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BIOCHEMICAL AND GENETIC STUDIES OF FUCOSYL- AND
SIALYLTRANSFERASES

City University of New York

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Biochemical and Genetic Studies of
Fucosyl- and Sialyltransferases

by

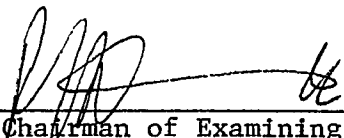
Rita Margolies

A dissertation submitted to the Graduate Faculty
in Biomedical Sciences in partial fulfillment of
the requirements for the degree of Doctor of
Philosophy, The City University of New York.

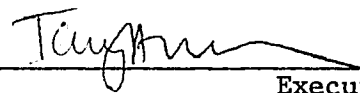
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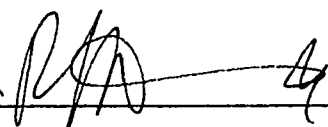
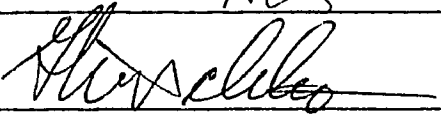
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ABSTRACT

BIOCHEMICAL AND GENETIC STUDIES OF
FUCOSYL- AND SIALYLTRANSFERASES

by

Rita Margolies

Advisor: Robert J. Desnick, M.D., Ph.D.

A major result of this study is the first report of $\alpha 1 \rightarrow 6$ fucosyltransferase in a tissue of human origin. This enzyme was characterized in human lymphoid lines and detected in human skin fibroblasts, kidney, spleen, adult and fetal intestine, and liver. An optimized assay method was developed that used asialo, agalactofetuin as acceptor. Lymphoid line $\alpha 1 \rightarrow 6$ fucosyltransferase had a pH optimum of 7.0 and was stimulated by Mg^{2+} and Ca^{2+} in the presence of Triton X-100. Apparent K_m 's for GDP-fucose and asialo, agalactofetuin were 66.6 μM and 0.24 mM, respectively.

Optimized assays for lymphoid line $\alpha 1 \rightarrow 2$ fucosyltransferase and sialyltransferase were also developed. $\alpha 1 \rightarrow 2$ fucosyltransferase had a pH optimum of between 6.5 and 7.0 when HEPES buffer was used. This enzyme was stimulated slightly by Mg^{2+} and Ca^{2+} and had an apparent K_m of 7.7 μM , for GDP-fucose and 0.326 mM for the acceptor, asialofetuin.

Sialyltransferase was assayed using asialofetuin as an acceptor. The enzyme had a strict requirement for divalent metal ion, 10 mM Mg^{2+} being optimal; however, enzyme activity also appeared to be stimulated by 20 mM EDTA and EGTA. It had an apparent K_m of 25 μM for CMP-sialic acid and a pH optimum of 6.

All three enzymes were membrane bound. When lymphoid line crude membrane preparations were treated with a combination of 0.3% deoxycholate

and 0.1% Triton X-100, over 90% of fucosyltransferase activity was released into the soluble phase. Detergent treatment also activated these enzymes to a considerable extent.

None of the detergents tested in this study, including Triton X-100, deoxycholate, Brij 35, Cutscum, and octylglucoside were able to solubilize sialyltransferase activity, and with the exception of Cutscum and octylglucoside, detergent treatment resulted in inhibition of enzyme activity.

The differential effects of sulfhydryl reagents, including mercaptoethanol, dithiothreitol, N-ethylmaleimide, iodoacetate, glutathione (reduced), and p-chloromercuribenzoate on fucosyl- and sialyltransferases from lymphoid lines, fibroblasts, and serum were examined. $\alpha 1 \rightarrow 2$ fucosyltransferase from all three sources was generally stimulated by sulfhydryl reagents with the exception that the serum enzyme was inhibited by N-ethylmaleimide. $\alpha 1 \rightarrow 6$ fucosyltransferase and sialyltransferase were unaffected or inhibited by sulfhydryl reagents. para-Chloromercuribenzoate was inhibitory to all three enzymes.

