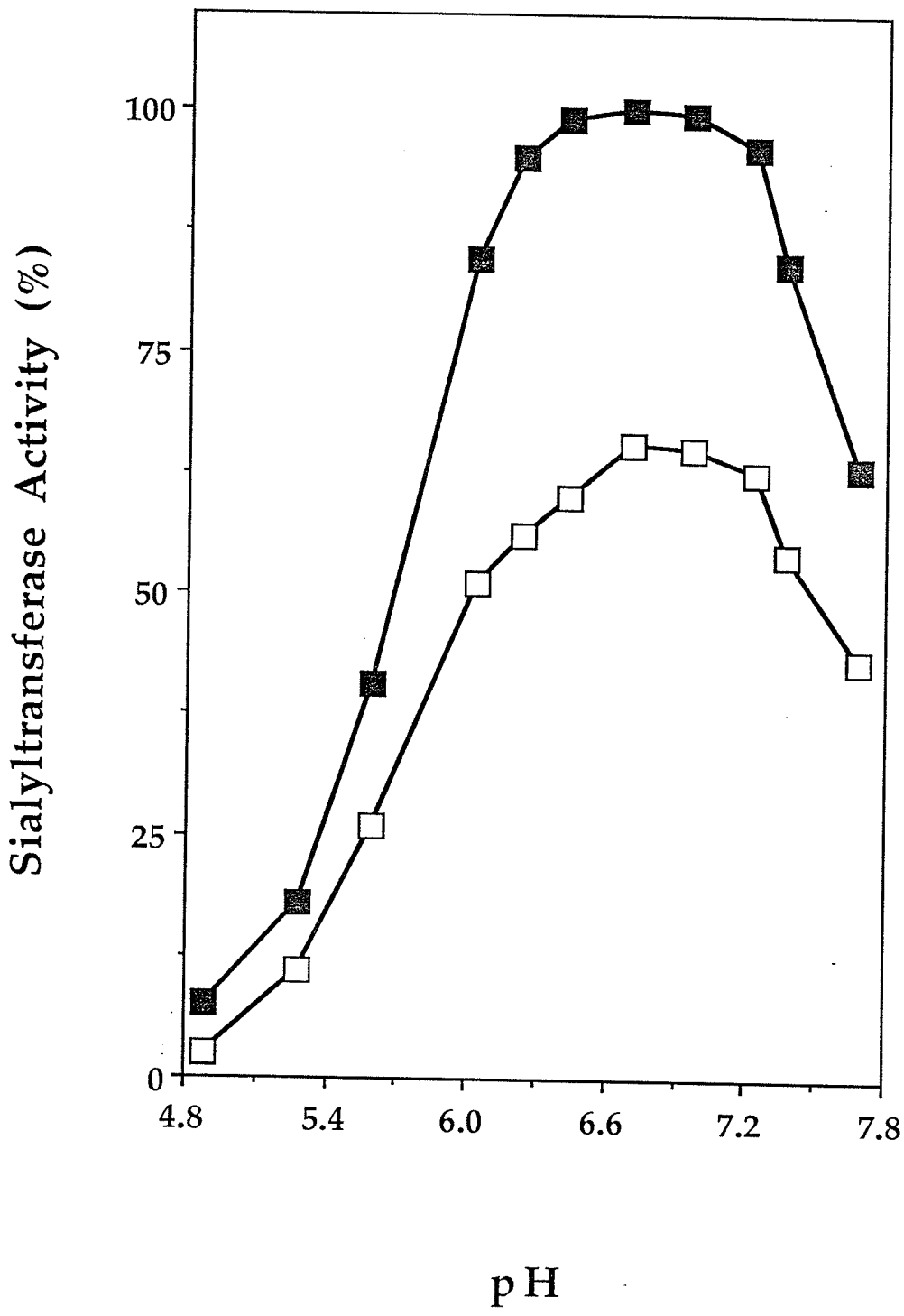
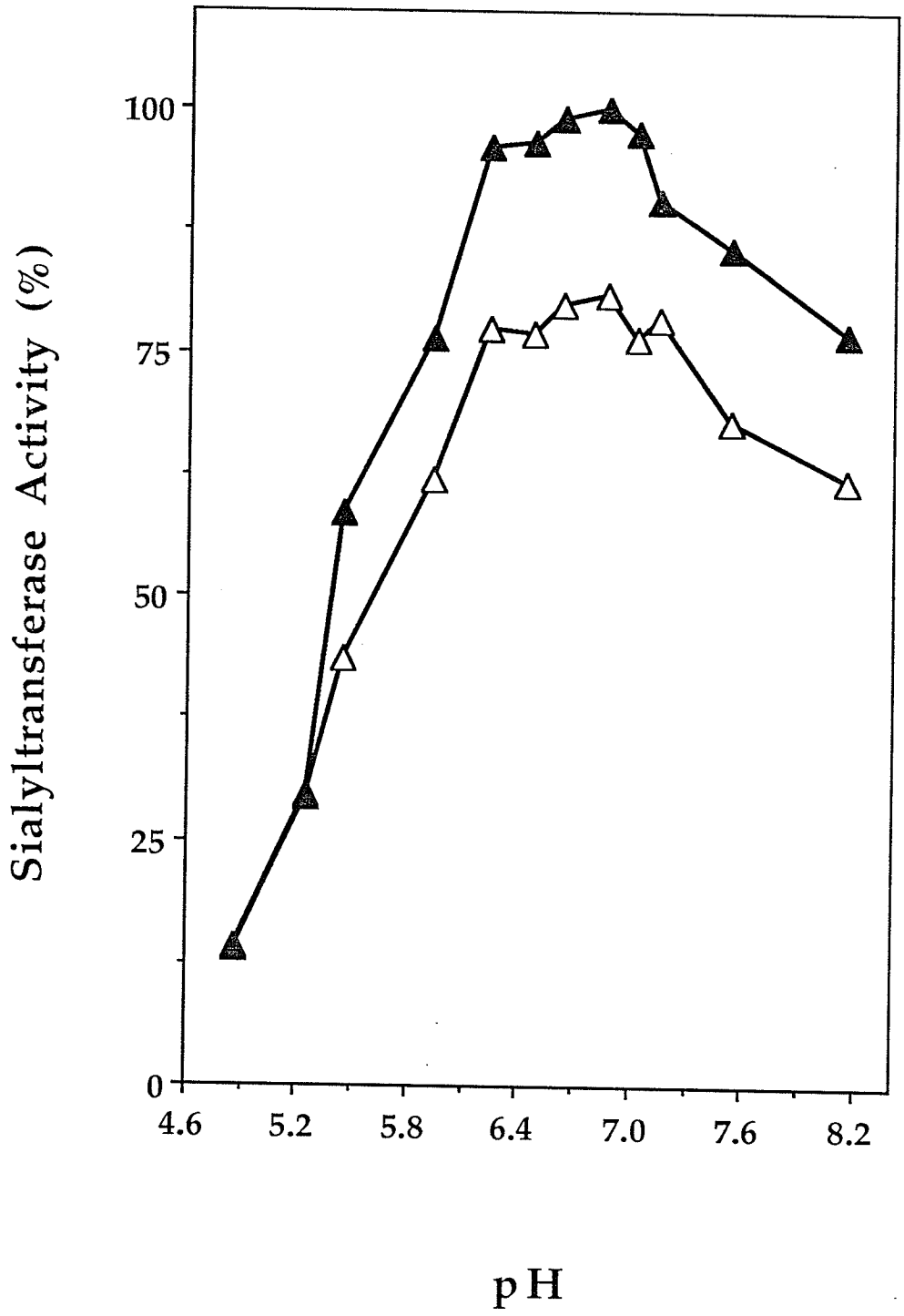

Sialyltransferase Activity (%)
100
75
50
25
0
4.8
5.4
6.0
6.6
7.2
7.8
pH

FIGURE 34

Effect of pH on Guinea Pig Serum Sialyltransferase Activities

Sialyltransferase activities present in serum from controls (Δ) and 96-hr inflamed guinea pigs (▲) were assayed in presence of 50 mM McIlvaine buffers of different pH, the values of which were measured at 37 ^{o}C. Results are expressed relative to the maximum amount of sialyltransferase activity present in serum obtained from inflamed animals. The 100% value was 126.0 pmol NeuAc transferred to rat asialo-α_1-AGP per ml serum per min. Each point represents the means from four to six separate determinations; reproducibility was within $\pm$ 10%.


Sialyltransferase Activity (%)
100
75
50
25
0
4.6
5.2
5.8
6.4
7.0
7.6
8.2
pH

and serum prepared from controls and from 48-hr inflamed rats were reported to have pH optima of 6.80 and 7.00, respectively (Kaplan *et al.*, 1983). Therefore, pH 6.80 was chosen to be the common pH at which rat, mouse and guinea pig serum and liver sialyltransferase activities would be measured in this study.

Characterization of Mouse and Guinea Pig Serum, Liver and Liver Slice Medium Sialyltransferase Activities

The hepatic N-linked Galβ1,4GlcNAc α2,6-sialyltransferase was found to be responsible for the elevated sialyltransferase activities present in serum and liver slice medium prepared from turpentine-inflamed mice and guinea pigs. Confirmation of the N-linked nature of the enzyme was achieved by using rat asialo-α_1-AGP as the macromolecular acceptor, as was done in the enzyme assays described above (Figures 27 - 30) showing that sialyltransferase was as an acute phase reactant in the mouse and the guinea pig. Since rat α_1-AGP possesses only asparagine-linked oligosaccharide chains (Yoshima *et al.*, 1981), use of rat asialo-α_1-AGP as the acceptor of N-acetylneuraminic acid transfer insured that only N-linked sialyltransferase activity was being measured.

In each of the five bi- or triantennary complex oligosaccharide chains of rat α_1-AGP the penultimate galactose residue is attached in either a β1,3 or a β1,4 linkage to N-acetylglucosamine. N-acetylneuraminic acid is present in one of three linkages: NeuAcα2,3Gal, NeuAcα2,6Gal, or Galβ1,3(NeuAcα2,6)GlcNAc (Yoshima *et al.*, 1981). Synthesis of the terminal α2,3 and α2,6 sialic acid linkages of the N-linked oligosaccharides of α_1-AGP is performed in rat liver by two distinct enzymes, the α2,3 and the α2,6 sialyltransferases (Hudgin and Schachter, 1972). These sialyltransferases may be

distinguished by using the disaccharide lactose (Galβ1,4Glc) as the low molecular weight acceptor. Prolonged incubation of α2,3 and α2,6 sialyltransferases with lactose results in the formation of NeuAcα2,3Galβ1,4Glc and NeuAcα2,6Galβ1,4Glc sialyllactose isomers, respectively, which may be readily separated using a chromatographic technique (Hudgin and Schachter, 1972; Weinstein *et al.*, 1982a; Kaplan *et al.*, 1983). This procedure was used to distinguish between α2,3 and α2,6 linkage specificities of the sialyltransferase activities found in mouse and guinea pig liver, serum and liver slice medium. Samples were incubated for 16 hr in the presence of unlimiting concentrations of lactose and CMP-[^{14}C]-NeuAc, and sialyllactose isomer separation was performed by descending paper chromatography in ethyl acetate:pyridine:water (12:5:4, v/v/v). Measurement of α2,3 and α2,6 sialyllactose isomer peaks identified by radiochromatogram scanning was achieved by individually counting 0.5 X 8 cm strips that had been cut from appropriate regions of the paper chromatograms (see Methods). Figures 35 - 37 show histograms obtained from the incubation of control and 72-hr inflamed-mouse liver, serum and liver slice medium samples with lactose. Control-mouse liver contained a significantly greater amount of α2,3-sialyltransferase activity than α2,6-sialyltransferase activity. Turpentine-induced inflammation caused an elevation in both enzyme activities, however the extent of increase of α2,3-sialyltransferase activity was marginal compared to that of α2,6 enzyme activity. In contrast to control-mouse liver, control-mouse serum and liver slice medium exhibited predominantly α2,6-sialyltransferase activity. Both sialyltransferase activities were present in inflamed-mouse serum and liver slice medium, however the amount of α2,6-sialyltransferase activity greatly exceeded the amount of α2,3-sialyltransferase activity. Lactose incubations with inflamed-mouse serum and liver slice medium showed

FIGURE 35

Sialyllactose Isomers Produced by Mouse Liver Sialyltransferase Activities

Histogram of sialyllactose isomers produced by control-mouse (broken line) and 72-hr inflamed-mouse (solid line) liver sialyltransferase activities. 6'SL and 3'SL indicate the positions of the 6'(NeuAcα2,6Galβ1,4Glc) and 3'(NeuAcα2,3Galβ1,4Glc) isomers, respectively. Chromatography in ethyl actetate:pyridine:water (12:5:4, v/v/v) was for five days, and sialyllactose isomer peaks identified by radiochromatogram scanning were measured by counting appropriate 0.5 X 8 cm strips of chromatograms in a liquid scintillation counter. Results are expressed relative to the maximum [^{14}C] dpm of the 6'SL isomer. The 100% value was 4495 dpm.

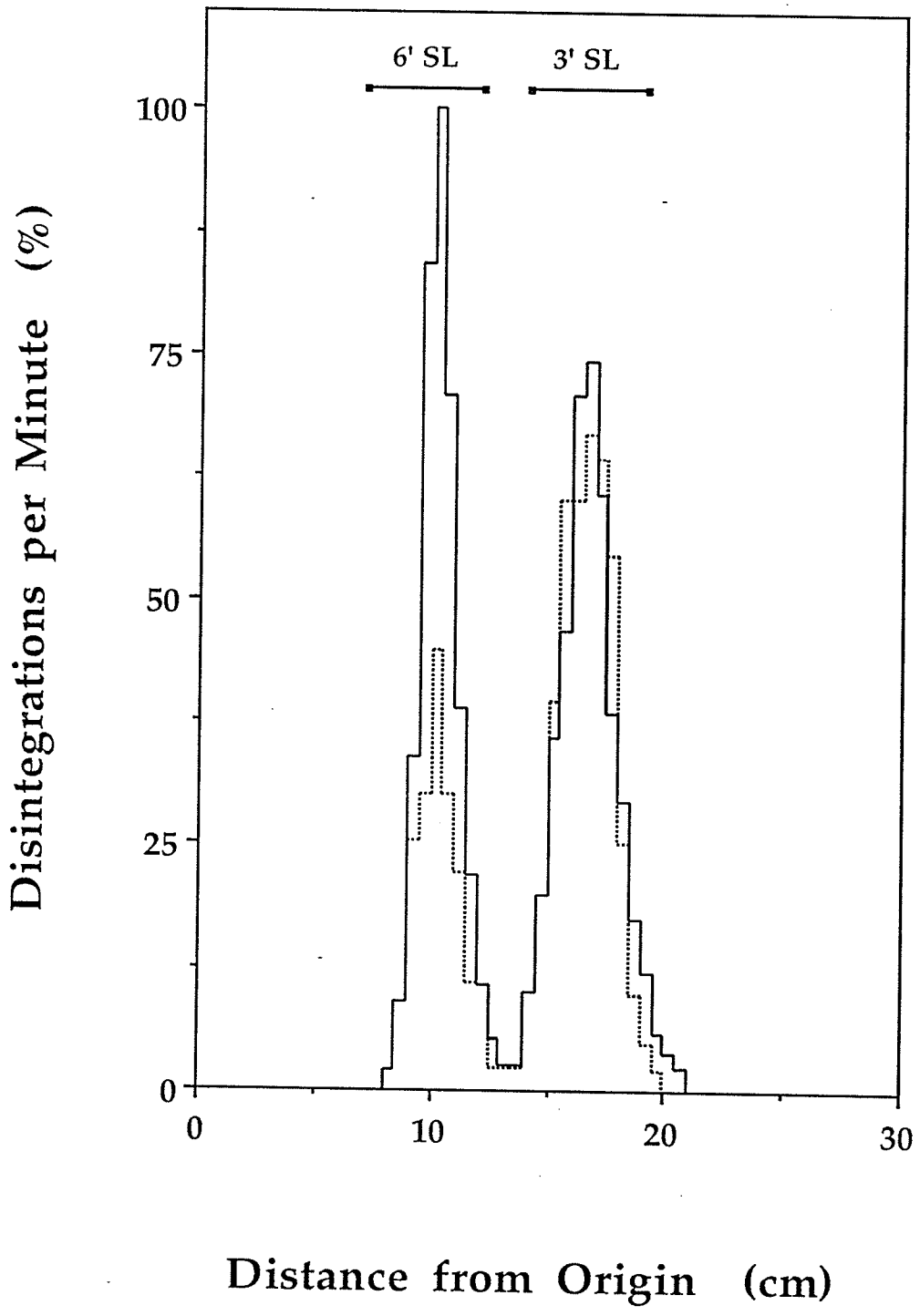

6' SL
3' SL
100
75
50
25
0
0
10
20
30
Disintegrations per Minute (%)
Distance from Origin (cm)

FIGURE 36

Sialyllactose Isomers Produced by Mouse Serum Sialyltransferase Activities

Histogram of sialyllactose isomers produced by control-mouse (broken line) and 72-hr inflamed-mouse (solid line) serum sialyltransferase activities. 6'SL and 3'SL indicate the positions of the 6'(NeuAcα2,6Galβ1,4Glc) and 3'(NeuAcα2,3Galβ1,4Glc) isomers, respectively. Chromatography was as described in the legend for Figure 35. Results are expressed relative to the maximum [^{14}C] dpm of the 6'SL isomer. The 100% value was 3784 dpm.

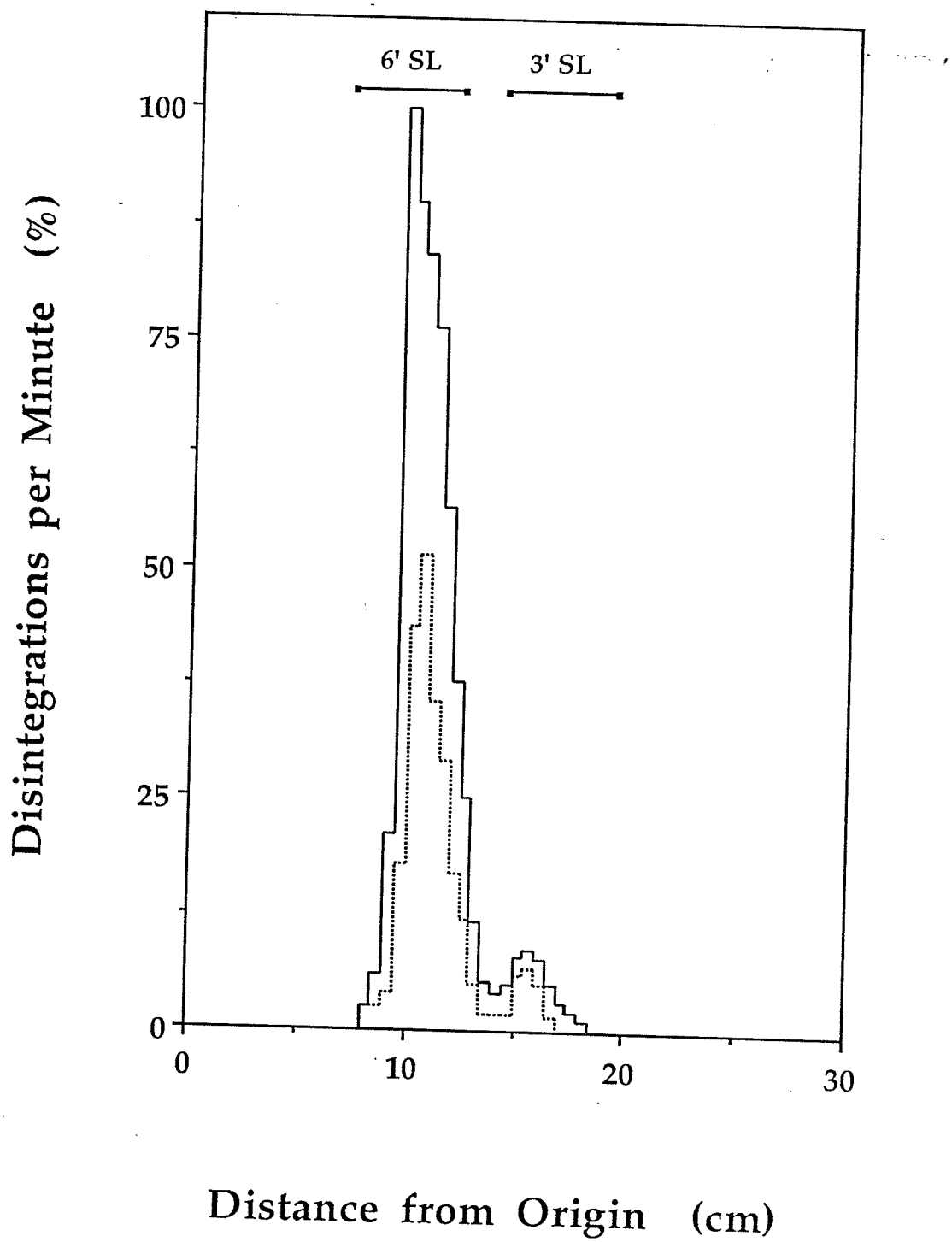
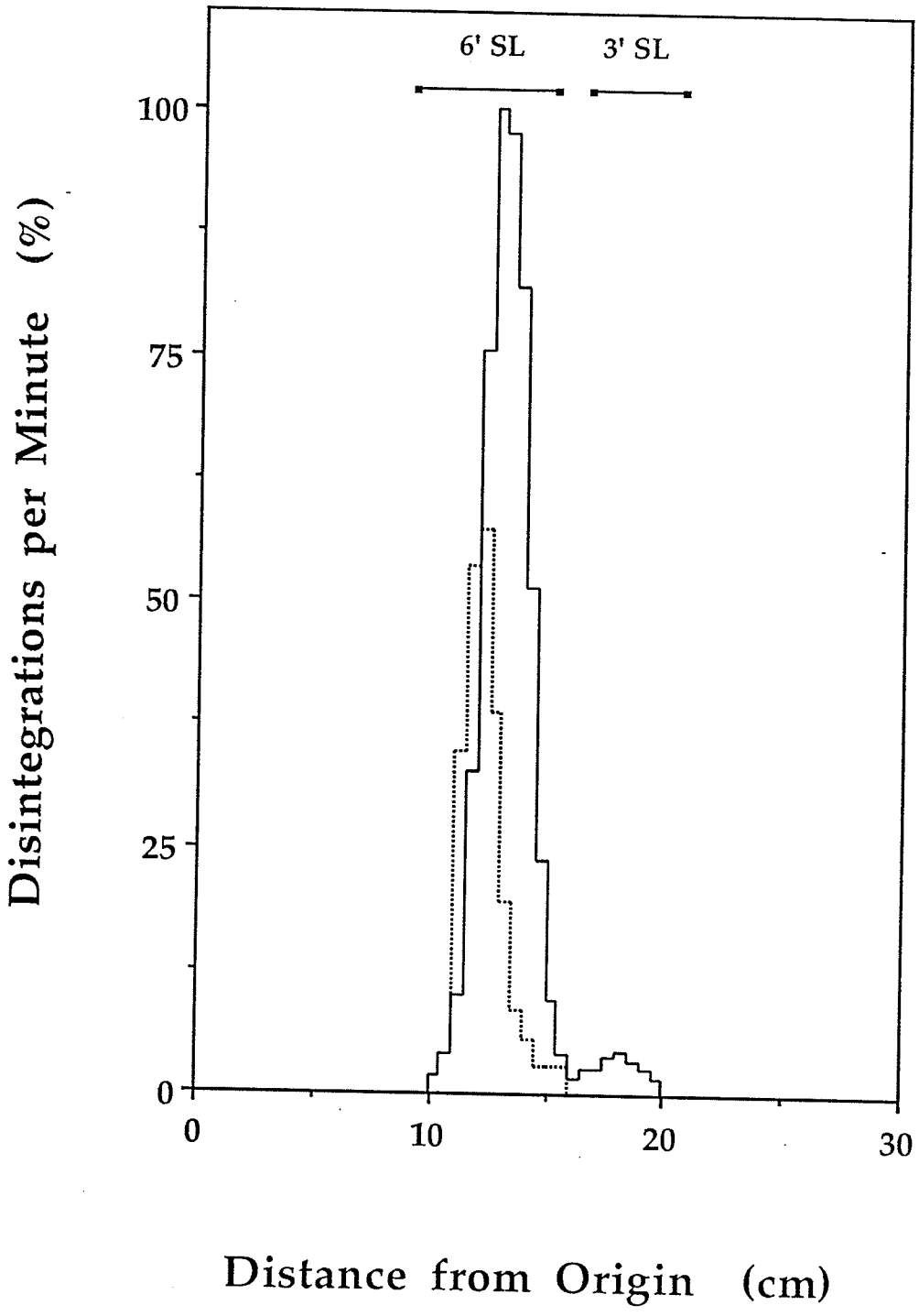

6' SL
3' SL
100
75
50
25
0
0
10
20
30
Disintegrations per Minute (%)
Distance from Origin (cm)

FIGURE 37

Sialyllactose Isomers Produced by Mouse Liver Slice Medium Sialyltransferase Activities

Histogram of sialyllactose isomers produced by control-mouse (broken line) and 72-hr inflamed-mouse (solid line) liver slice medium sialyltransferase activities. 6'SL and 3'SL indicate the positions of the 6'(NeuAcα2,6Galβ1,4Glc) and 3'(NeuAcα2,3Galβ1,4Glc) isomers, respectively. Chromatography was as described in the legend for Figure 35. Results are expressed relative to the maximum [^{14}C] dpm of the 6'SL isomer. The 100% value was 2197 dpm.


6' SL
3' SL
100
75
50
25
0
0
10
20
30
Disintegrations per Minute (%)
Distance from Origin (cm)

substantial increases only in NeuAcα2,6Galβ1,4Glc isomer formation which demonstrated that inflammation caused significant release only of α2,6-sialyltransferase activity. Histograms obtained from the incubation of liver, serum and liver slice medium samples from controls and from 96-hr inflamed guinea pigs with lactose are shown in Figures 38 - 40. Control-guinea pig liver exhibited both α2,3- and α2,6-sialyltransferase activities; however, in contrast to the mouse, the amount of α2,6-sialyltransferase activity in control-guinea pig liver was significantly greater than the amount of α2,3-sialyltransferase activity. Both enzyme activities were increased in 96-hr inflamed-guinea pig liver, however the increase in α2,6-sialyltransferase activity was more pronounced. Control-guinea pig serum and liver slice medium contained substantially greater amounts of α2,6-sialyltransferase activity than α2,3-sialyltransferase activity. As was found in the mouse, inflammation in the guinea pig caused significant increases in serum and liver slice medium α2,6-sialyltransferase activities. However, the extent of increase of α2,6-sialyltransferase activities in guinea pig serum and liver slice medium due to turpentine-induced inflammation was not as pronounced as that of α2,6-sialyltransferase activities in mouse serum and liver slice medium. Additional experiments were carried out in which mouse and guinea pig sialyltransferase activities towards lactose were examined with respect to time of inflammation. Liver samples from mice that had been suffering from turpentine-induced inflammation for 12, 24 and 48 hr were found to have proportionately greater amounts of α2,3- and α2,6-sialyltransferase activities. However, mouse serum samples obtained under similar conditions showed consistent increases only in α2,6-sialyltransferase activity. Similar results were obtained from experiments using liver and

FIGURE 38

Sialyllactose Isomers Produced by Guinea Pig Liver Sialyltransferase Activities

Histogram of sialyllactose isomers produced by control-guinea pig (broken line) and 96-hr inflamed-guinea pig (solid line) liver sialyltransferase activities. 6'SL and 3'SL indicate the positions of the 6'(NeuAcα2,6Galβ1,4Glc) and 3'(NeuAcα2,3Galβ1,4Glc) isomers, respectively. Chromatography was as described in the legend for Figure 35. Results are expressed relative to the maximum [^{14}C] dpm of the 6'SL isomer. The 100% value was 5951 dpm.

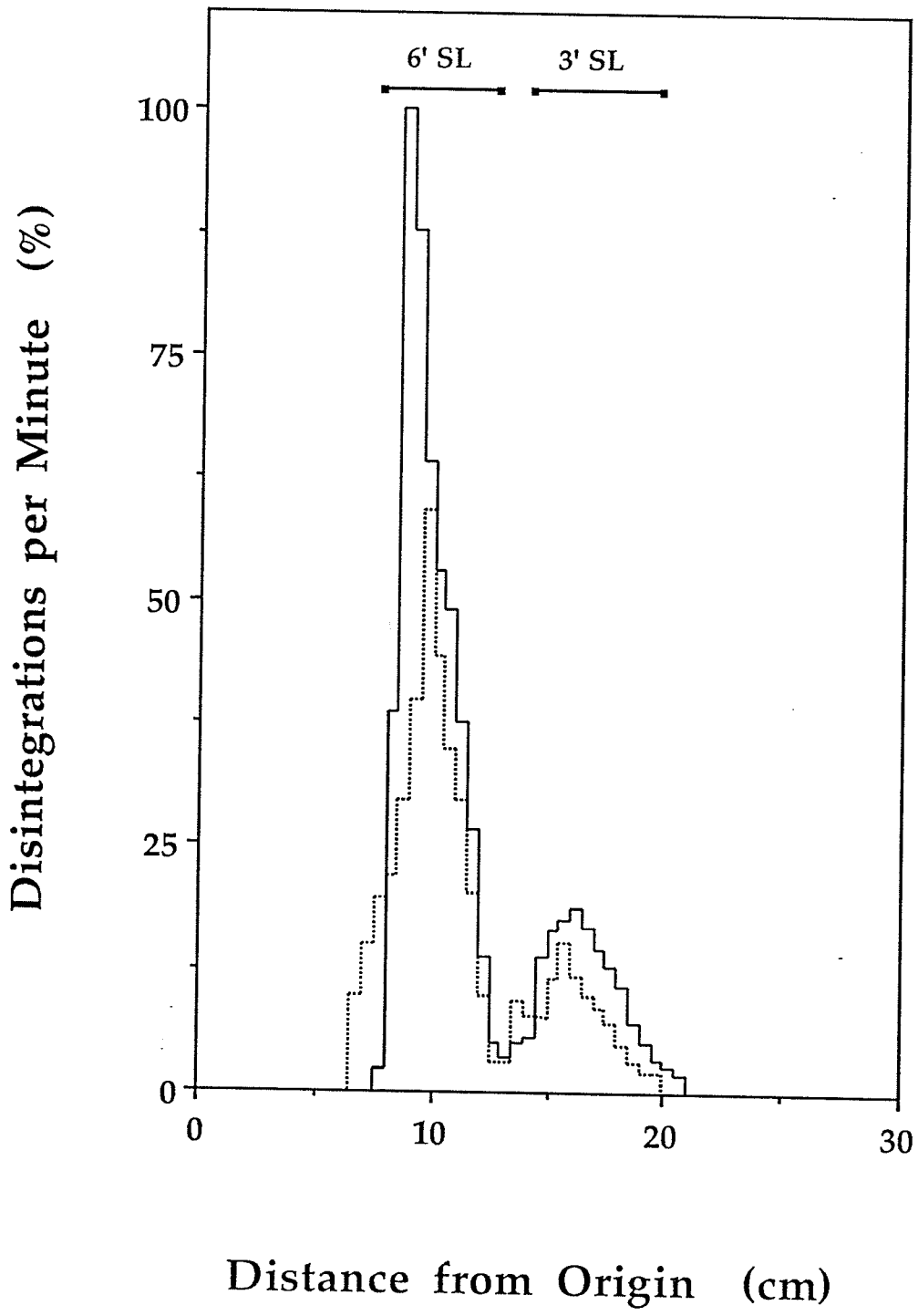

6' SL
3' SL
100
75
50
25
0
0
10
20
30
Disintegrations per Minute (%)
Distance from Origin (cm)

FIGURE 39

Sialyllactose Isomers Produced by Guinea Pig Serum Sialyltransferase Activities

Histogram of sialyllactose isomers produced by control-guinea pig (broken line) and 96-hr inflamed-guinea pig (solid line) serum sialyltransferase activities. 6'SL and 3'SL indicate the positions of the 6'(NeuAcα2,6Galβ1,4Glc) and 3'(NeuAcα2,3Galβ1,4Glc) isomers, respectively. Chromatography was as described in the legend for Figure 35. Results are expressed relative to the maximum [^{14}C] dpm of the 6'SL isomer. The 100% value was 8754 dpm.

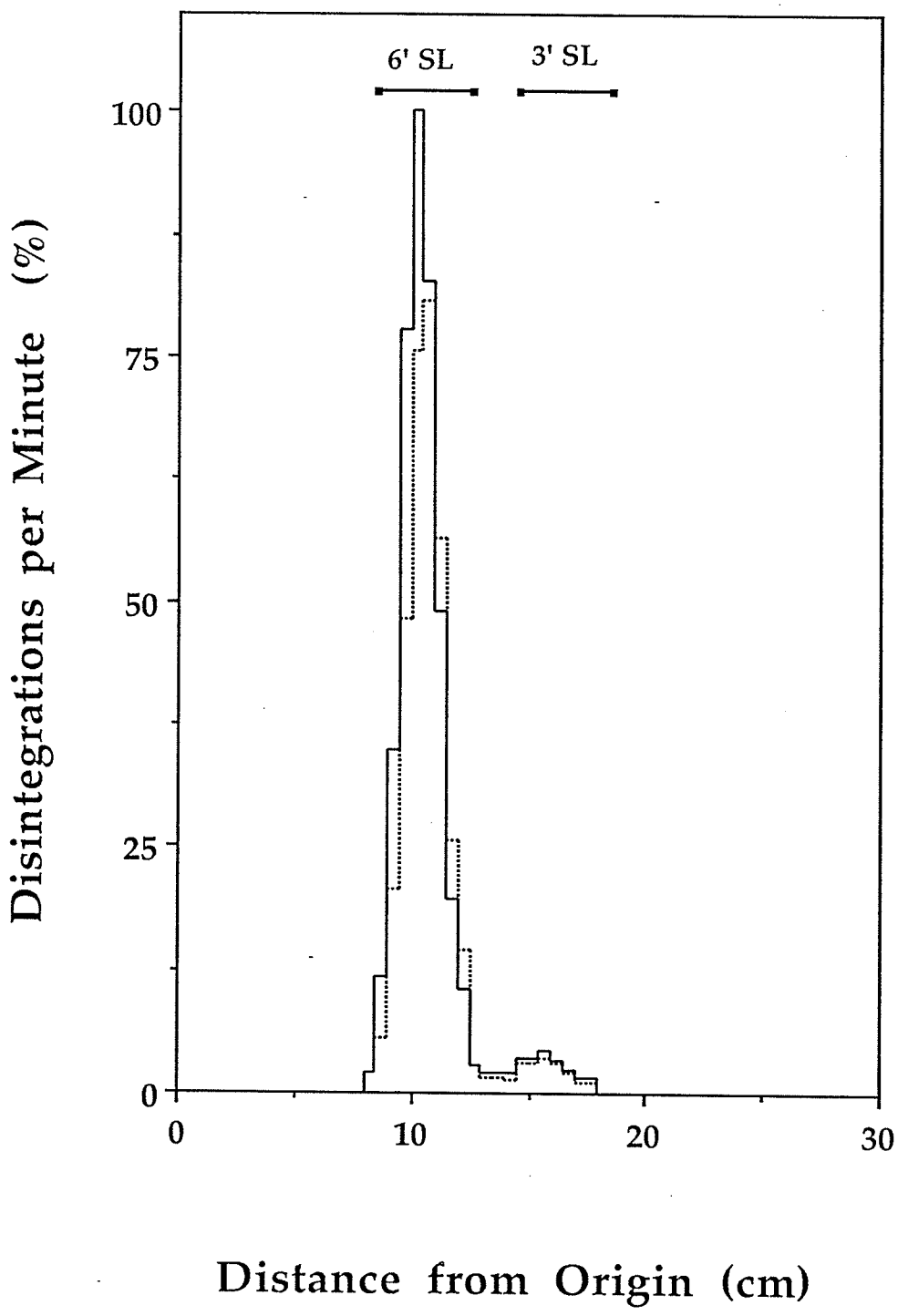
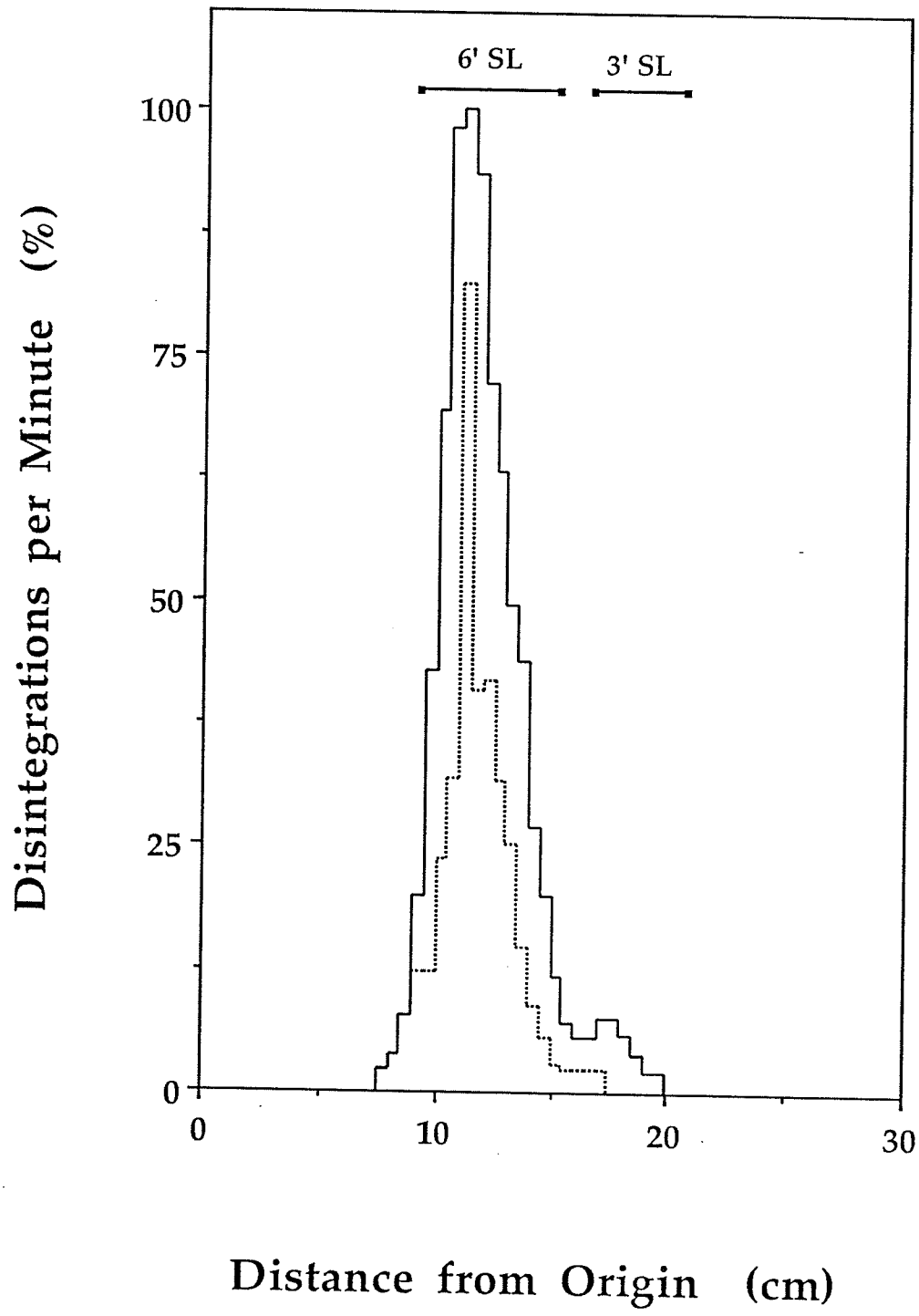

6' SL
3' SL
100
75
50
25
0
0
10
20
30
Disintegrations per Minute (%)
Distance from Origin (cm)

FIGURE 40

Sialyllactose Isomers Produced by Guinea Pig Liver Slice Medium Sialyltransferase Activities

Histogram of sialyllactose isomers produced by control-guinea pig (broken line) and 96-hr inflamed-guinea pig (solid line) liver slice medium sialyltransferase activities. 6'SL and 3'SL indicate the positions of the 6'(NeuAcα2,6Galβ1,4Glc) and 3'(NeuAcα2,3Galβ1,4Glc) isomers, respectively. Chromatography was as described in the legend for Figure 35. Results are expressed relative to the maximum [^{14}C] dpm of the 6'SL isomer. The 100% value was 2321 dpm.


6' SL
3' SL
100
75
50
25
0
0
10
20
30
Disintegrations per Minute (%)
Distance from Origin (cm)

serum samples from guinea pigs that had been suffering from turpentine-induced inflammation for 24, 48 and 72 hr.

Two rat hepatic N-linked sialyltransferases, the Galβ1,3(4)GlcNAc α2,3 and the Galβ1,4GlcNAc α2,6 enzymes, have been purified to homogeneity and characterized (Weinstein *et al.*, 1982a, b). The availability of rabbit antiserum containing antibodies to rat hepatic Galβ1,4GlcNAc α2,6 sialyltransferase provided a means to further characterize the α2,6-sialyltransferase activities present in serum and liver slice medium samples prepared from experimentally-inflamed rats, mice and guinea pigs. Thirty μl samples were preincubated for 45 min with 1.0 μl aliquots of antiserum to inactivate the Galβ1,4GlcNAc α2,6-sialyltransferase, and then assayed for enzyme activity using lactose as the low molecular weight acceptor (see Methods). Figures 41, 42 and 43 show the effect of pretreatment with antiserum on sialyllactose isomer formation by sialyltransferase activities present in serum obtained from 48-hr inflamed rats, 72-hr inflamed mice and 96-hr inflamed guinea pigs, respectively. In each species inactivation of Galβ1,4GlcNAc α2,6-sialyltransferase activity caused a substantial reduction in the amount of NeuAcα2,6Galβ1,4Glc isomer formed, and this effect was most pronounced in the rat and the mouse. Similar results were obtained with liver slice medium samples prepared from experimentally-inflamed animals. Control experiments were done in order to determine if inhibition of NeuAcα2,6Galβ1,4Glc isomer formation was directly due to antibody inactivation of the Galβ1,4GlcNAc α2,6 enzyme, and it was found that preincubation of rat, mouse and guinea pig serum and liver slice medium samples with normal rabbit serum did not influence sialyllactose isomer formation.

FIGURE 41

Sialyllactose Isomers Produced by Rat Serum Sialyltransferase Activities Following Preincubation with Antiserum Containing Antibodies to Rat Hepatic Galβ1,4GlcNAc α2,6-Sialyltransferase

Histogram of sialyllactose isomers produced by sialyltransferase activities in 48-hr inflamed-rat serum that had previously been incubated for 45 min in the presence (broken line) or absence (solid line) of rabbit antiserum raised against rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase. Chromatography was as described in the legend for Figure 35. 6'SL and 3'SL indicate the positions of the 6'(NeuAcα2,6Galβ1,4Glc) and 3'(NeuAcα2,3Galβ1,4Glc) isomers, respectively. Results are expressed relative to the maximum [^{14}C] dpm of the 6'SL isomer produced by serum that had not been pretreated with antiserum. The 100% value was 3943 dpm.