Dithiothreitol almost totally inhibited lymphoid lines $\alpha 1 \rightarrow 6$ fucosyltransferase while stimulating the $\alpha 1 \rightarrow 2$ enzyme twofold. This provides a way of distinguishing between these two cellular enzymes.

In light of proposals that cystic fibrosis is the result of abnormal glycosylation mechanisms, the fucosyl- and sialyltransferases were examined in lymphoid lines derived from obligate heterozygotes and homozygotes and compared with those from normal individuals with respect to enzyme activities and their ratios. No differences among the groups could be detected. Fibroblasts from patients suffering from cystic fibrosis were also examined, and again no differences could be detected.

These enzymes were also assayed in lymphoid lines from individuals with a variety of metabolic defects, including fucosidosis, cystathioninuria, homocystinuria, hypercholesterolemia, and Sanfillipo syndrome. No striking differences were found with respect to enzyme activities or their ratios.

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-1-
INTRODUCTION

The study of glycoprotein structure, function, and biosynthesis is a relatively new area of endeavour. Although the first recognition of mucus as an entity distinct from other types of proteins took place in 1805, it was not until 1962 (1) that sufficient chemical and structural data were accumulated to allow an acceptable definition of the term "glycoproteins." The definition formulated by Gottschalk reads: "...conjugated proteins containing as prosthetic group(s) one or more heterosaccharides, usually branched with a relatively low number of sugar residues, lacking a serially repeating unit and bound covalently to the polypeptide chain." From this definition, glycoproteins are clearly contrasted with mucopolysaccharides or proteoglycans such as hyaluronic acid, chondroitin sulfate, heparin, chitin, etc., all of which have linear repeating heterosaccharide moieties and high carbohydrate content.

Glycoproteins are found in almost all extra- and intracellular fluids and in cell membranes. Tables 1 and 2 provide representative lists of glycoproteins from various sources. They appear to have diverse functions ranging from physical protection of mucous membranes to molecular recognition and mediation of cell-cell adhesion.

Work carried out in the last 10-15 years has elucidated the nature of the glycopeptide bond, the structure of oligosaccharide moieties, biosynthetic mechanisms, especially the involvement of intracellular membranes, and the physiological role of the oligosaccharide moiety.

The first half of this introduction briefly describes the most important aspects of this work; the second half deals specifically with fucosylation and sialylation of glycoproteins, particularly as it relates to disease states.

TABLE 1
Glycoproteins that Contain the N-linked
Glycopeptide Bond^a

Human IgE, IgG, IgA, and IgM	Bovine RNase
Human chorionic gonadotropin	Porcine RNase
Human transferrin	Hen ovalbumin
Human ceruloplasm	Rat α -lactalbumin
Human, bovine, and porcine thyroglobulin	Bovine and mouse IgG
Human α_2 macroglobulin	Human lactoferrin
Human α_1 acid glycoprotein	Bovine fetuin
Bovine visual pigment	

^aFrom Ref. 8

TABLE 2
Glycoproteins that Contain the O-Linked
Glycopeptide Bond^a

Submaxillary mucins, ovine, bovine, murine, porcine,
human, canine

Antifreeze glycoprotein of Antarctic fish

Human IgA, IgG, and IgM

β -subunit of human chorionic gonadotropin

Fetuin

Human gastric mucin

Core region of human and hog blood group
substances

^aFrom Ref. 8

I. Glycoprotein Chemistry

Glycoproteins are divided into two principal types depending on the carbohydrate to amino acid linkages. The first type to be discussed is the N-acetylglucosaminy-l-asparagine linkage, which is generally found in serum proteins; the other type is the N-acetylgalactosaminy-l-serine (threonine) linkage found in mucins and the blood group substances.