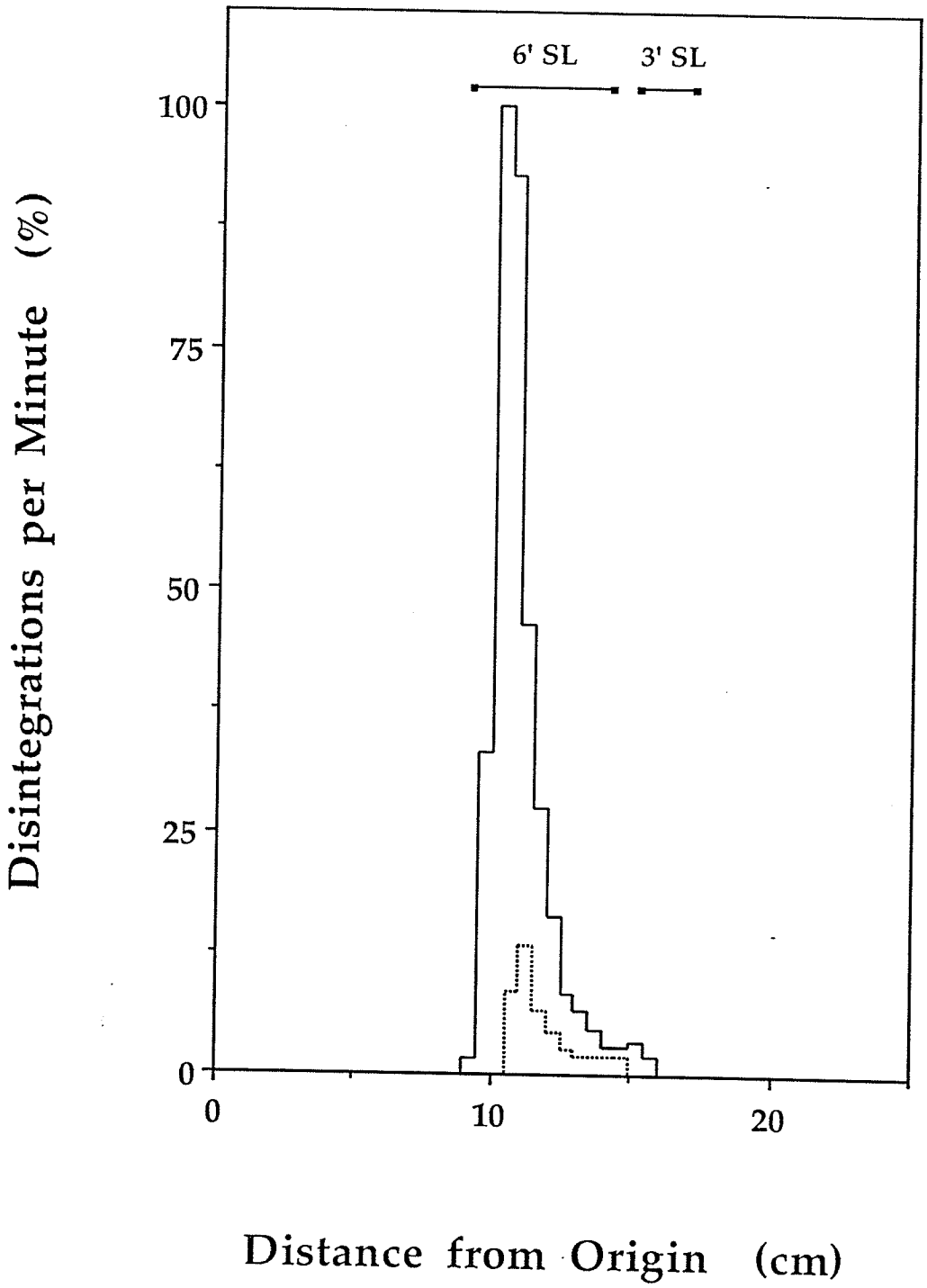

6' SL
3' SL
100
75
50
25
0
0
10
20
Disintegrations per Minute (%)
Distance from Origin (cm)

FIGURE 42

Sialyllactose Isomers Produced by Mouse Serum Sialyltransferase Activities Following Preincubation with Antiserum Containing Antibodies to Rat Hepatic Galβ1,4GlcNAc α2,6-Sialyltransferase

Histogram of sialyllactose isomers produced by sialyltransferase activities in 72-hr inflamed-mouse serum that had previously been incubated for 45 min in the presence (broken line) or absence (solid line) of rabbit antiserum raised against rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase. Chromatography was described in the legend for Figure 35. 6'SL and 3'SL indicate the positions of the 6'(NeuAcα2,6Galβ1,4Glc) and 3'(NeuAcα2,3Galβ1,4Glc) isomers, respectively. Results are expressed relative to the maximum [^{14}C] dpm of the 6'SL isomer produced by serum that had not been pretreated with antiserum. The 100% value was 2145 dpm.

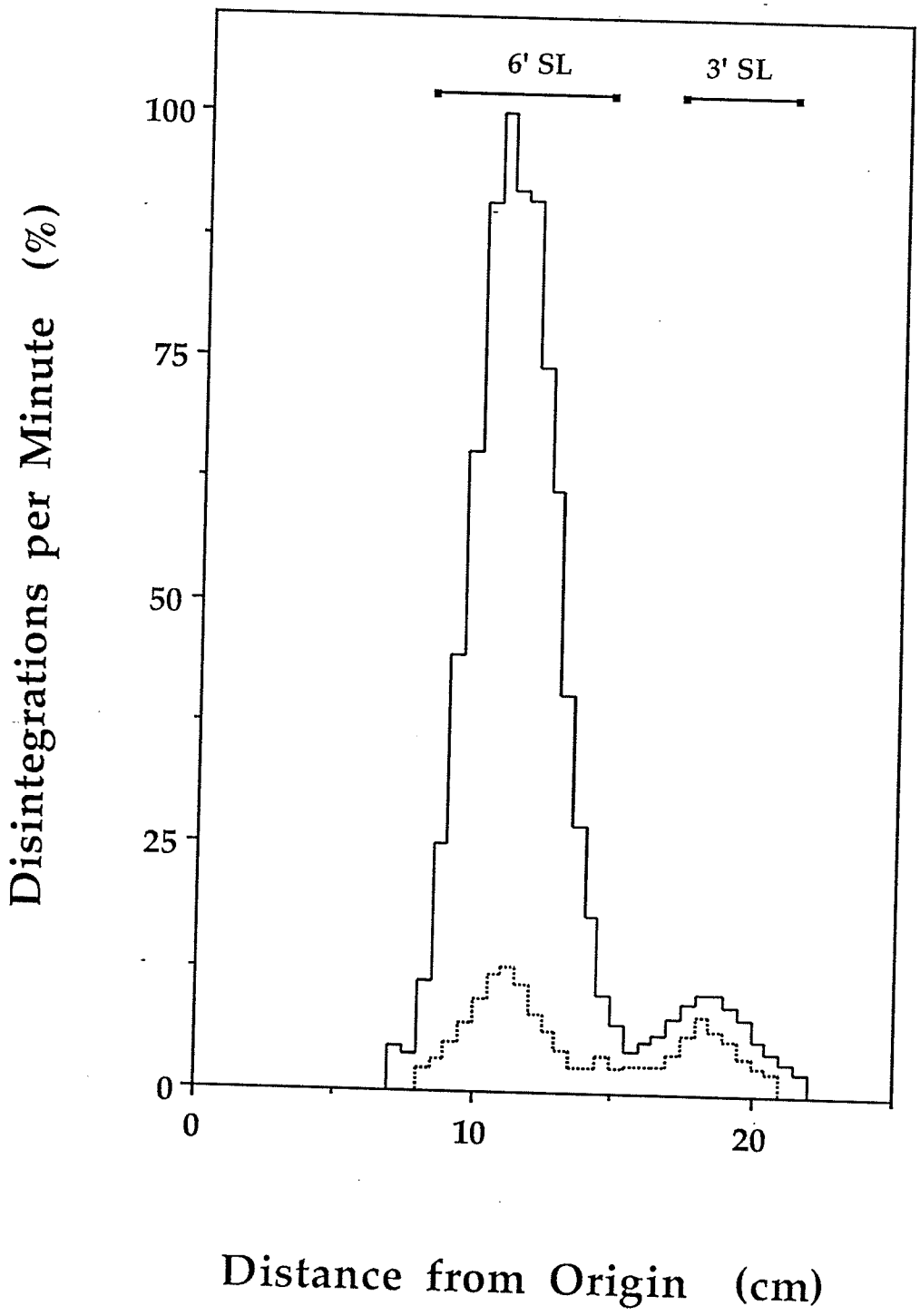
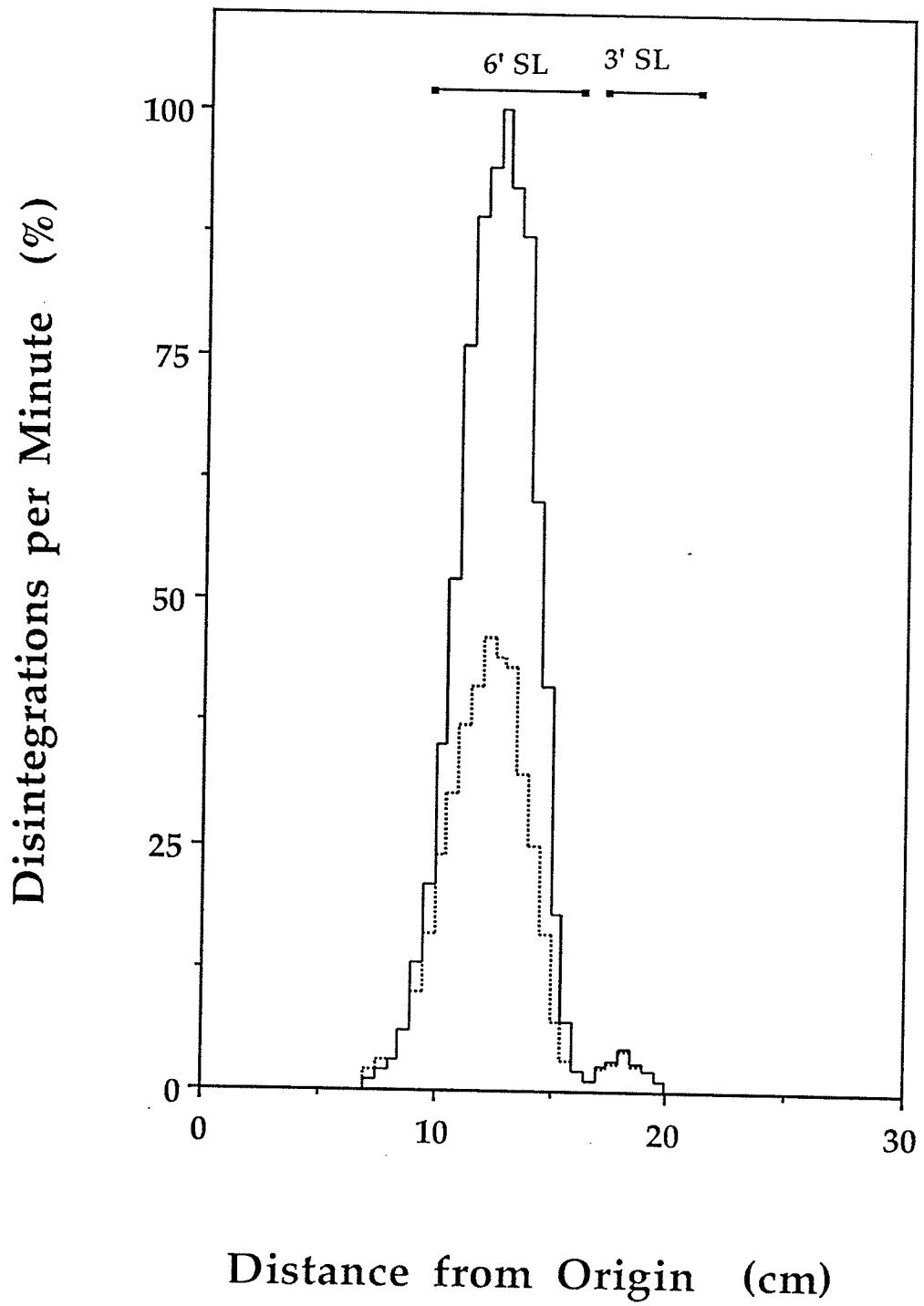

6' SL
3' SL
100
75
50
25
0
0
10
20
Disintegrations per Minute (%)
Distance from Origin (cm)

FIGURE 43

Sialyllactose Isomers Produced by Guinea Pig Serum Sialyltransferase Activities Following Preincubation with Antiserum Containing Antibodies to Rat Hepatic Galβ1,4GlcNAc α2,6-Sialyltransferase

Histogram of sialyllactose isomers produced by sialyltransferase activities in 96-hr inflamed-guinea pig serum that had previously been incubated for 45 min in the presence (broken line) or absence (solid line) of rabbit antiserum raised against rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase. Chromatography was described in the legend for Figure 35. 6'SL and 3'SL indicate the positions of the 6'(NeuAcα2,6Galβ1,4Glc) and 3'(NeuAcα2,3Galβ1,4Glc) isomers, respectively. Results are expressed relative to the maximum [^{14}C] dpm of the 6'SL isomer produced by serum that had not been pretreated with antiserum. The 100% value was 7659 dpm.


6' SL
3' SL
100
75
50
25
0
0
10
20
30
Disintegrations per Minute (%)
Distance from Origin (cm)

Additional evidence to support the conclusion that the sialyltransferase which was an acute phase reactant in the mouse and the guinea pig was the hepatic Galβ1,4GlcNAc α2,6-sialyltransferase was achieved by investigating the effect of rabbit antiserum raised against rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase on mouse and guinea pig N-linked sialyltransferase activities. Thirty μl volumes of mouse and guinea pig liver, serum, and liver slice medium samples were preincubated for 45 min with increasing amounts of antiserum up to 2.00 μl, following which the experimental samples were assayed for enzyme activity using rat asialo-α_1-AGP as acceptor (see Methods). Tables 11 and 12 show the effect of pretreatment with antiserum on sialyltransferase activities in liver, serum and liver slice medium prepared from mice and guinea pigs, respectively. Exposure of experimental samples to increasing amounts of antiserum caused a graded inhibition in mouse and guinea pig sialyltransferase activities. Approximately 90% of the total N-linked sialyltransferase activity present in serum and liver slice medium obtained from inflamed mice was inhibited by preincubation of experimental samples with 0.10 μl aliquots of antiserum. Treatment of liver samples prepared from 72-hr inflamed mice with 1.00 μl aliquots of antiserum produced a greater than 85% inhibition in total N-linked sialyltransferase activity. Preincubation of liver, serum and liver slice medium samples obtained from control mice with antiserum also caused significant reductions in sialyltransferase activities, however the extent of inhibition was not as dramatic as with experimental samples prepared from turpentine-inflamed mice. Guinea pig liver, serum and liver slice medium N-linked sialyltransferase activities were also substantially inhibited by pretreatment with antiserum, with enzyme activities in samples obtained from experimentally-inflamed animals being inhibited to a greater extent.

TABLE 11

EFFECT OF PREINCUBATION WITH ANTISERUM CONTAINING ANTIBODIES AGAINST RAT HEPATIC Galβ1,4GlcNAc α2,6-SIALYLTRANSFERASE ON MOUSE LIVER, SERUM AND LIVER SLICE MEDIUM SIALYLTRANSFERASE ACTIVITIES

VOLUME ANTISERUM (μl)	SIALYLTRANSFERASE ACTIVITY (units)					
	LIVER		SERUM		MEDIUM	
	Control	Inflamed	Control	Inflamed	Control	Inflamed
0	35	100	28	100	40	100
0.02	30	90	24	70	32	85
0.05	18	64	10	48	18	55
0.10	12	58	8	12	10	10
0.20	12	40	-	-	-	-
1.00	12	16	-	-	-	-

Liver, serum and liver slice medium samples obtained from control and 72-hr inflamed mice were preincubated with rabbit antiserum containing antibodies to rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase for 45 min, following which sialyltransferase activities were assayed using rat asialo-α1-AGP as acceptor. Results are expressed relative to the amount of sialyltransferase activity present in inflamed-mice samples that had not been pretreated with antiserum. Units of sialyltransferase activity are pmol NeuAc transferred per mg liver protein, or per ml serum or medium, per min. The 100% values were 14.0, 26.8 and 11.9 units for liver, serum and liver slice medium, respectively. Results represent the means from three separate determinations; reproducibility was within ± 10%.

TABLE 12

EFFECT OF PREINCUBATION WITH ANTISERUM CONTAINING ANTIBODIES AGAINST RAT HEPATIC Galβ1,4GlcNAc α2,6-SIALYLTRANSFERASE ON GUINEA PIG LIVER, SERUM AND LIVER SLICE MEDIUM SIALYLTRANSFERASE ACTIVITIES

VOLUME ANTISERUM (μl)	SIALYLTRANSFERASE ACTIVITY (units)					
	LIVER		SERUM		MEDIUM	
	Control	Inflamed	Control	Inflamed	Control	Inflamed
0	83	100	68	100	51	100
0.02	86	95	58	98	45	94
0.04	74	98	50	80	39	79
0.08	61	81	33	60	32	54
1.00	55	84	28	50	31	52
1.50	48	70	21	35	32	58
2.00	-	52	-	30	-	-

Liver, serum and liver slice medium samples obtained from controls and from 96-hr inflamed guinea pigs were preincubated with rabbit antiserum containing antibodies to rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase for 45 min, following which sialyltransferase activities were assayed using rat asialo-α1-AGP as acceptor. Results are expressed relative to the amount of sialyltransferase activity present in inflamed-guinea pig samples that had not been pretreated with antiserum. Units of sialyltransferase activity are pmol NeuAc transferred per mg liver protein, or per ml serum or medium, per min. The 100% values were 24.4 159.4 and 19.1 units for liver, serum and liver slice medium, respectively. Results represent the means from three separate determinations; reproducibility was within ± 10%.

However, guinea pig sialyltransferase activities were less sensitive to antiserum than mouse sialyltransferase activities. Ten times the amount of antiserum that was sufficient to cause a 90% inhibition in sialyltransferase activities in inflamed-mouse serum and liver slice medium produced only a 50% inhibition in sialyltransferase activities in inflamed-guinea pig serum and liver slice medium. Two μl aliquots of antiserum caused the total sialyltransferase activities in inflamed-guinea pig serum and liver to be reduced 70% and 50%, respectively. Control experiments were conducted to determine the effect of non-immune rabbit serum on mouse and guinea pig N-linked sialyltransferase activities, and it was found that preincubation of liver, serum and liver slice medium samples with normal rabbit serum did not cause detectable changes in sialylation of rat asialo-α_1-AGP.

Kinetic Properties of Mouse and Guinea Pig Liver and Serum Sialyltransferase Activities

The kinetic behavior of N-linked sialyltransferase activities in liver and serum obtained from control and experimentally-inflamed mice and guinea pigs was investigated in order to determine if inflammation caused significant changes in catalytic performance. Table 13 shows the apparent K_m and V_{max} values of mouse and guinea pig liver and serum sialyltransferase activities exhibited towards rat asialo-α_1-AGP and CMP-NeuAc. Turpentine-induced inflammation was found to have only a small effect on apparent K_m values. Apparent K_m values for mouse liver and serum sialyltransferase activities determined with respect to rat asialo-α_1-AGP were within the ranges 18 - 24 μM and 24 - 30 μM, respectively. Guinea pig sialyltransferase activities exhibited slightly higher apparent K_m values towards this macromolecular acceptor; the ranges of apparent K_m values for guinea pig

TABLE 13

APPARENT K_M AND V_{MAX} VALUES FOR MOUSE AND GUINEA PIG LIVER AND SERUM SIALYLTRANSFERASE ACTIVITIES

	SUBSTRATE			
	CMP-NeuAc		Asialo-α_1-AGP	
ENZYME SOURCE	K_m (μM)	V_{max}	K_m (μM)	V_{max}
Mouse Liver				
Control	67 ± 6	20 ± 2	24 ± 1	6 ± 1
72-hr Inflamed	88 ± 6	48 ± 4	18 ± 1	16 ± 1
Mouse Serum				
Control	51 ± 2	25 ± 1	30 ± 2	18 ± 1
72-hr Inflamed	53 ± 1	84 ± 2	24 ± 1	56 ± 2
Guinea Pig Liver				
Control	73 ± 7	19 ± 3	21 ± 3	75 ± 7
96-hr Inflamed	92 ± 11	27 ± 3	42 ± 5	96 ± 7
Guinea Pig Serum				
Control	65 ± 6	211 ± 18	61 ± 6	28 ± 3
96-hr Inflamed	63 ± 4	257 ± 14	67 ± 5	39 ± 2

Activities towards CMP-NeuAc and rat asialo-α_1-AGP were determined for the sialyltransferase activities present in liver and serum samples obtained from controls, and from 72-hr inflamed mice and 96-hr inflamed guinea pigs. Apparent K_m values for rat asialo-α_1-AGP were calculated assuming a molecular weight of 34,600 Da (Jamieson *et al.*, 1972; Kaplan *et al.*, 1983). Values of V_{max} are in arbitrary units. Results represent the means from four to six separate determinations ± SD.

liver and serum sialyltransferase activities were 21 - 42 μM and 61 - 67 μM, respectively. Apparent K_m values of mouse and guinea pig liver and serum sialyltransferase activities exhibited towards rat asialo-α_1-AGP were higher than those of rat liver and serum sialyltransferase activities, which were reported to be in the ranges 10 - 12 μM and 7 - 10 μM, respectively (Kaplan *et al.*, 1983). This showed that mouse and guinea pig sialyltransferase activities have lower substrate affinities for rat asialo-α_1-AGP. Mouse and guinea pig liver and serum sialyltransferase activities were found to have similar apparent K_m values towards CMP-NeuAc. Apparent K_m values for liver and serum sialyltransferase activities were in the ranges 67 - 92 μM and 51 - 65 μM, respectively. These apparent K_m values were also higher than those exhibited by rat liver and serum sialyltransferase activities, which were found to be in the ranges 25 - 32 μM and 16 - 29 μM, respectively (Kaplan *et al.*, 1983). Values of V_{max} for mouse and guinea pig sialyltransferase activities determined with respect to either rat asialo-α_1-AGP or CMP-NeuAc were increased by turpentine-induced inflammation. In addition, increases in V_{max} values were two-fold greater for mouse sialyltransferase activities than for guinea pig sialyltransferase activities. Lack of significant change in apparent K_m values, coupled with observed increases in V_{max} values, suggested that the increases in sialyltransferase activities in mouse and guinea pig liver and serum caused by turpentine-induced inflammation were due largely to an increase in enzyme presence and not changes in substrate affinities.

INVESTIGATION INTO THE MECHANISM OF RELEASE OF HEPATIC Galβ1,4GlcNAc α2,6-SIALYLTRANSFERASE FROM MOUSE AND GUINEA PIG LIVER GOLGI MEMBRANES

Effect of pH on Release of Sialyltransferase and Galactosyltransferase from Mouse and Guinea Pig Liver Golgi Membranes

Confirmation that hepatic Galβ1,4GlcNAc α2,6-sialyltransferase was also an acute phase reactant in the mouse and the guinea pig prompted experiments to be undertaken to determine if a pH-sensitive proteolytic event was also involved in release of this glycosyltransferase from mouse and guinea pig liver Golgi membranes. Figures 44 and 45 show the effect of pH on release of sialyltransferase from Golgi vesicles prepared from control-mouse and 72-hr inflamed-mouse livers, and from control-guinea pig and 96-hr inflamed-guinea pig livers, respectively. Golgi membranes were treated with ultrasonic vibrations for 30 s, incubated for 30 min in the presence of equimolar McIlvaine sodium phosphate - citrate buffers of different pH values, and solubilized sialyltransferase activities were assayed using rat asialo-α_1-AGP as acceptor (see Methods). As was found for the rat, reduction in the pH of the incubation medium caused substantial amounts of sialyltransferase to be released from mouse and guinea pig Golgi membranes. Although greater amounts of enzyme were released from Golgi membranes obtained from livers from inflamed animals, a similar response to pH was exhibited by Golgi membranes prepared from livers from both controls and inflamed animals. Significant release of sialyltransferase from mouse Golgi membranes occurred over a narrow range of pH between 4.2 and 5.0, with maximum release occurring at pH 4.6. At pH 4.6, the amount of sialyltransferase released from Golgi membranes prepared from control-mouse and 72-hr inflamed-mouse livers was ten and thirty times,

FIGURE 44

Effect of pH on Release of Sialyltransferase and Galactosyltransferase from Mouse Liver Golgi Membranes

Golgi membranes were exposed to ultrasonic vibrations for 30 s and then incubated for 30 min in the presence of 50 mM McIlvaine buffers of different pH. At the end of incubation pH was adjusted to 7.0, particulate matter was removed by centrifugation, and supernatants were assayed for glycosyltransferase activities. Sialyltransferase (○,●) and galactosyltransferase (Δ,▲) activities released from Golgi vesicles prepared from livers from controls (open symbols) and from 72-hr inflamed mice (closed symbols) are expressed relative to the amount of sialyltransferase activity present in the supernatant obtained from the incubation at pH 4.6 of Golgi membranes prepared from inflamed- mouse livers. The 100% value was 2654 pmol NeuAc transferred to rat asialo-α_1-AGP per mg Golgi protein per hr. Results represent the means from four separate determinations; reproducibility was within $\pm$ 10%.

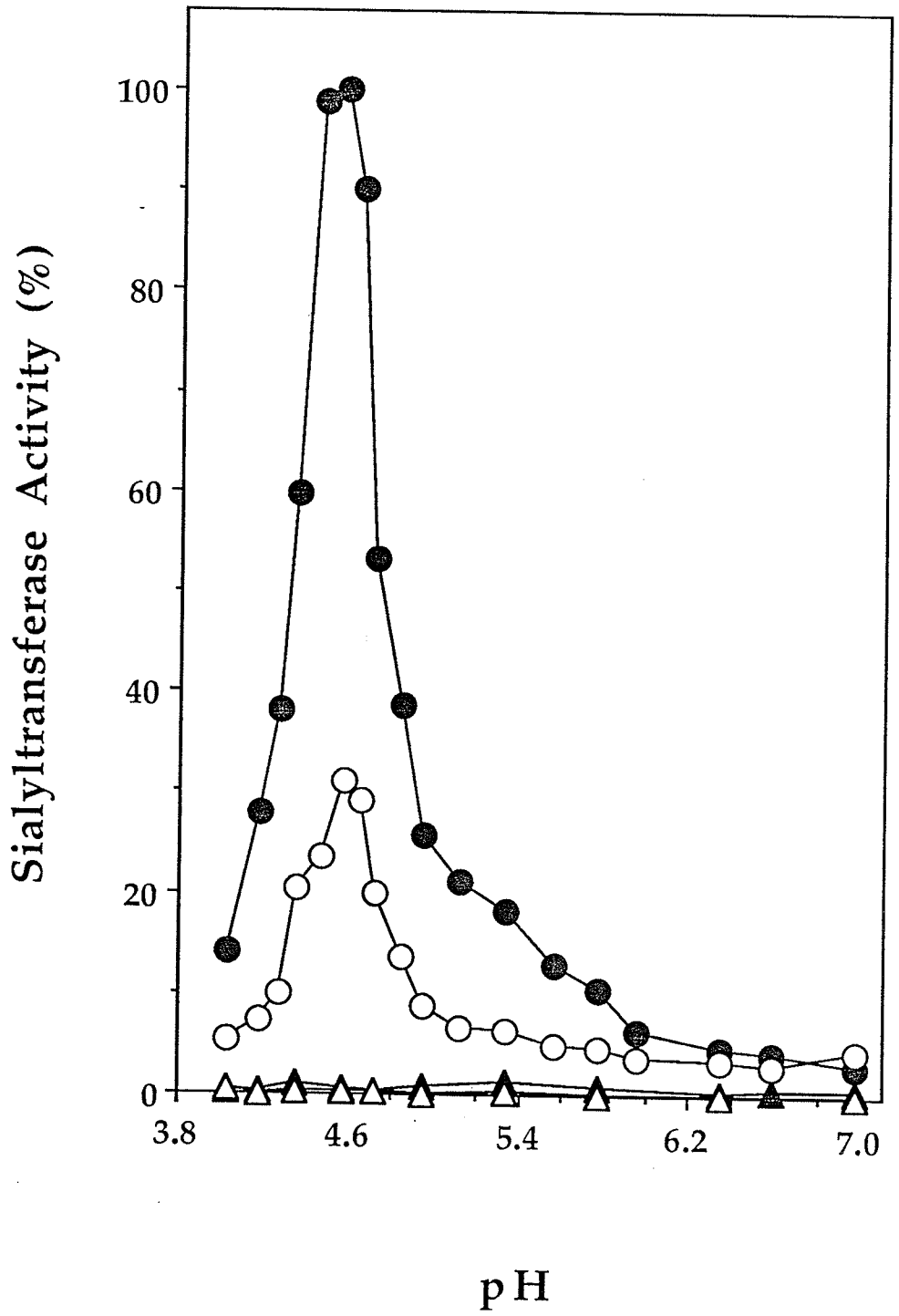
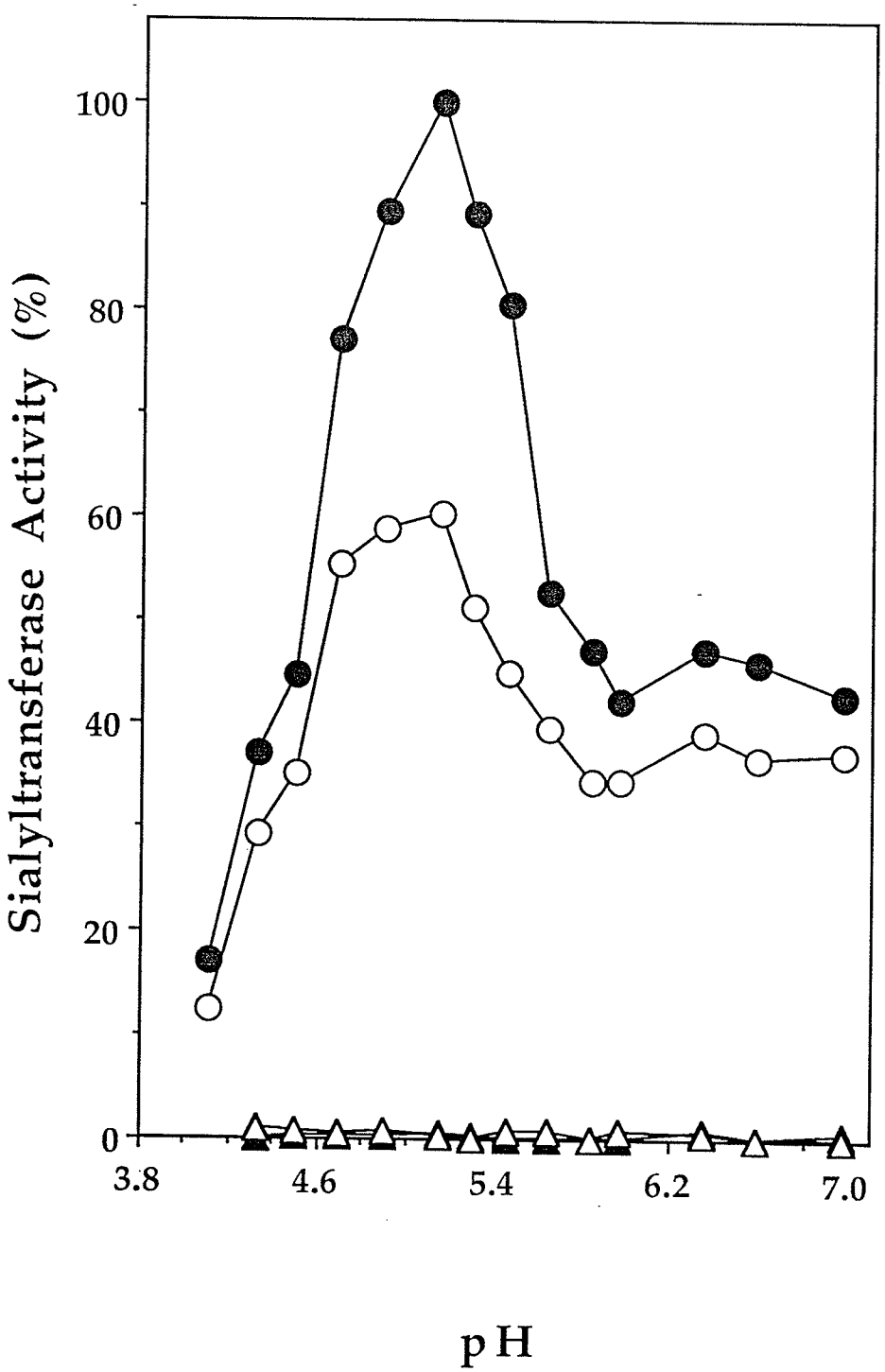

Sialyltransferase Activity (%)
100
80
60
40
20
0
3.8
4.6
5.4
6.2
7.0
pH

FIGURE 45

Effect of pH on Release of Sialyltransferase and Galactosyltransferase from Guinea Pig Liver Golgi Membranes

Golgi membranes were exposed to ultrasonic vibrations for 30 s and then incubated for 30 min in the presence of 50 mM McIlvaine buffers of different pH. At the end of incubation pH was adjusted to 7.0, particulate matter was removed by centrifugation, and supernatants were assayed for glycosyltransferase activities. Sialyltransferase (○,●) and galactosyltransferase (Δ,▲) activities released from Golgi vesicles prepared from livers from controls (open symbols) and from 96-hr inflamed-guinea pigs (closed symbols) are expressed relative to the amount of sialyltransferase activity present in the supernatant obtained from the incubation at pH 5.2 of Golgi membranes prepared from inflamed-guinea pig livers. The 100% value was 3151 pmol NeuAc transferred to rat asialo-α_1-AGP per mg Golgi protein per hr. Results represent the means from four separate determinations; reproducibility was within $\pm$ 10%.


Sialyltransferase Activity (%)
100
80
60
40
20
0
3.8
4.6
5.4
6.2
7.0
pH

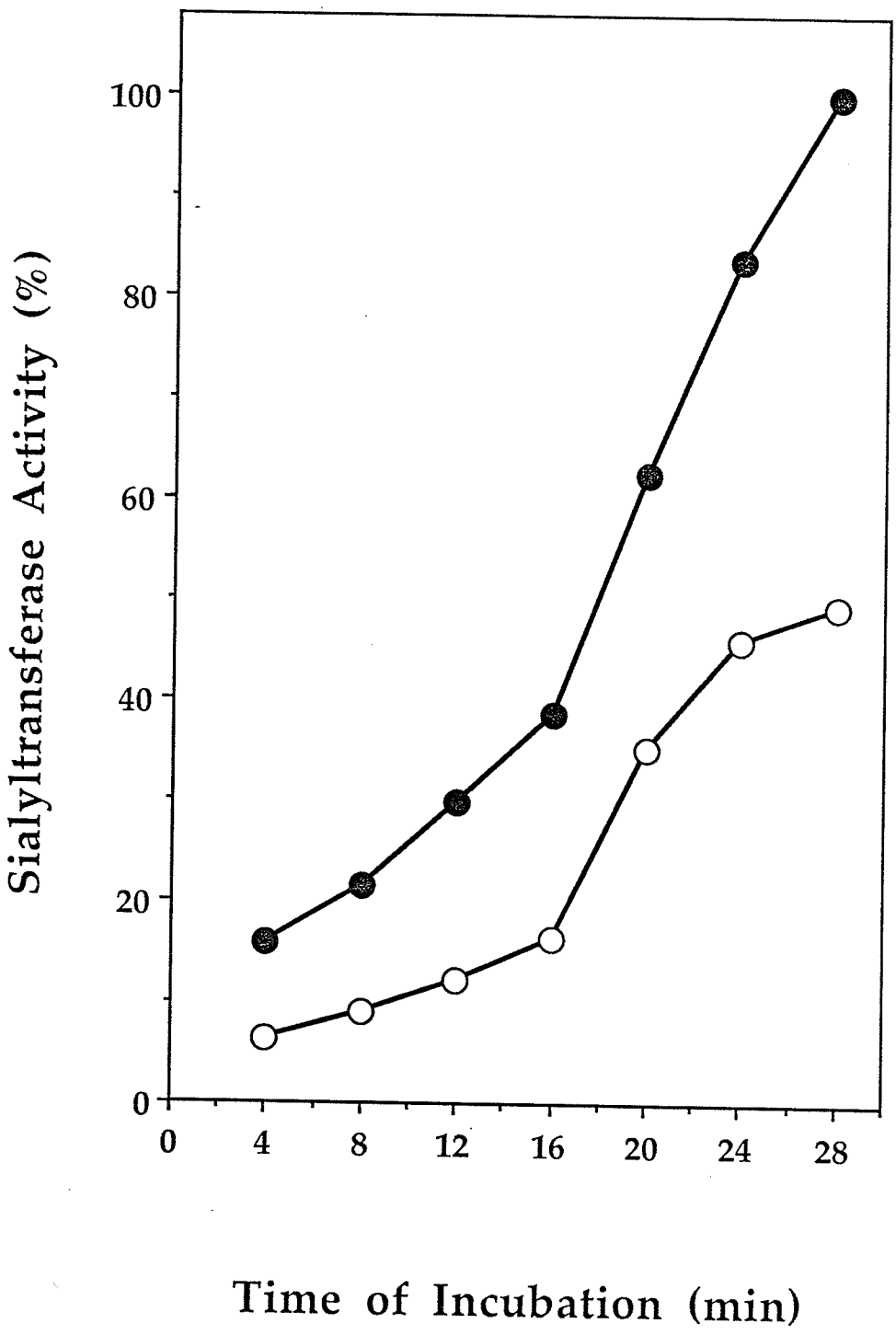
respectively, the amount of sialyltransferase released from similarly-prepared Golgi membranes at pH 7.0. In contrast to the mouse, significant increases in the release of sialyltransferase from guinea pig Golgi membranes occurred over a broader range of pH between 4.6 and 5.8. Maximum enzyme release from guinea pig Golgi membranes was found at pH 5.2, and approximately twice the amount of sialyltransferase was released at pH 5.2 than at pH 7.0. As in the case of the rat, it was found that treatment of mouse and guinea pig Golgi membranes with ultrasonic vibrations for 30 s prior to incubation at reduced pH was essential for release of significant amounts of sialyltransferase. Sialyltransferase activities released from mouse and guinea pig Golgi membranes incubated at reduced pH were characterized using antiserum containing antibodies to rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase. Sialyltransferase activities in medium obtained from the incubation of mouse Golgi membranes at pH 4.6 and the incubation of guinea pig Golgi membranes at pH 5.2 were assayed before and after a 45-min incubation with antiserum using rat asialo-α_1-AGP as acceptor (see Methods). Pretreatment with antiserum inhibited over 90% and 70% of mouse and guinea pig solubilized N-linked sialyltransferase activities, respectively. Lowering of the pH of the incubation medium did not cause significant release of galactosyltransferase from mouse and guinea pig Golgi membranes. Examination of the glycosyltransferase activities that remained associated with mouse and guinea pig Golgi membranes following incubation showed that as the pH of the incubation medium was lowered only the amounts of membrane-bound sialyltransferase activities were reduced.

Release of sialyltransferase from mouse and guinea pig Golgi membranes incubated at reduced pH was found to be time-dependent. Figure 46 shows that the amount of sialyltransferase released at pH 4.6 from Golgi

FIGURE 46

Effect of Incubation Time on Release of Sialyltransferase from Mouse Liver Golgi Membranes Incubated at Reduced pH

Golgi membranes were exposed to ultrasonic vibrations for 30 s and then incubated at pH 4.6 for up to 30 min. At the end of incubation pH was adjusted to 7.0, particulate matter was removed by centrifugation, and supernatants were assayed for glycosyltransferase activities. Sialyltransferase activities released from Golgi vesicles prepared from livers from controls (○) and from 72-hr inflamed-mice (●) are expressed relative to the amount of sialyltransferase activity present in the supernatant obtained from the 30-min incubation of Golgi membranes prepared from inflamed-mouse livers. The 100% value was 2214 pmol NeuAc transferred to rat asialo-α_1-AGP per mg Golgi protein per hr. Results represent the means from four separate determinations; reproducibility was within $\pm$ 12%.


Sialyltransferase Activity (%)
100
80
60
40
20
0
0
4
8
12
16
20
24
28
Time of Incubation (min)

vesicles prepared from livers from controls and from 72-hr inflamed mice increased with time of incubation up to 30 min, with greater amounts of enzyme being released from Golgi vesicles that had been obtained from livers from inflamed mice. Similar results were found for the release of sialyltransferase at pH 5.2 from Golgi vesicles prepared from control-guinea pig and 96-hr inflamed-guinea pig livers, as shown in Figure 47. As was found for the rat, an incubation time of 30 min was sufficient to release approximately 70 - 80% of membrane-bound sialyltransferase activity from mouse and guinea pig Golgi membranes.

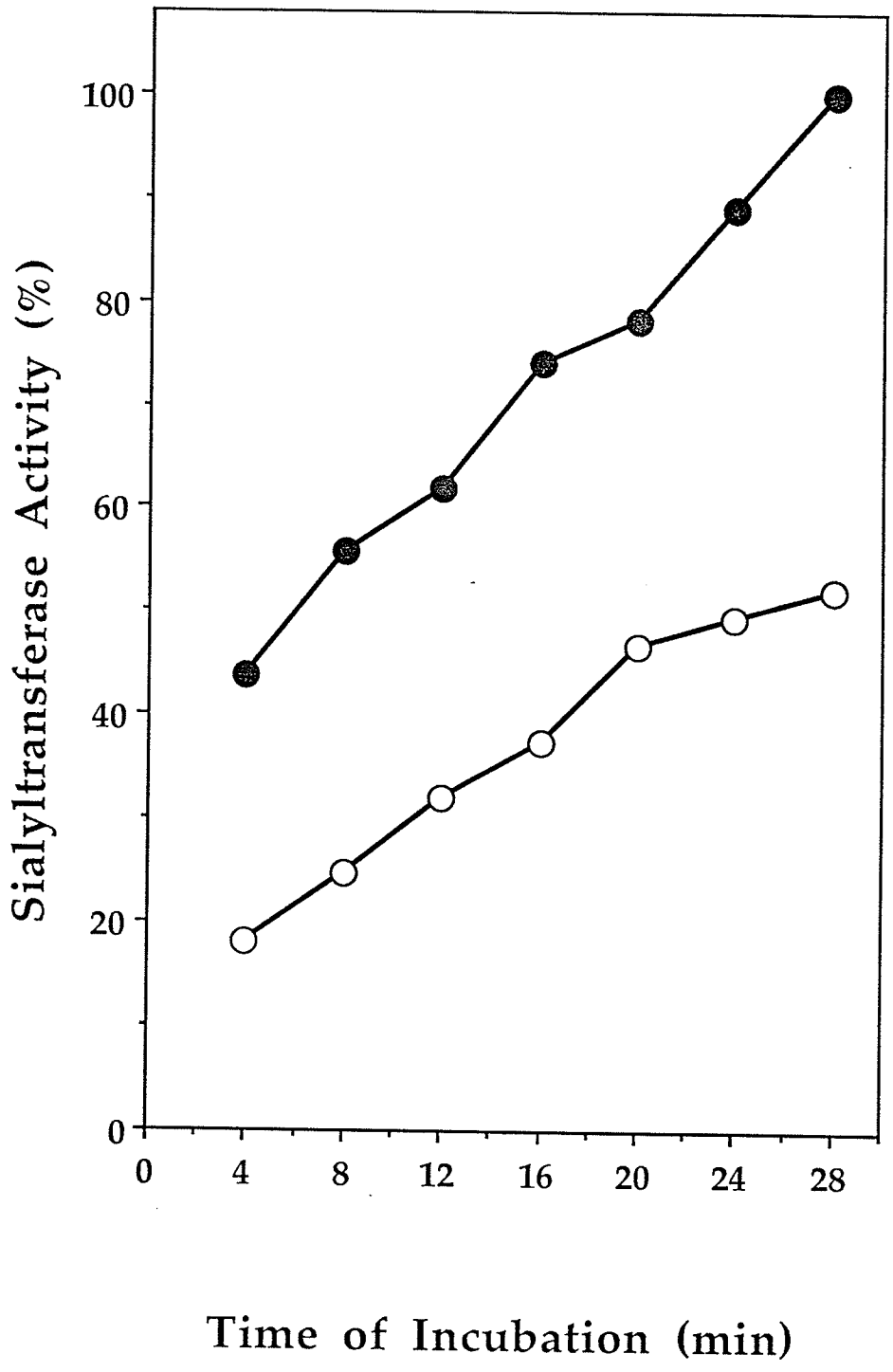
Effect of Pepstatin A on Release of Sialyltransferase from Mouse and Guinea Pig Liver Golgi Membranes

Preferential release of mouse and guinea pig hepatic Galβ1,4GlcNAc α2,6-sialyltransferase from permeabilized Golgi membranes incubated at reduced pH suggested the involvement of an endogenous proteinase. In order to characterize this proteinase activity, the effect of proteinase inhibitors on release of sialyltransferase from mouse and guinea pig Golgi membranes was investigated. Permeabilized Golgi vesicles prepared from livers from controls, and from 72-hr inflamed-mice and 96-hr inflamed-guinea pigs, were incubated at pH 4.6 (mouse) or pH 5.2 (guinea pig) for 30 min in the presence of 10^{-3} M concentrations of leupeptin, antipain, aprotinin, bestatin and pepstatin A, or 100 μg/ml concentrations of soybean trypsin inhibitor, α_1-antitrypsin and α_2-M (see Methods). As was found for the rat, significant inhibition of sialyltransferase release from mouse and guinea pig Golgi membranes only occurred in the presence of pepstatin A. Inhibition of mouse and guinea pig sialyltransferase release in the presence of pepstatin A was examined by investigating the effect of 10^{-4} to 10^{-11} M concentrations of

FIGURE 47

Effect of Incubation Time on Release of Sialyltransferase from Guinea Pig Liver Golgi Membranes Incubated at Reduced pH

Golgi membranes were exposed to ultrasonic vibrations for 30 s and then incubated at pH 5.2 for up to 30 min. At the end of incubation pH was adjusted to 7.0, particulate matter was removed by centrifugation, and supernatants were assayed for glycosyltransferase activities. Sialyltransferase activities released from Golgi vesicles prepared from livers from controls (O) and from 96-hr inflamed guinea pigs (●) are expressed relative to the amount of sialyltransferase activity present in the supernatant obtained from the 30-min incubation of Golgi membranes prepared from inflamed-guinea pig livers. The 100% value was 2752 pmol NeuAc transferred to rat asialo-α_1-AGP per mg Golgi protein per hr. Results represent the means from four separate determinations; reproducibility was within $\pm$ 12%.