A. The N-Linked Glycopeptide Bond

This linkage was first established in ovalbumin in 1961 by Marshall, Neuberger, and co-workers (2-4) and by several others. Since that time many other glycoproteins (see Table 1 for a representative list) have been shown to contain this linkage.

This type of glycopeptide bond is characterized by alkali stability, and the heterosaccharides associated with it contain the common core region shown in Fig. 1, made up of N-acetyl chitobiose and three mannose residues. N-acetylgalactosamine is never found in these oligosaccharides. Figure 2 shows the two sorts of heterosaccharides associated with this linkage, the high-mannose type, which contains the core region and up to eight additional mannoses, and the complex type, which contains additional N-acetylglucosamine and galactose and possibly fucose and/or sialic acid.

B. The O-Linked Glycopeptide Bond

This bond is an alkali-labile, O-glycosidic linkage between the proximal N-acetylgalactosamine and the β -hydroxyamino acids serine and threonine; the carbohydrate chain associated with this linkage is characterized by the absence of mannosyl residues and by structural microheterogeneity. The structure of a typical O-glycosidically linked chain is shown in Fig. 3.

While this bond is characteristically found in mucins, the blood

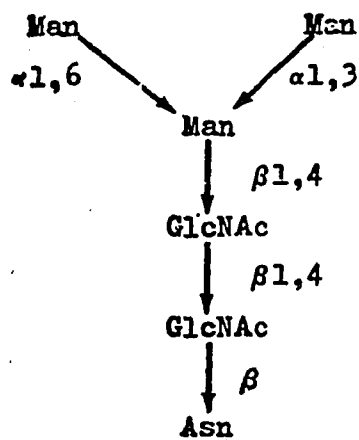
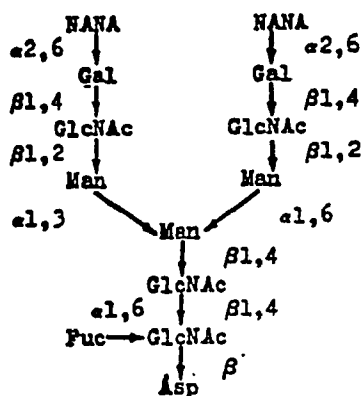
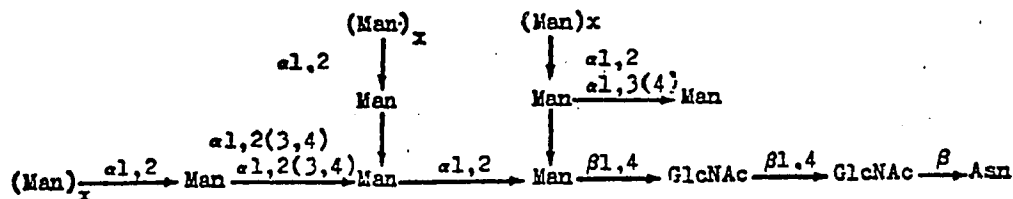


Fig. 1. Core region of N-glycosidically linked oligosaccharides. Abbreviations: Asn, asparagine; GlcNAc, N-acetylglucosamine; Man, mannose.

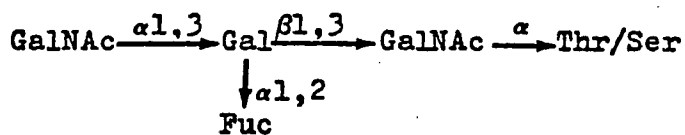


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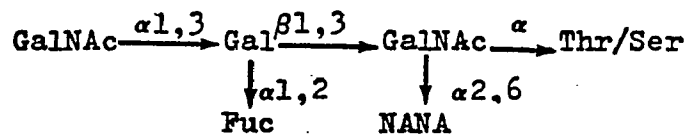


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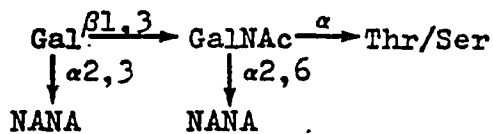
Fig. 2. Structures of N-glycosidically linked glycopeptides. (a) structure proposed for human IgG, IgA, IgE glycopeptides (9-11); this structure is an example of the complex type of oligosaccharide. (b) Glycopeptide derived from thyroglobulin (12) is an example of the high mannose type oligosaccharide. Abbreviations: Man, mannose; Gal, galactose; Fuc, fucose; NANA, sialic acid; Asn, asparagine.