Sialyltransferase Activity (%)
100
80
60
40
20
0
0
4
8
12
16
20
24
28
Time of Incubation (min)

pepstatin A on sialyltransferase release from Golgi membranes prepared from livers from controls and from turpentine-inflamed mice and guinea pigs. Figure 48 shows the effect of pepstatin A on release of sialyltransferase from control-mouse and 72-hr inflamed-mouse permeabilized Golgi membranes incubated for 30 min at pH 4.6. Pepstatin A was found to be a very effective inhibitor of the release of mouse Golgi membrane-bound sialyltransferase. Increases in the concentration of pepstatin A from 10^{-9} to 10^{-7} M caused dramatic decreases in the amount of solubilized sialyltransferase activity in the medium. Concentrations of pepstatin A from 10^{-7} to 10^{-4} M resulted in a greater than 90% inhibition in release of sialyltransferase from Golgi membranes prepared from livers from both controls and inflamed mice. The effect of pepstatin A on release of sialyltransferase at pH 5.2 from permeabilized Golgi vesicles prepared from livers from controls and 96-hr inflamed guinea pigs is shown in Figure 49. Sialyltransferase release from guinea pig Golgi membranes decreased as the concentration of pepstatin A in the incubation medium was increased from 10^{-11} M to 10^{-8} M; concentrations of pepstatin A greater than 10^{-8} M did not result in increases in inhibition of enzyme release. Pepstatin A was not as potent an inhibitor of sialyltransferase release from guinea pig Golgi membranes as it was of sialyltransferase release from mouse Golgi membranes. Whereas 10^{-7} M pepstatin A essentially eliminated release of sialyltransferase from mouse Golgi membranes, concentrations of 10^{-7} to 10^{-4} M pepstatin A were only effective in inhibiting release of sialyltransferase from guinea pig Golgi membranes by approximately 50%. Control experiments were conducted to determine the effect of DMSO, the solvent used to dissolve pepstatin A, on release of sialyltransferase from Golgi membranes and on solubilized sialyltransferase activities. DMSO inhibited by less than 15% the amount of

FIGURE 48

Effect of Pepstatin A on Release of Sialyltransferase from Mouse Liver Golgi Membranes

Golgi membranes were exposed to ultrasonic vibrations for 30 s and then incubated for 30 min at pH 4.6 in the presence of increasing amounts of pepstatin A dissolved in DMSO. At the end of incubation pH was adjusted to 7.0, particulate matter was removed by centrifugation and supernatants were assayed for sialyltransferase activities. Sialyltransferase activities released from Golgi membranes prepared from livers from controls (O) and from 72-hr inflamed mice (●) are expressed relative to the amount of sialyltransferase activity present in the supernatant obtained from the incubation in the presence of DMSO of Golgi membranes prepared from inflamed-mouse livers. The 100% value was 2800 pmol NeuAc transferred to rat asialo-α_1-AGP per mg Golgi protein per hr. Results represent the means from six separate determinations; reproducibility was within $\pm$ 10%.

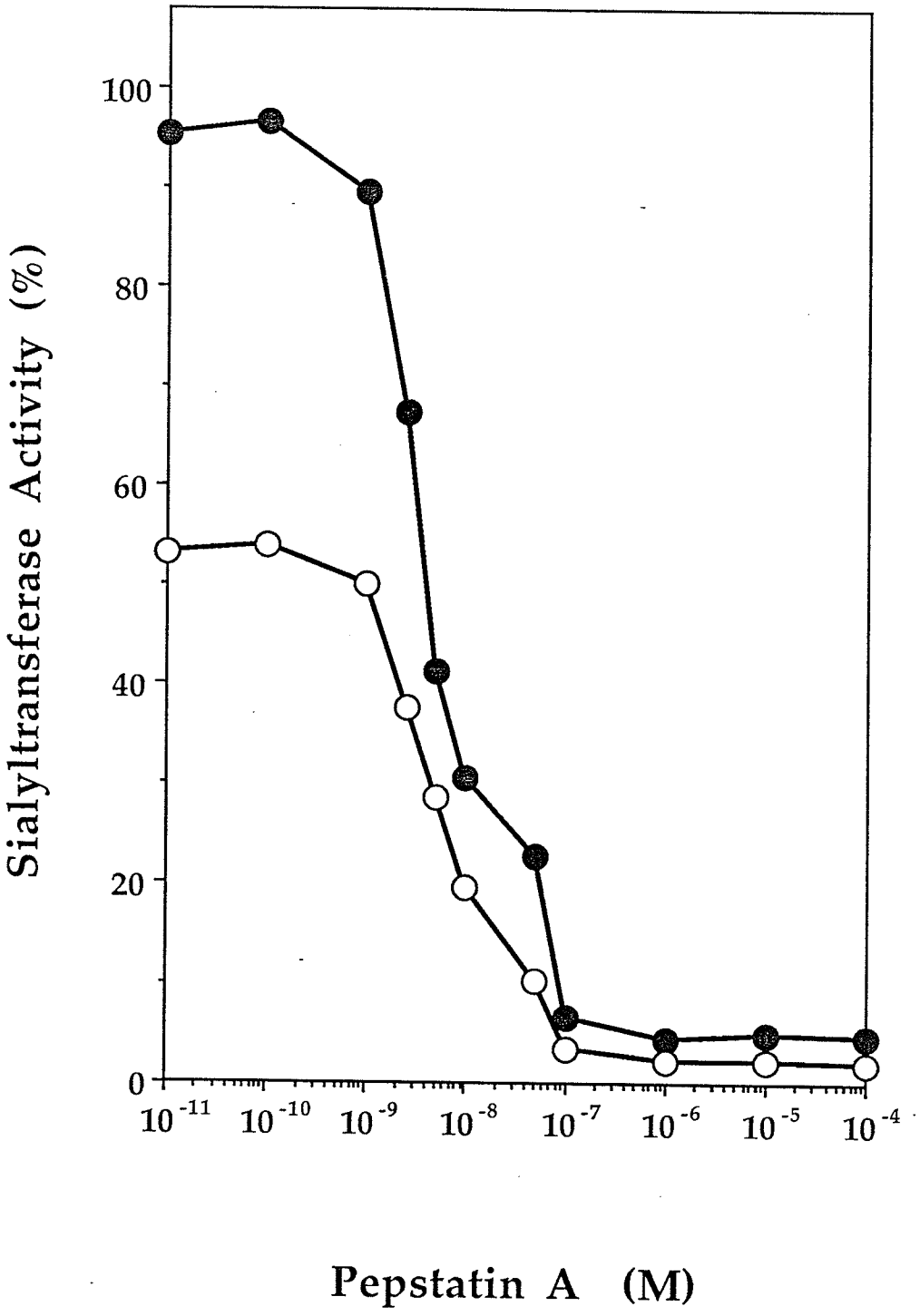
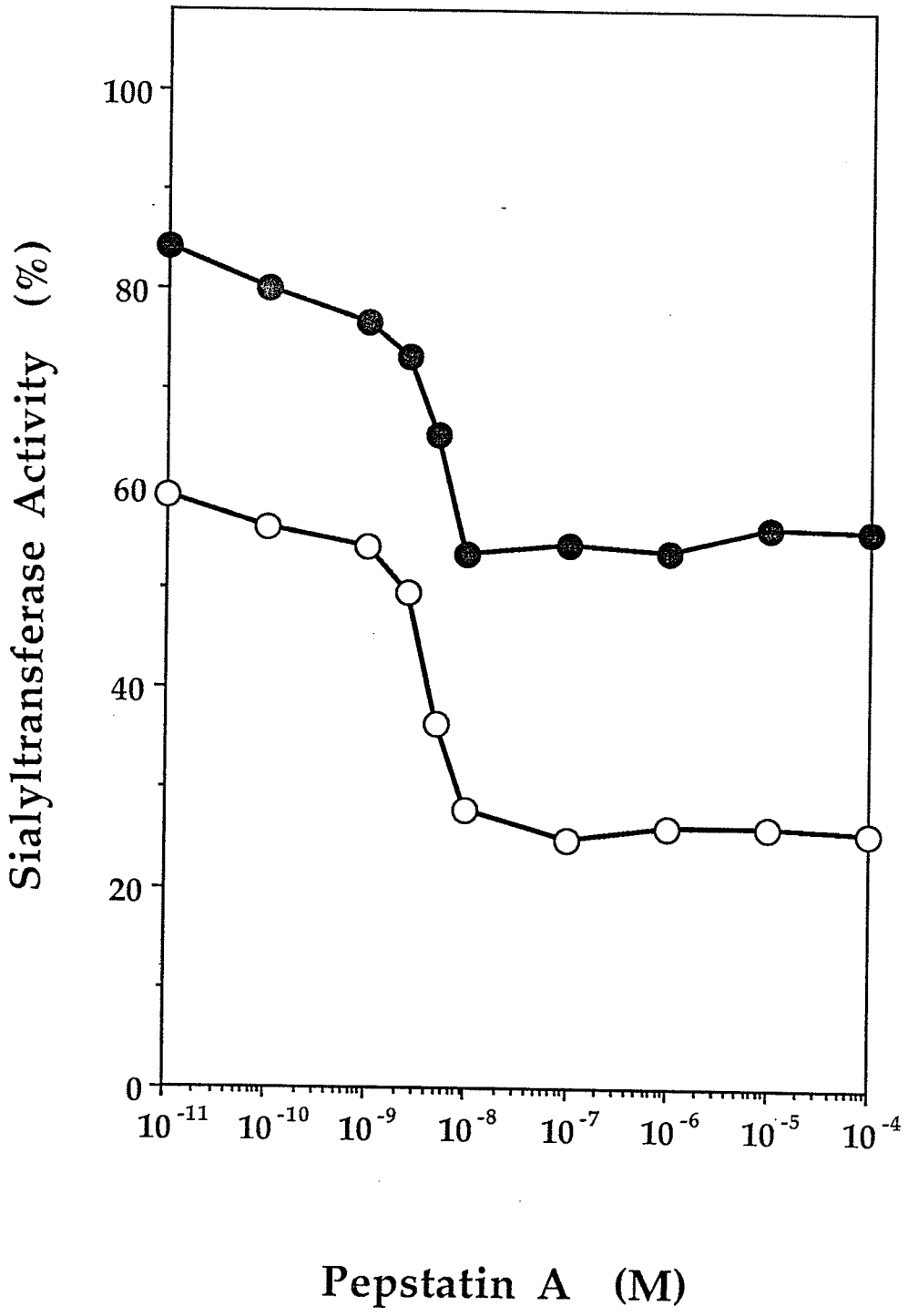

Sialyltransferase Activity (%)
100
80
60
40
20
0
10 -11
10 -10
10 -9
10 -8
10 -7
10 -6
10 -5
10 -4
Pepstatin A (M)

FIGURE 49

Effect of Pepstatin A on Release of Sialyltransferase from Guinea Pig Liver Golgi Membranes

Golgi membranes were exposed to ultrasonic vibrations for 30 s and then incubated for 30 min at pH 5.2 in the presence of increasing amounts of pepstatin A dissolved in DMSO. At the end of incubation pH was adjusted to 7.0, particulate matter was removed by centrifugation and supernatants were assayed for sialyltransferase activities. Sialyltransferase activities released from Golgi membranes prepared from livers from controls (O) and from 96-hr inflamed guinea pigs (●) are expressed relative to the amount of sialyltransferase activity present in the supernatant obtained from the incubation in the presence of DMSO of Golgi membranes prepared from inflamed-guinea pig livers. The 100% value was 3250 pmol NeuAc transferred to rat asialo-α_1-AGP per mg Golgi protein per hr. Results represent the means from six separate determinations; reproducibility was within $\pm$ 10%.


Sialyltransferase Activity (%)
100
80
60
40
20
0
10 -11
10 -10
10 -9
10 -8
10 -7
10 -6
10 -5
10 -4
Pepstatin A (M)

sialyltransferase released from mouse and guinea pig Golgi membranes, but did not adversely affect solubilized sialyltransferase activities.

Effect of Exogenous Cathepsin D on Release of Sialyltransferase from Mouse and Guinea Pig Liver Golgi Membranes

In order to confirm the involvement of a cathepsin D-like proteinase in the release of mouse and guinea pig hepatic Galβ1,4GlcNAc α2,6-sialyltransferase from Golgi membranes, experiments were conducted in which Golgi membranes were treated with antiserum to cathepsin D prior to incubation at reduced pH, or were incubated at reduced pH in the presence of exogenous bovine spleen cathepsin D as had been done in the experiments with the rat. Rabbit antiserum raised against rat liver lysosomal cathepsin D apparently did not cross-react with mouse and guinea pig Golgi membrane endogenous cathepsin D, and therefore it was ineffective in inhibiting sialyltransferase release. However, incubation of mouse and guinea pig Golgi membranes at reduced pH in the presence of exogenous cathepsin D did result in enhancement of sialyltransferase release. Table 14 shows the effect of exposure for up to 8 min to 1 μg bovine spleen cathepsin D per mg Golgi membrane protein on release of sialyltransferase from mouse and guinea pig permeabilized Golgi vesicles incubated at pH 4.6 and 5.2, respectively. Increased presence of cathepsin D and increased time of exposure to cathepsin D both caused increased amounts of sialyltransferase to be released from Golgi membranes prepared from livers from controls, and from livers from 72-hr inflamed mice and 96-hr inflamed guinea pigs. In addition, exogenous bovine spleen cathepsin D was equally effective in enhancing sialyltransferase release from both mouse and guinea pig Golgi membranes. Although in the rat it was found that incubation periods up to 12 min at pH 5.6 in the presence of

TABLE 14

EFFECT OF EXOGENOUS CATHEPSIN D ON RELEASE OF SIALYLTRANSFERASE FROM MOUSE AND GUINEA PIG LIVER GOLGI MEMBRANES

	SIALYLTRANSFERASE ACTIVITY (pmol / mg Golgi protein / hr)			
	Control Golgi		Inflamed Golgi	
Cathepsin D.....	-	+	-	+
Mouse Golgi, pH 4.6				
4 min	195 ± 12	286 ± 15	335 ± 23	470 ± 32
8 min	282 ± 10	355 ± 24	644 ± 42	798 ± 38
Guinea Pig Golgi, pH 5.2				
4 min	350 ± 15	409 ± 20	402 ± 19	564 ± 28
8 min	428 ± 25	591 ± 30	809 ± 52	1044 ± 68

Golgi membranes prepared from livers from controls and from 72-hr inflamed mice and 96-hr inflamed guinea pigs were exposed to ultrasonic vibrations for 30 s and then incubated at pH 4.6 (mice) or pH 5.2 (guinea pigs) for the times indicated either alone (-), or in the presence of 1 μg bovine spleen cathepsin D per mg Golgi membrane protein (+). At the end of incubation pH was adjusted to 7.0, particulate material was removed by centrifugation and supernatants were assayed for sialyltransferase activities. Results represent the means from four separate determinations ± SD.

exogenous cathepsin D resulted in significant increases in solubilized sialyltransferase activity, in the mouse and the guinea pig it was found that incubation periods up to 12 min at pH 4.6 and pH 5.2, respectively, caused lesser amounts of sialyltransferase activity to be detected in the medium because greater amounts of solubilized enzyme were being degraded. As in the case of the rat, mouse and guinea pig Golgi galactosyltransferase was resistant to proteolysis by cathepsin D under the experimental conditions used, and the bulk of mouse and guinea pig galactosyltransferase activities remained membrane-bound following incubation of Golgi membranes at reduced pH.

Evidence that Sialyltransferase and Cathepsin D are Localized in the Same Compartments of Mouse and Guinea Pig Liver Golgi Apparatus

Release of sialyltransferase from mouse and guinea pig Golgi membranes by the action of endogenous Golgi cathepsin D was found to be an intraluminal proteolytic event using the same approach developed for studies with the rat. This was demonstrated by the inability of pepstatin A in the incubation medium to substantially inhibit proteolysis of sialyltransferase within intact mouse and guinea pig Golgi vesicles incubated at reduced pH. Tables 15 and 16 show the effect of incubation in the presence of pepstatin A on release of sialyltransferase within intact control-mouse and 72-hr inflamed-mouse Golgi vesicles, and within intact control-guinea pig and 96-hr inflamed-guinea pig Golgi vesicles, respectively. Mouse Golgi vesicles were incubated for 4 and 8 min at pH 4.6 either alone or in the presence of 10^{-6} M pepstatin A, following which the pH was adjusted to 7.0 and N-linked sialyltransferase activities in the medium were assayed before and after ultrasonication for 30 s. Guinea pig Golgi vesicles were similarly treated,

TABLE 15

EFFECT OF INCUBATION OF INTACT MOUSE LIVER GOLGI MEMBRANES WITH PEPSTATIN A FOLLOWED BY ULTRASONICATION TO RELEASE INTRALUMINAL SIALYLTRANSFERASE

		SIALYLTRANSFERASE ACTIVITY (pmol / mg Golgi protein / hr)			
		Control Golgi		Inflamed Golgi	
Conditions	Incubation time	4 min	8 min	4 min	8 min
pH 4.6					
Before Sonication		72 ± 4	108 ± 6	102 ± 7	173 ± 12
After Sonication		107 ± 9	213 ± 15	316 ± 21	675 ± 42
pH 4.6 + Pepstatin A					
Before Sonication		17 ± 2	41 ± 3	36 ± 3	94 ± 5
After Sonication		77 ± 6	136 ± 10	224 ± 18	448 ± 28

Golgi membranes prepared from livers from controls and from 72-hr inflamed mice were incubated for 4 min and 8 min at pH 4.6 either alone, or in the presence of 10^{-6} M pepstatin A. At the end of incubation pH was adjusted to 7.0, 400 µl portions were removed from each sample, centrifuged to remove particulate matter, and supernatants were assayed for sialyltransferase activity. The remaining material was then ultrasonicated for 30 s to release any sialyltransferase that was no longer membrane-bound, centrifuged and supernatants were assayed for sialyltransferase activity. Results represent the means from three separate determinations ± SD.

TABLE 16

EFFECT OF INCUBATION OF INTACT GUINEA PIG LIVER GOLGI MEMBRANES WITH PEPSTATIN A FOLLOWED BY ULTRASONICATION TO RELEASE INTRALUMINAL SIALYLTRANSFERASE

	SIALYLTRANSFERASE ACTIVITY (pmol / mg Golgi protein / hr)			
	Control Golgi		Inflamed Golgi	
Conditions / Incubation time	4 min	16 min	4 min	16 min
pH 5.2				
Before Sonication	117 ± 6	167 ± 11	236 ± 12	280 ± 16
After Sonication	708 ± 44	833 ± 51	1691 ± 96	1960 ± 114
pH 5.2 + Pepstatin A				
Before Sonication	96 ± 7	87 ± 4	130 ± 8	174 ± 18
After Sonication	671 ± 41	680 ± 49	1456 ± 64	1556 ± 87

Golgi membranes prepared from livers from controls and from 96-hr inflamed guinea pigs were incubated for 4 min and 16 min at pH 5.2 either alone, or in the presence of 10^{-6} M pepstatin A. At the end of incubation pH was adjusted to 7.0, 400 µl portions were removed from each sample, centrifuged to remove particulate matter, and supernatants were assayed for sialyltransferase activity. The remaining material was then ultrasonicated for 30 s to release any sialyltransferase that was no longer membrane-bound, centrifuged and supernatants were assayed for sialyltransferase activity. Results represent the means from three separate determinations ± SD.

except that incubation was at pH 5.2 for 4 and 16 min (see Methods). Increase in time of incubation at reduced pH resulted in increased proteolysis of mouse and guinea pig Golgi membrane-bound sialyltransferase. Ultrasonication of intact Golgi vesicles caused substantial increases in the levels of solubilized sialyltransferase activities detected in the medium, which confirmed the intraluminal orientation of mouse and guinea pig Golgi membrane-bound sialyltransferase. Permeabilized Golgi vesicles obtained from livers from inflamed mice and guinea pigs released greater amounts of sialyltransferase than did permeabilized Golgi vesicles prepared from livers from controls under the same experimental conditions. Although exposure of intact mouse and guinea pig Golgi vesicles to pepstatin A caused inhibition of intraluminal proteolysis of membrane-bound sialyltransferase, the amount of inhibition was not substantial. Incubation of intact mouse Golgi vesicles in the presence of pepstatin A resulted in a 29 - 37% reduction in sialyltransferase activities assayed following ultrasonication. Guinea pig solubilized sialyltransferase activities detected in the medium after ultrasonication were reduced 6 - 19% by the presence of this proteinase inhibitor. These data strongly suggested that, as in the case of the rat, mouse and guinea pig endogenous Golgi cathepsin D was located within the same compartments of the liver Golgi apparatus as membrane-bound Galβ1,4GlcNAc α2,6-sialyltransferase.

Mouse and Guinea Pig Liver Golgi Membrane Cathepsin D Activities

Cathepsin D activities in mouse and guinea pig liver Golgi membrane suspensions were investigated in order to determine if they were increased by turpentine-induced inflammation and if they were associated with membrane-bound mannose 6-phosphate receptors, as was found with the rat

system. Tables 17 and 18 show the effect of treatment with ultrasonic vibrations, Triton-X-100 and mannose 6-phosphate on release of cathepsin D activities from control-mouse and 72-hr inflamed-mouse Golgi membranes, and from control-guinea pig and 96-hr inflamed-guinea pig Golgi membranes, respectively. Golgi membranes prepared from livers from inflamed mice and guinea pigs contained greater amounts of cathepsin D activities than Golgi membranes obtained from livers from controls. Ultrasonication for 30 s of intact mouse and guinea pig Golgi vesicles only caused marginal release of cathepsin D activities, and 80 - 90% of the total cathepsin D activities exhibited by intact mouse and guinea pig Golgi vesicles were released following treatment with Triton-X-100. Ultrasonication for 30 s of mouse and guinea pig Golgi vesicles followed by incubation for 30 min in the presence of 5 mM mannose 6-phosphate caused the release of 62 - 73% of the total membrane-bound cathepsin D activities. These data strongly supported the conclusion that, as was found in the rat, mouse and guinea pig endogenous cathepsin D activities were largely associated with membrane-bound mannose 6-phosphate receptors.

CHARACTERIZATION OF HEPATIC Galβ1,4GlcNAc α2,6-SIALYLTRANSFERASE RELEASED FROM MOUSE AND GUINEA PIG LIVER GOLGI MEMBRANES AT REDUCED pH

Immunoblot Analysis of Sialyltransferase Released from Mouse and Guinea Pig Liver Golgi Membranes at Reduced pH

In order to affirm that a proteolytic event was involved in the release of mouse and guinea pig hepatic Galβ1,4GlcNAc α2,6-sialyltransferases from Golgi membranes incubated at reduced pH, the molecular weights of

TABLE 17

CATHEPSIN D ACTIVITIES IN INTACT MOUSE LIVER GOLGI MEMBRANES AND SUPERNATANT FRACTIONS PREPARED BY ULTRASONICATION, EXTRACTION WITH TRITON X-100 AND INCUBATION WITH MANNOSE 6-PHOSPHATE

Sample	CATHEPSIN D ACTIVITY (ΔA_{280} / ml / min) Control Golgi	Inflamed Golgi
Intact Golgi	3.77 ± 0.23	4.70 ± 0.28
Supernatant Fractions		
Ultrasonicated Golgi	0.22 ± 0.01	0.69 ± 0.03
Triton X-100	3.06 ± 0.16	4.33 ± 0.26
Mannose 6-phosphate	2.34 ± 0.11	3.41 ± 0.22

Golgi membranes prepared from livers from controls and from 72-hr inflamed mice were assayed directly for cathepsin D activities ("intact Golgi" samples). Golgi membranes were then either exposed to ultrasonication for 30 s ("ultrasonicated Golgi" samples) or extracted with 0.1% Triton X-100 ("Triton X-100" samples), following which particulate material was removed by centrifugation and supernatants were assayed for cathepsin D activities. Golgi membranes were also ultrasonicated for 30 s and then incubated for 30 min in the presence of 5 mM mannose 6-phosphate, following which particulate material was removed by centrifugation and supernatants were assayed for cathepsin D activities ("mannose 6-phosphate" samples). Supernatants obtained from centrifugation of intact Golgi membranes, and centrifugation of intact Golgi membranes that had been incubated with mannose 6-phosphate contained low levels of cathepsin D activities, and these activities were subtracted from those present in "ultrasonicated Golgi" and "mannose 6-phosphate" samples, respectively. Results represent the means of three separate determinations ± SD.

TABLE 18

CATHEPSIN D ACTIVITIES IN INTACT GUINEA PIG LIVER GOLGI MEMBRANES AND SUPERNATANT FRACTIONS PREPARED BY ULTRASONICATION, EXTRACTION WITH TRITON X-100 AND INCUBATION WITH MANNOSE 6-PHOSPHATE

Sample	CATHEPSIN D ACTIVITY (ΔA_{280} / ml / min) Control Golgi	Inflamed Golgi
Intact Golgi	2.84 ± 0.13	3.90 ± 0.20
Supernatant Fractions		
Ultrasonicated Golgi	0.26 ± 0.02	0.99 ± 0.05
Triton X-100	2.24 ± 0.10	3.30 ± 0.19
Mannose 6-phosphate	1.92 ± 0.08	2.75 ± 0.12

Golgi membranes prepared from livers from controls and from 96-hr inflamed guinea pigs were treated as described in the legend for Table 17. Results represent the means of three separate determinations ± SD.

membrane-bound and solubilized forms of sialyltransferase were determined by immunoblot analysis of SDS-gels using rabbit antiserum containing antibodies to the rat hepatic Galβ1,4GlcNAc α2,6-sialyltransferase. Figure 50 shows that whereas the predominant membrane-bound form of sialyltransferase present within Golgi membranes obtained from 72 hr inflamed-mouse livers exhibited a molecular weight of 49,000 Da, that contained within Golgi membranes purified from 96 hr inflamed-guinea pig livers had a lower molecular weight of approximately 42,000 Da. However, the solubilized forms of sialyltransferase released upon incubation of mouse and guinea pig permeabilized Golgi membranes at pH 4.6 and 5.2, respectively, displayed a similar molecular weight of 38,000 Da.

Kinetic Properties of Mouse and Guinea Pig Liver Golgi Membrane-Bound and Released Sialyltransferase

Demonstration of the intraluminal orientation of the catalytic domains of mouse and guinea pig hepatic Golgi Galβ1,4GlcNAc α2,6-sialyltransferases combined with electrophoretic data on membrane-bound and solubilized enzyme forms (see above) suggested that, as in the rat, the catalytic domains of mouse and guinea pig α2,6 sialyltransferases are separated from membrane-anchor domains by intervening linker domains. The kinetic properties of mouse and guinea pig Golgi membrane-bound and solubilized sialyltransferase activities were therefore investigated in order to determine if proteolysis of membrane-bound enzyme caused significant changes in catalytic efficiency. Table 19 shows the apparent K_m values towards CMP-NeuAc and rat asialo-α_1-AGP determined for mouse Golgi membrane-bound sialyltransferase activities and solubilized sialyltransferase activities released from permeabilized Golgi vesicles during incubation at pH 4.6. Apparent K_m

FIGURE 50

Immunoblot of Membrane-Bound and Solubilized Mouse and Guinea Pig Liver Golgi Sialyltransferases

Intact Golgi membranes prepared from livers from 48-hr inflamed rats, 72-hr inflamed mice and 96-hr inflamed guinea pigs were extracted with Triton X-100 to release the membrane-bound sialyltransferase, which was compared with solubilized sialyltransferase released from rat, mouse and guinea pig Golgi membranes by sonication followed by incubation at pH 5.6, 4.6 and 5.2, respectively, for 30 min. Samples containing membrane-bound and solubilized sialyltransferases were subjected to SDS-gel electrophoresis for 1 hr using a Bio-Rad Mini-Protean II Slab Cell system, and immunoblot analysis was performed using antiserum to rat liver Golgi Galβ1,4GlcNAc α2,6-sialyltransferase (see Methods). Tracks 1, 3 5, rat, mouse and guinea pig Golgi membrane-bound sialyltransferases, respectively; Tracks 2, 4, 6, sialyltransferase released from rat, mouse and guinea pig Golgi membranes, respectively, by sonication and incubation at reduced pH. Molecular weight markers were as described in legend for Figure 23.

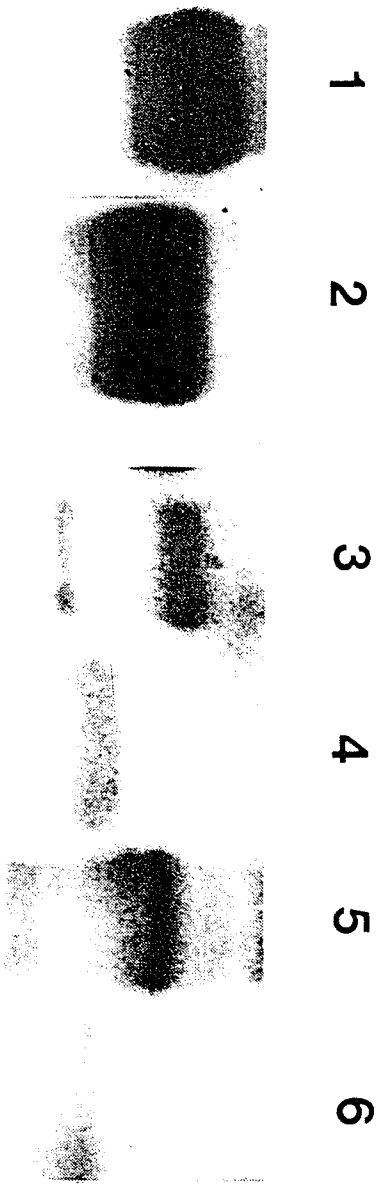

1
2
3
4
5
6

TABLE 19

APPARENT K_m VALUES FOR MOUSE LIVER GOLGI MEMBRANE-BOUND AND SOLUBILIZED SIALYLTRANSFERASE ACTIVITIES

Enzyme Source	CMP-NeuAc K_m (μM)	Asialo-α_1-Acid Glycoprotein K_m (μM)
Control Mouse		
Membrane-Bound	151 ± 12	35 ± 6
Solubilized	146 ± 10	37 ± 4
Inflamed Mouse		
Membrane-Bound	145 ± 9	28 ± 4
Solubilized	152 ± 10	33 ± 5

Golgi membranes prepared from livers from controls and from 72-hr inflamed mice were exposed to ultrasonic vibrations for 30 s and then incubated for 30 min at either pH 7.0 or 4.6. After incubation pH was adjusted to 7.0, and incubation mixtures were centrifuged to obtain pellet and supernatant samples. Activities towards CMP-NeuAc and rat asialo-α_1-acid glycoprotein were determined for sialyltransferase associated with pelleted Golgi membranes or present in supernatants obtained from centrifugation of Golgi membrane samples incubated at pH 7.0 and pH 4.6, respectively. K_m values for rat asialo-α_1-acid glycoprotein were calculated using a molecular weight of 34,600 Da (Jamieson *et al.*, 1972; Kaplan *et al.*, 1983). Results represent the means from three separate determinations ± SD.

values for control-mouse and 72-hr inflamed-mouse membrane-bound and solubilized sialyltransferase activities were very similar with respect to each substrate. The ranges of apparent K_m values determined for CMP-NeuAc and asialo-α_1-AGP were 145 - 152 μM and 28 - 37 μM, respectively. Values of apparent K_m for CMP-NeuAc for mouse membrane-bound and solubilized sialyltransferase activities were higher than those found for mouse liver and serum sialyltransferase activities, which were within the range 51 - 88 μM (see above). However, values of apparent K_m for asialo-α_1-AGP determined for mouse liver and serum sialyltransferase activities were within the range 18 - 30 μM (see above) and compared favorably with those determined for mouse Golgi membrane-bound and solubilized sialyltransferases. Table 20 shows the apparent K_m values for CMP-NeuAc and asialo-α_1-AGP determined for guinea pig membrane-bound sialyltransferase activities and solubilized sialyltransferase activities released during incubation at pH 5.2. As had been found in the mouse, control-guinea pig and 96-hr inflamed-guinea pig membrane-bound and solubilized sialyltransferase activities exhibited similar affinities with respect to each substrate. Values of K_m for CMP-NeuAc and rat asialo-α_1-AGP for guinea pig sialyltransferase activities were in the ranges 144 - 147 μM and 32 - 37 μM, and these compared very favorably with those determined for corresponding sialyltransferase activities in the mouse. In comparison, apparent K_m values for guinea pig liver and serum sialyltransferase activities towards CMP-NeuAc and rat asialo-α_1-AGP were within the ranges 63 - 92 μM and 21 - 67 μM, respectively (see above).

TABLE 20

APPARENT K_m VALUES FOR GUINEA PIG LIVER GOLGI MEMBRANE-BOUND AND SOLUBILIZED SIALYLTRANSFERASE ACTIVITIES

Enzyme Source	CMP-NeuAc K_m (μM)	Asialo-α_1-Acid Glycoprotein K_m (μM)
Control Guinea Pig		
Membrane-Bound	145 ± 7	32 ± 3
Solubilized	144 ± 9	34 ± 4
Inflamed Guinea Pig		
Membrane-Bound	147 ± 12	33 ± 2
Solubilized	147 ± 10	37 ± 5

Golgi membranes prepared from livers from controls and from 96-hr inflamed guinea pigs were exposed to ultrasonic vibrations for 30 s and then incubated for 30 min at either pH 7.0 or 5.2. After incubation pH was adjusted to 7.0, and incubation mixtures were centrifuged to obtain pellet and supernatant samples. Activities towards CMP-NeuAc and rat asialo-α_1-acid glycoprotein were determined for sialyltransferase associated with pelleted Golgi membranes or present in supernatants obtained from centrifugation of Golgi membrane samples incubated at pH 7.0 and pH 5.2, respectively. K_m values for rat asialo-α_1-acid glycoprotein were calculated using a molecular weight of 34,600 Da (Jamieson *et al.*, 1972; Kaplan *et al.*, 1983). Results represent the means from three separate determinations ± SD.

DISCUSSION

As mentioned in the Introduction, the majority of the acute phase reactants are glycoproteins that typically contain terminally-processed N-linked oligosaccharide side-chains (Hatton *et al.*, 1983; Gordon and Koj, 1985; Jamieson *et al.*, 1986). Amplification of hepatic glycosylation machinery involved in synthesis of N-linked oligosaccharides therefore must necessarily accompany the elevated synthesis and secretion of plasma glycoproteins during inflammation. Studies conducted in the Jamieson laboratory on the acute phase response in the rat have confirmed that injection of either turpentine or a cytokine preparation results in not only an increase in hepatic glycosyltransferase activities and corresponding nucleotide sugar pools, but also in dilation of the ER and proliferation of the Golgi apparatus which facilitate the handling of the significantly increased volume of secretory glycoprotein traffic (Turchen *et al.*, 1977; Jamieson *et al.*, 1983; Kaplan *et al.*, 1983; Woloski *et al.*, 1985a, 1986). Examination of the magnitude of response of sialyltransferase and galactosyltransferase levels to turpentine-induced inflammation in the rat revealed that enzyme activities towards the macromolecular acceptors rat asialo- and asialo-agalacto-α_1-AGP increased approximately three-fold in the liver within 48 hr following injection of turpentine (Turchen *et al.*, 1977; Kaplan *et al.*, 1983). Characterization of hepatic sialyltransferase activity using the low molecular weight acceptor lactose demonstrated that inflammation promoted a significantly greater increase in α2,6-sialyltransferase activity than in α2,3-sialyltransferase activity (Kaplan *et al.*, 1983). Surprisingly however, the anticipated increase in liver sialyltransferase activity during inflammation was found to be accompanied by the unanticipated five-fold enhancement in serum sialyltransferase

activity. Use of the *in vitro* liver slice system confirmed not only that the liver was the source of the elevated serum sialyltransferase activity, but also that inflammation did not result in the secretion of significant amounts of hepatic galactosyltransferase activity. Moreover, use of lactose to determine the nature of the linkage formed provided evidence that an α2,6-sialyltransferase was responsible for the elevation in serum sialyltransferase activity towards the large molecular weight acceptor asialo-α_1-AGP (Kaplan *et al.*, 1983).

In order to follow up on this earlier work, studies were undertaken in this thesis to determine the mechanism of release of this newly-identified acute phase reactant, α2,6-ST, from the Golgi membrane during inflammation. Experimental design was predicated on a proposed topological model (Figure 11) of the α2,6-ST which defined this glycosyltransferase to be comprised of a globular, luminally-oriented catalytic domain, an extended linker region and a hydrophobic anchor that serves to attach the enzyme to the Golgi membrane lipid bilayer. It was hypothesized that release of the α2,6-ST from the liver during inflammation was caused by limited proteolytic cleavage of the linker region, which resulted in secretion into the extracellular space of a lower molecular weight form of the enzyme that possessed an intact catalytic domain.

Results obtained from *in vitro* experiments using rat liver Golgi membranes show that the Galβ1,4GlcNAc α2,6-sialyltransferase, but not the GlcNAc β1,4-galactosyltransferase or the Galβ1,3(4)GlcNAc α2,3-sialyltransferase, is particularly sensitive to proteolysis under conditions of reduced pH. Maximum release of sialyltransferase from sonicated Golgi membranes occurred at pH 5.6, and approximately 70% of the membrane-bound enzyme was released under these conditions after 30 min of

incubation. Use of antiserum containing antibodies to the Galβ1,4GlcNAc α2,6-sialyltransferase purified from rat liver Golgi membranes (Weinstein *et al.*, 1982b) confirmed that exposure of Golgi membranes to reduced pH promoted specific release of the α2,6-ST. Permeabilization of the Golgi membrane was found to be a necessary prerequisite for subsequent retrieval in the supernatant of the bulk of the originally membrane-bound sialyltransferase, which confirmed that the enzyme had been previously attached to the intraluminal face of the lipid bilayer. Although significant amounts of sialyltransferase were released upon reduction of the pH of the incubation medium to less than pH 5.6, a greater proportion of the solubilized enzyme was subsequently degraded in the incubation medium. Therefore, pH 5.6 represents the pH at which, under the experimental conditions of the assay used, there is the least amount of proteolytic degradation of solubilized sialyltransferase in the incubation medium.

Golgi membranes prepared from livers excised from rats suffering from turpentine-induced inflammation for 36 hr were found to release approximately twice the amount of sialyltransferase activity at pH 5.6 than did Golgi membranes that had been isolated from livers from controls. A two-fold increase in rat liver homogenate sialyltransferase activity after 36 hr of turpentine-induced inflammation had previously been demonstrated (Kaplan *et al.*, 1983), and therefore the enhancement in liver homogenate sialyltransferase activity following the advent of injury appears to be due to an increase in the concentration of sialyltransferase within the Golgi apparatus. This in turn provides support for the conclusion that during the acute phase response the bulk of the nascent α2,6-ST is correctly targeted to the *trans* Golgi cisternae and the TGN wherein it is retained through physical attachment to the intraluminal face of the Golgi membrane. Release of this

glycosyltransferase from the liver during inflammation therefore is apparently not the result of direct passage of newly synthesized enzyme along the mainstream secretory pathway to the cell surface, but rather the consequence of a secondary, proteolytic event that takes place within the Golgi apparatus.

In order to provide additional evidence in support of the conclusion that during the acute phase response the increase in rat liver Golgi sialyltransferase activity is indeed due to an increase in enzyme presence within the Golgi apparatus, and is not the consequence of an increase in catalytic efficiency, a comparative kinetic analysis of the affinities exhibited by control-rat and inflamed-rat membrane-bound sialyltransferase towards CMP-NeuAc and asialo-α_1-AGP was performed. The results show that values of apparent K_m for either substrate are not altered by turpentine-induced inflammation. Values of apparent K_m were also determined for sialyltransferase that had been released from control-rat and inflamed-rat liver Golgi membranes at pH 5.6. Lack of significant discrepancies between kinetic parameters displayed by membrane-bound and solubilized sialyltransferase substantiated the conclusion that proteolytic cleavage at reduced pH of the hypothesized linker region did not result in the generation of a solubilized enzyme form that possessed a damaged catalytic domain.

The hypothesis that release of α2,6-ST from the Golgi membrane is strongly dependent upon maintenance of an acidic pH within the lumen of the sialyltransferase-containing Golgi compartment is supported by demonstration of the inhibitory effect of lysosomotropic agents on enzyme release from rat liver slices. Weakly basic amines such as ammonium chloride, methylamine and chloroquine, which were originally classified as lysosomotropic agents upon detection of their ability to perturb the acidic

internal milieu of lysosomes, have been found to accumulate in their protonated forms within all acidic compartments of the cell and to promote an elevation in intraluminal pH (de Duve *et al.*, 1974; Poole and Ohkuma, 1981; Maxfield, 1982). Electron microscopic examination of the morphological effect of ammonium chloride, methylamine and chloroquine on the Golgi apparatus in cultured rat hepatocytes and fibroblasts has revealed that a common structural alteration is dilation and swelling of the *trans* elements and associated vesicles (Wibo and Poole, 1974; Oda *et al.*, 1986). Similar findings have been reported on the effect of lysosomotropic agents on human fibroblast and HepG2 hepatoma cells (Anderson and Pathak, 1985; Schwartz *et al.*, 1985; Strous *et al.*, 1985). Physical distension of regions of the *trans* Golgi apparatus due to the accumulation of protonated lysosomotropic agents has revealed that these intracellular compartments are acidic, and maintenance of low intraluminal pH has been shown to be due to the activity of Golgi membrane-associated ATP-dependent H^+ pumps (Glickman *et al.*, 1983; Zhang and Schneider, 1983; Moriyama and Nelson, 1989). Results reported in this thesis show that presence in the incubation medium of 50 mM ammonium chloride, 50 mM methylamine or 1 mM chloroquine effectively abolishes sialyltransferase secretion from rat liver slices. Inhibition of enzyme release was rapidly reversed upon removal of lysosomotropic agents from the incubation medium, which revealed that these agents were not cytotoxic to the cells under the conditions used. Increased presence of protonated amines within the apparently acidic sialyltransferase-containing Golgi compartments necessarily would be expected to promote incremental rises in intraluminal pH. Consequently, exposure of liver slices to lysosomotropic agents would be expected to inhibit, in a concentration-dependent manner, the pH-dependent proteolysis of membrane-bound α2,6-

sialyltransferase. Results of experiments conducted with both control-rat and 36 hr inflamed-rat liver slices demonstrate that secretion of sialyltransferase is inhibited in a dose-dependent fashion by ammonium chloride, methylamine and chloroquine.

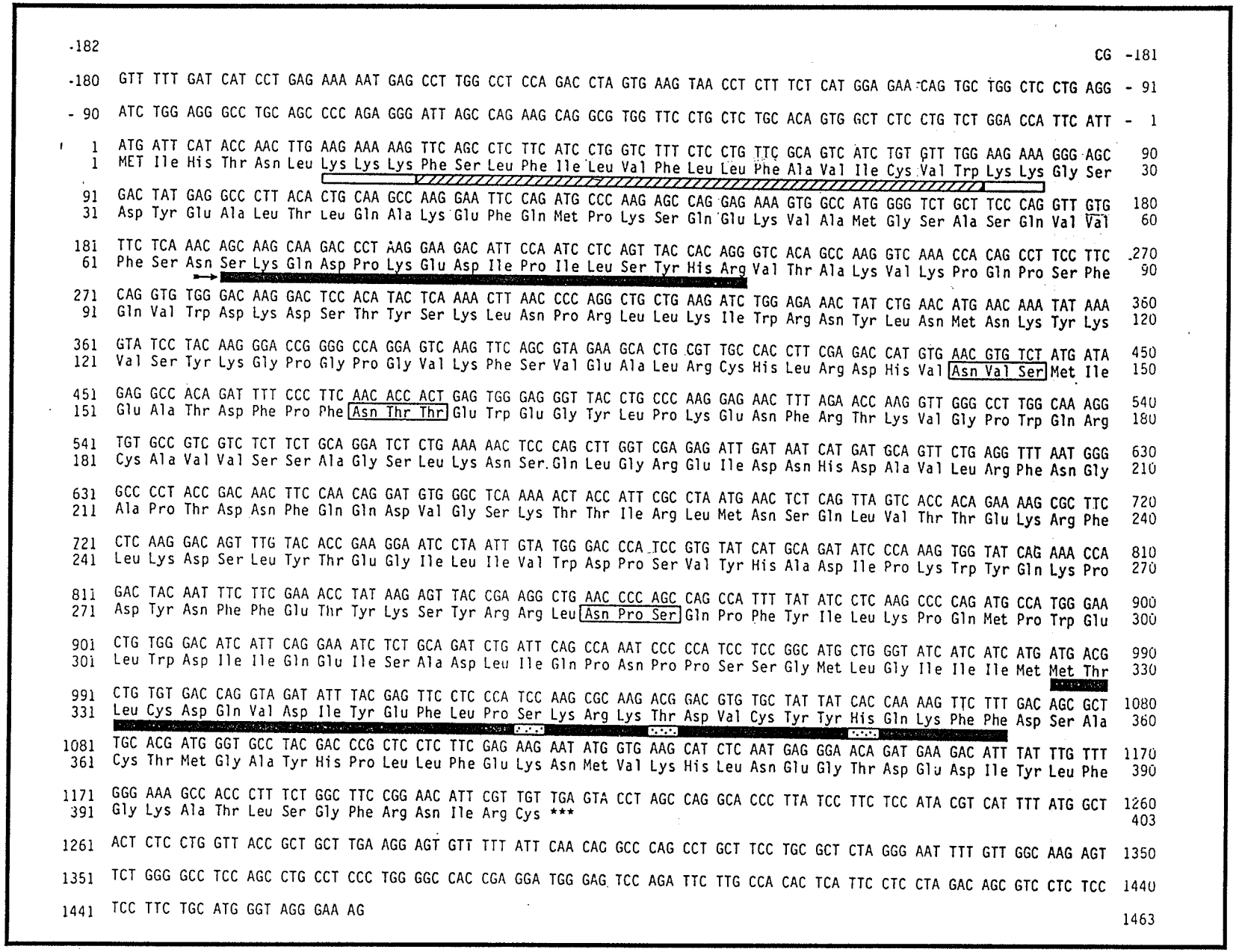
Elevation of intraluminal pH of *trans* Golgi cisternae due to the presence of protonated forms of lysosomotropic agents has been reported to have no effect on the terminal sialylation of rat α_1-PI in cultured rat hepatocytes, which has indicated that the integrity of *trans* Golgi glycosylation machinery remains unimpaired under these conditions (Oda *et al.*, 1986). However, presence of weakly basic amines has been found to severely inhibit the proteolytic conversion of precursor forms of albumin and complement component C3 in cultured rat hepatocytes, the consequence of which was demonstrated to be the increased presence in the incubation medium of proalbumin and pro-C3 (Oda and Ikehara, 1985; Oda *et al.*, 1986). Evidence in support of the conclusion that the inhibitory effect of lysosomotropic agents on sialyltransferase release from rat liver slices is indeed due to interference with proteolysis of the membrane-bound glycosyltransferase is provided by the finding that the presence of ammonium chloride, methylamine or chloroquine only slightly affected the secretion of α_1-AGP from rat liver slices. The terminally-sialylated N-linked glycoprotein α_1-AGP is not subject to proteolytic processing within the Golgi apparatus (Jamieson *et al.*, 1972a; Jamieson and Ashton, 1973; Ricca and Taylor, 1981; Reinke and Fiegelson, 1985), and the inability of lysosomotropic agents to inhibit its secretion from rat liver slices demonstrated that the presence of protonated amines does not cause impairment of physical transport through the Golgi apparatus to the cell surface along the mainstream secretory pathway.

Immunoblot analysis of SDS gels revealed that the molecular weight of the α2,6-ST associated with Golgi membranes isolated from livers from rats suffering from turpentine-induced inflammation for 36 hr is approximately 49,000 Da. Availability of the nucleotide sequence of cDNA clones of rat liver α2,6-ST has allowed deduction of the complete amino acid sequence (Figure 51) (Paulson *et al.*, 1987; Weinstein *et al.*, 1987). This Golgi glycosyltransferase is a polypeptide of 403 amino acids, with a predicted molecular weight of 46,700 Da based on amino acid content. Three potential N-linked glycosylation sites are present (residues 146-148, 158-160, 285-287), however use of N-glycosidase F to characterize the carbohydrate content of the α2,6-ST has revealed that only two of the asparagine sequons are utilized (Weinstein *et al.*, 1987). Presence of proline within the tripeptide sequence located at residues 285-287 indicates that this N-linked glycosylation site is most likely not utilized (Marshall, 1972, 1974; Bause and Lehle, 1979), and if so, then interestingly, the two N-linked oligosaccharide side-chains of sialyltransferase would be in close physical proximity. Correction of the predicted molecular weight of 46,700 Da to account for the addition of two N-linked carbohydrate groups of an estimated molecular weight of 6000 Da reveals that the true molecular weight of the mature, membrane-bound sialyltransferase would be approximately 52,700 Da. The reported molecular weight of rat liver membrane-bound α2,6-ST obtained from immunoblot analysis of SDS gels is 47,000 Da (Roth *et al.*, 1985; Weinstein *et al.*, 1987), which is similar to the molecular weight of 49,000 Da determined from electrophoresis studies undertaken in this thesis. The discrepancy between the experimentally observed molecular weights of the mature, membrane-bound α2,6-ST with the predicted molecular weight may be speculated to be due to the limitations inherent in the SDS electrophoresis of glycosylated proteins.

FIGURE 51

Amino Acid Sequence of Rat Liver Golgi Galβ1,4GlcNAc α2,6-sialyltransferase

The complete amino acid sequence of rat liver Golgi α2,6-ST was deduced from the nucleotide sequence of sialyltransferase cDNA. Residues underscored by a cross-hatched box represent the proposed signal-anchor sequence that is bordered by charged lysine residues. Residues underscored by black and stippled boxes represent peptide sequence overlaps and unidentified residues, respectively. Potential N-linked glycosylation sites are boxed, and the NH_2-terminus of purified sialyltransferase is indicated by an arrow. (Taken from Weinstein *et al.*, 1987).


-182
CG -181
-180 GTT TTT GAT CAT CCT GAG AAA AAT GAG CCT TGG CCT CCA GAC CTA GTG AAG TAA CCT CTT TCT CAT GGA GAA CAG TGC TGG CTC CTG AGG - 91
- 90 ATC TGG AGG GCC TGC AGC CCC AGA GGG ATT AGC CAG AAG CAG GCG TGG TTC CTG CTC TGC ACA GTG GCT CTC CTG TCT GGA CCA TTC ATT - 1
1 ATG ATT CAT ACC AAC TTG AAG AAA AAG TTC AGC CTC TTC ATC CTG GTC TTT CTC CTG TTC GCA GTC ATC TGT GTT TGG AAG AAA GGG AGC 90
1 MET Ile His Thr Asn Leu Lys Lys Lys Phe Ser Leu Phe Ile Leu Val Phe Leu Leu Phe Ala Val Ile Cys Val Trp Lys Lys Gly Ser 30
91 GAC TAT GAG GCC CTT ACA CTG CAA GCC AAG GAA TTC CAG ATG CCC AAG AGC CAG GAG AAA GTG GCC ATG GGG TCT GCT TCC CAG GTT GTG 180
31 Asp Tyr Glu Ala Leu Thr Leu Gln Ala Lys Glu Phe Gln Met Pro Lys Ser Gln Glu Lys Val Ala Met Gly Ser Ala Ser Gln Val Val 60
181 TTC TCA AAC AGC AAG CAA GAC CCT AAG GAA GAC ATT CCA ATC CTC AGT TAC CAC AGG GTC ACA GCC AAG GTC AAA CCA CAG CCT TCC TTC 270
61 Phe Ser Asn Ser Lys Gln Asp Pro Lys Glu Asp Ile Pro Ile Leu Ser Tyr His Arg Val Thr Ala Lys Val Lys Pro Gln Pro Ser Phe 90
271 CAG GTG TGG GAC AAG GAC TCC ACA TAC TCA AAA CTT AAC CCC AGG CTG CTG AAG ATC TGG AGA AAC TAT CTG AAC ATG AAC AAA TAT AAA 360
91 Gln Val Trp Asp Lys Asp Ser Thr Tyr Ser Lys Leu Asn Pro Arg Leu Leu Lys Ile Trp Arg Asn Tyr Leu Asn Met Asn Lys Tyr Lys 120
361 GTA TCC TAC AAG GGA CCG GGG CCA GGA GTC AAG TTC AGC GTA GAA GCA CTG CGT TGC CAC CTT CGA GAC CAT GTG AAC GTG TCT ATG ATA 450
121 Val Ser Tyr Lys Gly Pro Gly Pro Gly Val Lys Phe Ser Val Glu Ala Leu Arg Cys His Leu Arg Asp His Val Asn Val Ser Met Ile 150
451 GAG GCC ACA GAT TTT CCC TTC AAC ACC ACT GAG TGG GAG GGT TAC CTG CCC AAG GAG AAC TTT AGA ACC AAG GTT GGG CCT TGG CAA AGG 540
151 Glu Ala Thr Asp Phe Pro Phe Asn Thr Thr Glu Trp Glu Gly Tyr Leu Pro Lys Glu Asn Phe Arg Thr Lys Val Gly Pro Trp Gln Arg 180
541 TGT GCC GTC GTC TCT TCT GCA GGA TCT CTG AAA AAC TCC CAG CTT GGT CGA GAG ATT GAT AAT CAT GAT GCA GTT CTG AGG TTT AAT GGG 630
181 Cys Ala Val Val Ser Ser Ala Gly Ser Leu Lys Asn Ser Gln Leu Gly Arg Glu Ile Asp Asn His Asp Ala Val Leu Arg Phe Asn Gly 210
631 GCC CCT ACC GAC AAC TTC CAA CAG GAT GTG GGC TCA AAA ACT ACC ATT CGC CTA ATG AAC TCT CAG TTA GTC ACC ACA GAA AAG CGC TTC 720
211 Ala Pro Thr Asp Asn Phe Gln Gln Asp Val Gly Ser Lys Thr Thr Ile Arg Leu Met Asn Ser Gln Leu Val Thr Thr Glu Lys Arg Phe 240
721 CTC AAG GAC AGT TTG TAC ACC GAA GGA ATC CTA ATT GTA TGG GAC CCA TCC GTG TAT CAT GCA GAT ATC CCA AAG TGG TAT CAG AAA CCA 810
241 Leu Lys Asp Ser Leu Tyr Thr Glu Gly Ile Leu Ile Val Trp Asp Pro Ser Val Tyr His Ala Asp Ile Pro Lys Trp Tyr Gln Lys Pro 270
811 GAC TAC AAT TTC TTC GAA ACC TAT AAG AGT TAC CGA AGG CTG AAC CCC AGC CAG CCA TTT TAT ATC CTC AAG CCC CAG ATG CCA TGG GAA 900
271 Asp Tyr Asn Phe Phe Glu Thr Tyr Lys Ser Tyr Arg Arg Leu Asn Pro Ser Gln Pro Phe Tyr Ile Leu Lys Pro Gln Met Pro Trp Glu 300
901 CTG TGG GAC ATC ATT CAG GAA ATC TCT GCA GAT CTG ATT CAG CCA AAT CCC CCA TCC TCC GGC ATG CTG GGT ATC ATC ATC ATG ATG ACG 990
301 Leu Trp Asp Ile Ile Gln Glu Ile Ser Ala Asp Leu Ile Gln Pro Asn Pro Pro Ser Ser Gly Met Leu Gly Ile Ile Ile Met Met Thr 330
991 CTG TGT GAC CAG GTA GAT ATT TAC GAG TTC CTC CCA TCC AAG CGC AAG ACG GAC GTG TGC TAT TAT CAC CAA AAG TTC TTT GAC AGC GCT 1080
331 Leu Cys Asp Gln Val Asp Ile Tyr Glu Phe Leu Pro Ser Lys Arg Lys Thr Asp Val Cys Tyr Tyr His Gln Lys Phe Phe Asp Ser Ala 360
1081 TGC ACG ATG GGT GCC TAC GAC CCG CTC CTC TTC GAG AAG AAT ATG GTG AAG CAT CTC AAT GAG GGA ACA GAT GAA GAC ATT TAT TTG TTT 1170
361 Cys Thr Met Gly Ala Tyr His Pro Leu Leu Phe Glu Lys Asn Met Val Lys His Leu Asn Glu Gly Thr Asp Glu Asp Ile Tyr Leu Phe 390
1171 GGG AAA GCC ACC CTT TCT GGC TTC CGG AAC ATT CGT TGT TGA GTA CCT AGC CAG GCA CCC TTA TCC TTC TCC ATA CGT CAT TTT ATG GCT 1260
391 Gly Lys Ala Thr Leu Ser Gly Phe Arg Asn Ile Arg Cys *** 403
1261 ACT CTC CTG GTT ACC GCT GCT TGA AGG AGT GTT TTT ATT CAA CAC GCC CAG CCT GCT TCC TGC GCT CTA GGG AAT TTT GTT GGC AAG AGT 1350
1351 TCT GGG GCC TCC AGC CTG CCT CCC TGG GGC CAC CGA GGA TGG GAG TCC AGA TTC TTG CCA CAC TCA TTC CTC CTA GAC AGC GTC CTC TCC 1440
1441 TCC TTC TGC ATG GGT AGG GAA AG 1463

Examination of the NH_2-terminal sequence of the rat liver α2,6-ST has revealed the presence of a putative membrane-binding domain of 17 hydrophobic amino acids (residues 10-26) that is bounded on both sides by two or three positively-charged lysine residues. The first 9 amino acids therefore represent the NH_2-terminal cytoplasmic domain, and the remaining 377 amino acids culminating in the COOH-terminus (residues 27-403) constitute the large, luminally-oriented portion of the molecule that contains the catalytic site (Paulson *et al.*, 1987; Weinstein *et al.*, 1987). Chromatographic purification of the α2,6-ST from rat liver homogenates was reported to result in the isolation of a catalytically-active form of the enzyme that exhibited a molecular weight on SDS gels of 40,000 - 41,000 Da, which was in contrast to the molecular weight of approximately 47,000 Da displayed by the mature, membrane-bound enzyme form (Weinstein *et al.*, 1987; Roth *et al.*, 1985). NH_2-terminal analysis of the purified, solubilized sialyltransferase has revealed that it is a truncated form of the enzyme that is missing the 63-residue NH_2-terminal peptide that contains the membrane-binding domain. Conversion of the mature, membrane-bound form of the α2,6-ST to the lower molecular weight, solubilized form during the purification procedure revealed the sensitivity of this enzyme to proteinases present within purified Golgi preparations. Studies undertaken in this thesis demonstrate that incubation at pH 5.6 of permeabilized Golgi membranes prepared from livers obtained from experimentally-inflamed rats results in the generation of a catalytically-active form of the α2,6-ST that has a molecular weight of about 42,000 Da. Conversion of the mature, membrane-bound form of the α2,6-ST to the solubilized, secretory form following incubation at reduced pH therefore is indeed the result of a proteolytic event. Retention of full biological activity by the released, lower molecular weight enzyme form

allows confirmation that proteolysis involves the removal of a peptide of approximately 7,000 Da that is not part of the catalytic domain.

The proteinase involved in release of the α2,6-ST from rat liver Golgi membranes under conditions of reduced pH has been characterized to be an aspartic proteinase due to the significant inhibitory effect of pepstatin A on enzyme proteolysis. Pepstatin A was found to inhibit in a dose-dependent fashion the release of α2,6-ST from permeabilized Golgi membranes incubated at pH 5.6, with sialyltransferase release being inhibited to a greater extent from 36 hr inflamed-rat Golgi membranes than from control-rat Golgi membranes. Presence in the incubation medium of 10^{-8} M pepstatin A was found to be sufficient to inhibit by approximately 80% the total amount of sialyltransferase activity that had been observed to be released from inflamed-rat Golgi membranes incubated at reduced pH in the presence of the solvent DMSO alone.

Microbial-derived pepstatin A is a pentapeptide that contains two residues of a novel hydroxyamino acid (4S-amino-3S-hydroxy-6-methyl-heptanoic acid) (Umezawa *et al.*, 1970), one of which is responsible for allowing pepstatin A to achieve a tight-binding, one-to-one inhibition of aspartic proteinases due to its ability to mimic an enzyme-bound dipeptide tetrahedral intermediate and therefore act as a transition state analogue (Rich, 1986). Although pepstatin A is effective against renin and pepsin, it is in particular a very potent inhibitor of lysosomal cathepsin D (K_i = 10-10 M) (Knight and Barrett, 1976). The remarkable sensitivity of sialyltransferase proteolysis to pepstatin A indicated that the mechanism of proteinase inhibition was very efficient, and therefore the possibility that the proteinase involved was cathepsin D was probed with the use of antiserum containing antibodies to rat liver lysosomal cathepsin D. The results show that

pretreatment of Golgi membranes with increasing amounts of antiserum produced corresponding concentration-dependent decreases in the amount of sialyltransferase released at reduced pH, which confirmed the cathepsin D-like identity of the proteinase. Moreover, antiserum-induced inhibition of α2,6-ST release was more pronounced with Golgi membranes obtained from experimentally-inflamed rat livers. Participation of a cathepsin D-like proteinase in sialyltransferase proteolysis was additionally affirmed by demonstration of the ability of exogenous bovine spleen cathepsin D to significantly enhance enzyme release in a time-dependent manner from permeabilized Golgi membranes prepared from both control-rat and 36 hr inflamed-rat livers.

Enhanced release of the α2,6-ST from hepatic Golgi membranes during the acute phase response had been hypothesized to be the result of increased interaction between membrane-bound sialyltransferase and incorrectly-targeted nascent lysosomal proteinase, and therefore the Leelavathi (1970) method of Golgi membrane preparation, as modified by Moremen and Touster (1986) was chosen in order to minimize the amount of lysosomal contamination. However, the inadvertent incorporation within the purified Golgi membrane preparation of lysosomal enzymes released from lysosomes damaged during the necessary initial homogenization step cannot be precluded. In order to affirm that the cathepsin D-like proteinase involved in sialyltransferase proteolysis was indeed an endogenous Golgi proteinase, the ability of pepstatin A to inhibit the *in vitro* release of α2,6-ST from rat liver slices was investigated. The results show that incubation of both control-rat and 30-hr inflamed-rat liver slices in the presence of 10^{-4} M pepstatin A caused a significant inhibition in sialyltransferase secretion, with enzyme

release from liver slices obtained from experimentally-inflamed animals being inhibited to a greater extent.

The ability of pepstatin A to influence the secretion of sialyltransferase from rat liver slices revealed that the cathepsin D-like proteinase involved was indeed an endogenous component of the sialyltransferase-containing Golgi compartment. In order to affirm the intraluminal colocalization of these two enzymes within the same Golgi compartment, experiments were conducted in which intact Golgi vesicles were incubated at pH 5.6 in both the presence and the absence of 10^{-6} M pepstatin A. Following incubation, the amount of solubilized sialyltransferase present in the incubation medium was assayed before and after the Golgi vesicles were permeabilized by sonication. The results show that a significantly greater amount of solubilized sialyltransferase was present in the incubation medium after the intact Golgi vesicles had been disrupted by sonication, which confirmed earlier studies undertaken in this thesis to affirm the intraluminal location of the membrane-bound sialyltransferase. The amount of sialyltransferase released from permeabilized Golgi vesicles following incubation at reduced pH in the absence of pepstatin A was found to be only slightly greater than that observed to be released from intact Golgi vesicles incubated under similar conditions in the presence of pepstatin A. The reported slow penetration of lipid bilayers by pepstatin A (Dean, 1977) would preclude this inhibitor from gaining access into the lumen of intact Golgi vesicles within the limited time period of the assay, and therefore presence of pepstatin A in the incubation medium would only inhibit residual cathepsin D that might have been released during resuspension of the purified Golgi membrane pellet or that was exposed on "inside-out" Golgi vesicles. Inability of pepstatin A to substantially influence sialyltransferase proteolysis indicated that the bulk of

the proteinase was inaccessible to this inhibitor, which revealed the intraluminal location of the cathepsin D-like activity.

Direct assay of the endogenous Golgi cathepsin D-like proteinase using bovine hemoglobin as substrate showed that Golgi membranes prepared from rats suffering from turpentine-induced inflammation contained approximately twice the amount of cathepsin D-like activity that was found to be associated with Golgi membranes obtained from control-rat livers. Enhanced release of the α2,6-ST from inflamed-rat Golgi membranes incubated at reduced pH therefore may the composite result of an increased presence of both membrane-bound glycosyltransferase and endogenous proteinase. In order to determine whether the cathepsin D-like proteinase was bound to the luminal face of the Golgi membrane, or was present in a soluble form within the lumen of the Golgi compartment, intact Golgi vesicles were permeabilized by sonication and the amount of cathepsin D-like activity released into the incubation medium was determined. The results show that disruption of intact Golgi vesicles caused only a marginal release of cathepsin D-like proteinase from either control-rat or 36-hr inflamed-rat Golgi membranes, which suggested that this enzyme was membrane-bound. Affirmation of the membrane-bound nature of endogenous Golgi cathepsin D-like activity was afforded by the finding that the bulk of enzyme could be released following treatment with the detergent Triton X-100. Interestingly, incubation of permeabilized Golgi membranes at neutral pH in the presence of mannose 6-phosphate was found to promote a substantial increase in the amount of solubilized cathepsin D-like activity in the incubation medium. This finding indicated that a significant proportion of the endogenous Golgi proteinase was associated with membrane-bound MPR, which suggests that

nascent lysosomal cathepsin D enroute to the lysosome may represent the proteinase responsible for sialyltransferase proteolysis.

The proposed mechanism of release of the membrane-bound α2,6-ST hypothesized that the site of proteolytic cleavage was within an exposed linker region of the molecule that is located between the membrane-binding domain and the catalytic domain. Availability of the complete amino acid sequence of the rat liver Golgi membrane-bound α2,6-ST has shown the presence within the NH_2-terminal region of an intervening stretch of 37 amino acids (residues 27-63) between the putative membrane-binding domain (residues 10-26) and the NH_2-terminus of the purified, solubilized enzyme form (residue 63) (Figure 51) (Paulson *et al.*, 1987; Weinstein *et al.*, 1987), and therefore revealed the existence of a putative linker domain. Characterization of the proteinase involved in sialyltransferase proteolysis at reduced pH to be a cathepsin D-like proteinase, combined with determination that the enzyme form of sialyltransferase released was missing a 7000 Da peptide, inferred that within the vicinity of the NH_2-terminus of the chromatographically-purified, solubilized sialyltransferase was located a preferred cathepsin D-cleavage site. Conversion of the mature, membrane-bound sialyltransferase to the lower molecular weight, solubilized form during the enzyme purification procedure has been reported to be prevented by the addition of inhibitors of thiol and serine proteinases (Weinstein *et al.*, 1987). Therefore, the apparent site of sialyltransferase proteolysis by either thiol or serine proteinases under the near-neutral pH conditions of the chromatographic purification procedure (between residues 63 and 64) would not be expected to be identical to that of sialyltransferase proteolysis by an aspartic proteinase under conditions of acidic pH. Comparison of the amino acid sequences within the vicinity of peptide bonds preferentially cleaved by

either bovine spleen or rat liver cathepsin D has revealed the typical presence of a 7-residue motif (Imoto *et al.*, 1987; van Noort and van der Drift, 1989). Cleavage is between two hydrophobic amino acid residues (P_1 and P_1'), and the cleaved peptide bond is usually preceded by a third hydrophobic residue at the P_2 position. There is an absence of amino acid selectivity for the P_3' and P_4' sites, however either or both the P_2' and P_5' sites is commonly occupied by a charged, preferably basic amino acid residue. Examination of the primary sequence of the mature, membrane-bound α2,6-ST has revealed the presence of two possible cleavage sites, between residues 60 and 61, and between residues 74 and 75, each of which is contained within a 7-residue amino acid sequence that fulfills the statistically-determined specificity requirements of cathepsin D.

The results of experiments undertaken in this thesis that have been discussed above provide confirmation of a proteolytic model, proposed to explain the acute phase behaviour of rat liver Golgi α2,6-ST, which is predicated on the predicted membrane topology of this glycosyltransferase. Availability of the amino acid sequences of GlcNAc β1,4-galactosyl-transferase, Gal α1,3-galactosyltransferase, GlcNAc α1,3-fucosyltransferase, Gal α1,2-fucosyltransferase and Gal α1,3-N-acetylgalactosaminyltransferase determined from cDNA clones obtained from several sources has now revealed that, like the Galβ1,4GlcNAc α2,6-sialyltransferase, each of these terminal glycosyltransferases typically possesses a predicted topology which includes a short NH_2-terminal cytoplasmic tail, a signal-anchor domain and a large COOH-terminal domain that contains the catalytic site (Paulson and Colley, 1989; Narimatsu *et al.*, 1986; Joziasse *et al.*, 1989; Shaper *et al.*, 1986, 1988; Nakazawa *et al.*, 1988; Masri *et al.*, 1988; D'Agostaro *et al.*, 1989). Presence within the luminal domain of each glycosyltransferase of a possible "stem" or

linker region is believed to provide the membrane-bound enzyme with the flexibility to suitably orient its catalytic domain towards the carbohydrate side-chains of the glycoprotein substrate. Proteolytic cleavage of these linker regions would result, as has been shown in this thesis for the rat hepatic Golgi α2,6-ST, in the generation of catalytically-active, solubilized enzyme forms of lower molecular weight.

Size heterogeneity has been reported for not only sialyltransferases, but also for galactosyltransferases, fucosyltransferases, and N-acetylgalactos-aminyltransferases (Schachter and Roseman, 1980; Beyer *et al.*, 1981; Blanken and van den Eijnden, 1985; Elhammer and Kornfeld, 1986; Sugiura *et al.*, 1982), and generation of multiple - M_r, catalytically-active enzyme forms of glycosyltransferases purified from various animal tissues appears to be due to limited proteolysis. Purification of porcine submaxillary Galβ1,3GalNAc α2,3-sialyltransferase has been reported to result in the generation of two catalytically-active enzyme forms of molecular weight 49,000 and 44,000 Da. Loss of the 5000 Da peptide rendered the lower molecular weight form of this α2,3-sialyltransferase incapable of being incorporated into phospholipid vesicles, which inferred the absence of the membrane-binding domain (Sadler *et al.*, 1979; Westcott and Hill, 1985). Availability of the NH_2-terminal sequences of the membrane-bound and solubilized forms of the bovine GlcNAc β1,4-galactosyltransferase has recently provided direct evidence for the presence of a putative luminal linker region of at least 35 amino acid residues that is located between the hydrophobic signal-anchor domain and the catalytic domain (D'Agostaro *et al.*, 1989). Constitutive, selective proteolysis of this presumably exposed luminal linker region of the Golgi membrane-bound GlcNAc β1,4-galactosyltransferase may be the explanation for the slow secretion of a lower molecular weight enzyme form that has been

observed to occur in HeLa cells (Strous and Berger, 1982; Strous *et al.*, 1983). Sensitivity of GlcNAc β1,4-galactosyltransferase to limited proteolysis has been demonstrated by the *in vitro* conversion of the higher molecular weight form of the bovine milk enzyme to a variety of biologically-active, lower molecular weight forms using trypsin or bovine milk plasmin (Powell and Brew, 1974; Magee *et al.*, 1976; Strous, 1986).

Limited proteolysis of ER glucosidase II (Hino and Rothman, 1985) and Golgi mannosidase II (Moremen and Touster, 1986), which has been reported to result in the release of catalytically-active, lower molecular weight enzyme forms, has suggested the presence within these membrane-bound biosynthetic glycosidases of a distinct domain structure similar to that which has been predicted for the Golgi terminal glycosyltransferases. Studies conducted with rat liver Golgi mannosidase II have revealed that the mature, membrane-bound enzyme form of molecular weight 124,000 Da may be proteolytically degraded by trypsin and *Staphylococcus aureus* V8 proteinase to yield a biologically-active, lower molecular weight enzyme form of 115,000 Da. Alternatively, selective proteolysis of the higher molecular weight, membrane-bound form of mannosidase II by chymotrypsin, pronase and proteinase K enzyme was found to generate a 110,000 Da form that not only possessed an undamaged catalytic domain, but also was extremely resistant to further proteolytic degradation. Interestingly, the very same experimental conditions of limited proteolysis using chymotrypsin, which promoted the generation of the catalytically-active, 110,000 Da form of mannosidase II, were found to result in the proteolytic inactivation of membrane-bound GlcNAc β1,4-galactosyltransferase. Studies undertaken in this thesis have as well demonstrated a differential sensitivity of rat liver Golgi membrane-bound biosynthetic enzymes to proteolysis. However, in the case of limited

proteolysis by cathepsin D at pH 5.6, the results show that both the GlcNAcGalβ1,3(4) α2,3-sialyltransferase and the GlcNAc β1,4-galactosyl-transferase remain impervious to the action of this aspartic proteinase under the experimental conditions used for the assay. Lack of sensitivity of these two glycosyltransferases to selective proteolysis by cathepsin D may be speculated to be due to the absence within their putative linker regions of preferred cathepsin D-cleavage sites. Therefore, the preferential release of the catalytic domain of the membrane-bound α2,6-ST during inflammation, which allows this glycosyltransferase to exhibit acute phase behaviour, may in large part simply be the consequence of the fact that within its exposed linker region there exists a peptide bond that is extremely susceptible to cleavage by a cathepsin D-like proteinase under conditions of reduced pH. Although the results of this study demonstrate that of the three liver Golgi glycosyltransferases examined (α2,3-sialyltransferase, α2,6-sialyltransferase and β1,4-galactosyltransferase) only the α2,6-sialyltransferase is sensitive to cathepsin D proteolysis, it would be of interest to examine the sensitivity of additional terminal glycosyltransferases to this proteinase. Results of such experiments may demonstrate that during inflammation the increased presence within acidic Golgi compartments of cathepsin D promotes either the proteolytic inactivation, or the selective release of catalytically-active, lower molecular weight forms, of several membrane-bound biosynthetic enzymes.

The predicted membrane topology of the rat hepatic Golgi α2,6-ST, which includes the absence of a cleavable signal sequence, invites the speculation that, similar to what is believed to occur with other class II membrane proteins (von Heijne and Blomberg, 1979; Inouye and Halegoua, 1980; Engelman and Steitz, 1981; Shaw *et al.*, 1988), translocation of the

nascent α2,6-ST polypeptide involves insertion of the initial portion of the NH_2-terminus as a helical hairpin or loop into the ER such that the extreme end of the NH_2-terminus remains exposed on the cytoplasmic side of the lipid bilayer. Participation of the stretch of hydrophobic amino acid residues (residues 10-26) as both signal sequence and membrane-binding domain allows the generation of a flexible, luminally-oriented COOH-terminus that contains the catalytic site. Presumably, present within either the cytoplasmic tail, the transmembrane domain, the linker region or the catalytic domain is a retention signal that targets this enzyme to the appropriate compartment of the Golgi apparatus. Replacement of the first 57 amino acid residues of the rat hepatic α2,6-ST with the cleavable signal peptide of γ-interferon has been reported by Paulson's group to result in the constitutive secretion of this glycosyltransferase upon expression in CHO cells, which has demonstrated that the Golgi retention signal is not located within the catalytic domain of the enzyme (Colley *et al.*, 1989). In addition, results of preliminary experiments in which the secretion of recombinant forms of the α2,6-ST containing successively truncated NH_2-termini has been examined have suggested that present within the putative linker region is a Golgi retention signal (Colley and Paulson, 1990). These findings have allowed the proposal that following translocation of the entire polypeptide, the nascent enzyme is transported in a soluble form along the mainstream secretory pathway through the ER to the Golgi apparatus, wherein it is caused to be detained in the correct compartment upon interaction with a specific Golgi membrane-bound receptor. Recently, comparative analysis of the primary sequences of several Golgi glycosyltransferases involved in transfer of N-acetylneuraminic acid, galactose and N-acetylgalactosamine has revealed the existence of the common peptide motif (Ser/Thr)-X-(Glu/Gln)-(Arg/Lys) that typically is

located in the vicinity of a more NH_2-terminus Met-Pro sequence (Bendiak, 1990). Although the importance of this peptide motif in localization of glycosyltransferases within the Golgi apparatus remains to be verified, it is of interest to note that in the case of the rat liver α2,6-ST, the peptide sequence Met-Pro-Lys-Ser-Gln-Glu-Lys (residues 44 - 50) occurs approximately within the center of the putative linker region. Existence of Golgi receptors for the membrane-bound glycosyltransferases and glycosidases would ensure the maintenance of strategic compartmentalization of these biosynthetic enzymes that has been observed in numerous cell types. Attachment of a particular glycosyltransferase or glycosidase to a putative Golgi membrane-bound receptor need not necessarily prevent limited proteolysis of the linker region. However, physical presence of a receptor may serve to protect the bound enzyme from selective proteolysis by a specific proteinase should access to a suitable cleavage site within the linker region be sterically precluded.

Elucidation of the mechanism which allows preferential release of the rat hepatic Golgi α2,6-ST into the serum during the acute phase response has served to confirm the domain structure of this membrane-bound glycosyltransferase which has been predicted from primary sequence analysis. Moreover, evidence that during turpentine-induced inflammation there apparently is an increase in a pH-dependent interaction between a Golgi membrane-bound, biosynthetic glycosyltransferase and a catalytically-active proteinase which appears to be a mistargeted lysosomal enzyme has provided insight into the altered cell biology of the acute phase hepatocyte of the rat. In order to affirm the inter-species nature of the phenomenon of the acute phase response of the Galβ1,4GlcNAc α2,6-sialyltransferase, studies were undertaken in this thesis to investigate the effect of turpentine-induced inflammation on this glycosyltransferase in the mouse and the guinea pig.

The experimental design used in this comparative study incorporated first the characterization of the acute phase behaviour of sialyltransferase in these two species, following which the participation of a cathepsin D-like proteinase in sialyltransferase proteolysis was examined using procedures that had been developed during the investigation of the mechanism of release of the hepatic Golgi α2,6-ST in the rat.

The results show that the liver Golgi Galβ1,4GlcNAc α2,6-sialyltransferase is an acute phase reactant in both the mouse and the guinea pig, with maximum enzyme secretion occurring 72 hr and 96 hr, respectively, following the injection of turpentine. The acute phase behaviour of the α2,6-ST in both species was found to be predicated on the sensitivity of the membrane-bound enzyme to proteolysis at reduced pH. Maximum release of mouse and guinea pig sialyltransferase from permeabilized Golgi membranes was found to occur at pH 4.6 and 5.2, respectively. Immunoblot analysis of SDS gels revealed that the membrane-bound enzyme forms of mouse and guinea pig α2,6-ST have molecular weights of 49,000 and 42,000 Da, respectively. In contrast, the solubilized enzyme forms of mouse and guinea pig α2,6-ST were found to have a similar molecular weight of 38,000 Da, which demonstrates that generation of the catalytically-active, lower molecular weight forms of these sialyltransferases by proteolysis at reduced pH involves removal from the mouse and guinea pig membrane-bound enzyme of 11,000 and 4,000 Da peptides, respectively. Release of mouse and guinea pig sialyltransferase from liver Golgi membranes at reduced pH was significantly inhibited by pepstatin A, and promoted by exogenous bovine spleen cathepsin D. Moreover, the cathepsin D-like proteinase responsible for proteolysis of mouse and guinea pig α2,6-ST was found to be associated with membrane-bound MPR, which suggests that it is the lysosomal enzyme.

Exposure of the rat, mouse and guinea pig liver Golgi membrane-bound Galβ1,4GlcNAc α2,6-sialyltransferase to catalytically-active cathepsin D-like proteinase, which explains the acute phase behaviour of this glycosyltransferase, is an intriguing phenomenon in terms of the cell biology of the acute phase hepatocyte. Ability of an aspartic proteinase of low pH optimum to be catalytically-active within the sialyltransferase-containing compartment presupposes the presence of an acidic luminal pH, and this has been affirmed by studies undertaken in this thesis on the effect of lysosomotropic agents on sialyltransferase secretion from rat liver slices. Moreover, analysis of the pH profiles of sialyltransferase release from rat, mouse and guinea pig liver Golgi membranes suggests that substantial proteolysis of this membrane-bound glycosyltransferase would require that the pH of the sialyltransferase-containing compartment be significantly less than pH 6 - 6.5, which is believed to be the luminal pH of the acidic regions of the *trans* Golgi and the TGN under normal conditions (Griffiths and Simons, 1986). Maintenance of low pH within acidic regions of the Golgi apparatus has been shown to be due to the presence of ATP-dependent H^+ pumps (Glickman *et al.*, 1983; Zhang and Schneider, 1983; Moriyama and Nelson, 1989), and therefore it may be speculated that enhanced proteolysis of membrane-bound sialyltransferase by cathepsin D-like proteinase during the acute phase response is facilitated by an inflammation-induced increase in the activity of the Golgi membrane H^+-translocating ATPase. Results of preliminary experiments conducted to determine the effect of exogenous ATP on sialyltransferase proteolysis have revealed that, at low concentrations of nucleotide, comparatively greater incremental increases in sialyltransferase proteolysis were observed to take place within control-rat intact Golgi vesicles incubated at neutral pH for short periods of time than within 36 hr inflamed-

rat intact Golgi vesicles under the same experimental conditions. These data suggest that, at the start of experimentation, the luminal pH of control-rat intact Golgi vesicles was higher than that of Golgi vesicles prepared from experimentally-inflamed rats. Activity of Golgi membrane ATP-dependent H^+ pumps notwithstanding, a decrease in luminal pH of the sialyltransferase-containing compartment during inflammation may be speculated to be an unavoidable consequence of an increased presence of both CMP-NeuAc and sialylated glycoproteins. Should the number of sialylated molecules within the sialyltransferase-containing compartment be itself primarily responsible for inducing transient reductions in luminal pH, then it would be expected that during inflammation a time-dependent increase in local volume of sialylated glycoprotein secretory traffic would be accompanied by a corresponding time-dependent decrease in luminal pH. Procedures have been established for the use of fluorescent lysosomotropic agents such as acridine orange to determine the luminal pH of various intracellular compartments (Gluck *et al.*, 1982; Adelsberg *et al.*, 1989), and it would be of interest to apply similar experimental protocols to assess the luminal pH of intact Golgi vesicles prepared from animals that had been suffering from inflammation for different time intervals. The differences in the time following the onset of inflammation at which, for example, rat and mouse serum sialyltransferase levels have been observed to reach a maximum, perhaps may be correlated to the time required for the luminal pH of the sialyltransferase-containing compartments to reach the values of pH required by the respective endogenous cathepsin D-like proteinases to achieve maximum activity.

Evidence that turpentine-induced inflammation promotes an increase in endogenous Golgi cathepsin D-like proteinase activities in the rat, mouse

and guinea pig, combined with the finding that a significant amount of this proteinase activity appears to be associated with MPRs, suggests that enhanced sialyltransferase proteolysis during the acute phase response is due to an increased presence within the sialyltransferase-containing compartments of the Golgi apparatus of nascent lysosomal cathepsin D. Studies on the biosynthesis of cathepsin D in porcine spleen and cultured human fibroblasts have demonstrated that this lysosomal enzyme is synthesized as a large molecular weight prepropolypeptide. Cleavage of the signal propeptide occurs upon completion of translocation into the ER, and removal of the propeptide by cysteine proteinases is believed to take place within the acidic environment of the mature lysosome (Erickson and Blobel, 1979; Erickson *et al.*, 1981; Giselmann *et al.*, 1985). Retention of the activation propeptide, which physically blocks the active site, precludes enzyme activity of newly-synthesized cathepsin D during its transport from the ER along the biosynthetic lysosomal pathway to the lysosome (Tang and Wong, 1987). However, evidence that procathepsin D is capable of autocatalysis upon incubation at acidic pH (Hasilik *et al.*, 1982; Capony *et al.*, 1987), allows the possibility that premature release of lysosomal procathepsin D from MPRs and activation of the proenzyme may occur during inflammation within the apparently abnormally acidic sialyltransferase-containing compartments of the Golgi apparatus.

In conclusion, data reported in this thesis strongly support the ideas expressed in the model presented in Figure 11. The acute phase behaviour of the liver Golgi Galβ1,4GlcNAc α2,6-sialyltransferase in the rat, mouse and guinea pig is clearly the result of a pH-dependent, proteolytic event which is catalyzed by a cathepsin D-like proteinase and which generates a catalytically-active, lower molecular weight sialyltransferase that is released into the

extracellular space. Although evidence suggests that lysosomal cathepsin D is responsible for cleavage of this membrane-bound sialyltransferase, it remains to be clarified if presence of catalytically-active cathepsin D in the sialyltransferase-containing Golgi compartment is due to an error in intracellular lysosomal enzyme trafficking and/or an abnormal, inflammation-induced reduction in intraluminal pH. Alternatively, during the acute phase state there may occur an error in intracellular targeting of the α2,6-ST which causes it to be present in significant amounts within acidic, endosomal compartments. Although there are many unanswered questions, it is hoped that future work on cathepsin D and α2,6-ST targeting within the central vacuolar system during the acute phase response may throw light on this intriguing problem. There is also the question of the biological function during inflammation of a high plasma concentration of the Galβ1,4GlcNAc α2,6-sialyltransferase, but this is beyond the scope of this thesis and is clearly a subject for future study.

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