(a)



(b)



(c)

Fig. 3. Typical O-glycosidically linked oligosaccharides from (a) and (b) A⁺ porcine submaxillary mucin (13), and (c) fetuin (6). Abbreviations: Thr, threonine; Ser, serine; GalNAc, N-acetylgalactosamine; Gal, galactose; Fuc, fucose; NANA, sialic acid.

group substances, and cell surface glycoproteins, a number of glycoproteins usually considered to be serum glycoproteins, such as IgG (5), fetuin (6), and thyroglobulin (7), have been shown to contain both sorts of linkages. Table 2 contains a list of glycoproteins that contain this linkage.

An O-glycosidic bond between xylose and threonine occurs in proteoglycans of connective tissue, such as chondroitin sulfate, heparin, etc.

II. Biosynthesis of Glycoproteins

Although the area of glycoprotein biosynthesis was almost entirely unexplored until 15 years ago, the basic outline, particularly for the N-linked glycoproteins, has now been established and confirmed by a number of laboratories using techniques borrowed from many areas of biochemistry.

The following sections describe the biosynthesis of glycoproteins in order that the reader can become familiar with these most recent developments and see where the fucosyl- and sialyltransferases fit into the overall biosynthetic scheme.

The synthesis of the protein backbone of both secretory (14) and transmembrane glycoproteins (15-17) begins on free ribosomes, which then most likely bind to the rough endoplasmic reticulum (RER). As the nascent protein elongates, it presumably traverses the RER membrane into the cisternae (16-19), where glycosylation is initiated (14-17) for the N-linked proteins, and then moves through the smooth endoplasmic reticulum (SER) to the golgi apparatus where glycosylation is completed.

A. Synthesis and Attachment of the Core Region-N-Glycopeptide Bond Formation

Synthesis and attachment of the core region takes place in the RER

(14-17) by the pathways shown in Fig. 4. This is a summary of the work of many laboratories (20). Its most important features are as follow:

(a) Synthesis of the entire core region takes place on dolichol phosphate. The completed oligosaccharide is then transferred en toto to the protein backbone by a peptididyl-N-acetylglucosaminyl transferase. (b) The inclusion of glucose in the lipid-linked intermediate; although this sugar is never found in the completed glycoprotein, it has been shown that glucose-containing oligosaccharides are preferentially transferred. (c) Mannose is almost exclusively transferred to the dolichol-carbohydrate intermediate in the form of dolichol-P-P-mannose. (d) Excess mannose residues are added to the dolichol intermediate. After it is transferred to the protein backbone, the oligosaccharide chain is processed to remove the extra mannose and glucose.

The possible function of core glycosylation is discussed in Sec.

III.A.

B. O-Glycopeptide Bond Formation

The synthesis of O-glycosidically linked oligosaccharides appears to proceed by sequential transfer of sugars from sugar nucleotides to the growing oligosaccharide chains. The reactions are catalyzed by a series of enzymes that are part of what has been called the multiglycosyltransferase system (21). Each enzyme utilizes the product of the preceding enzyme as its substrate, which in turn generates the substrate for the next enzyme.

N-acetylgalactosamine is the first monosaccharide residue transferred during the biosynthesis of the O-type glycoprotein. Hagopian and Eylar (22) have shown that in bovine submaxillary gland the transferase catalyzing this reaction is highly specific, requiring intact deglyco-

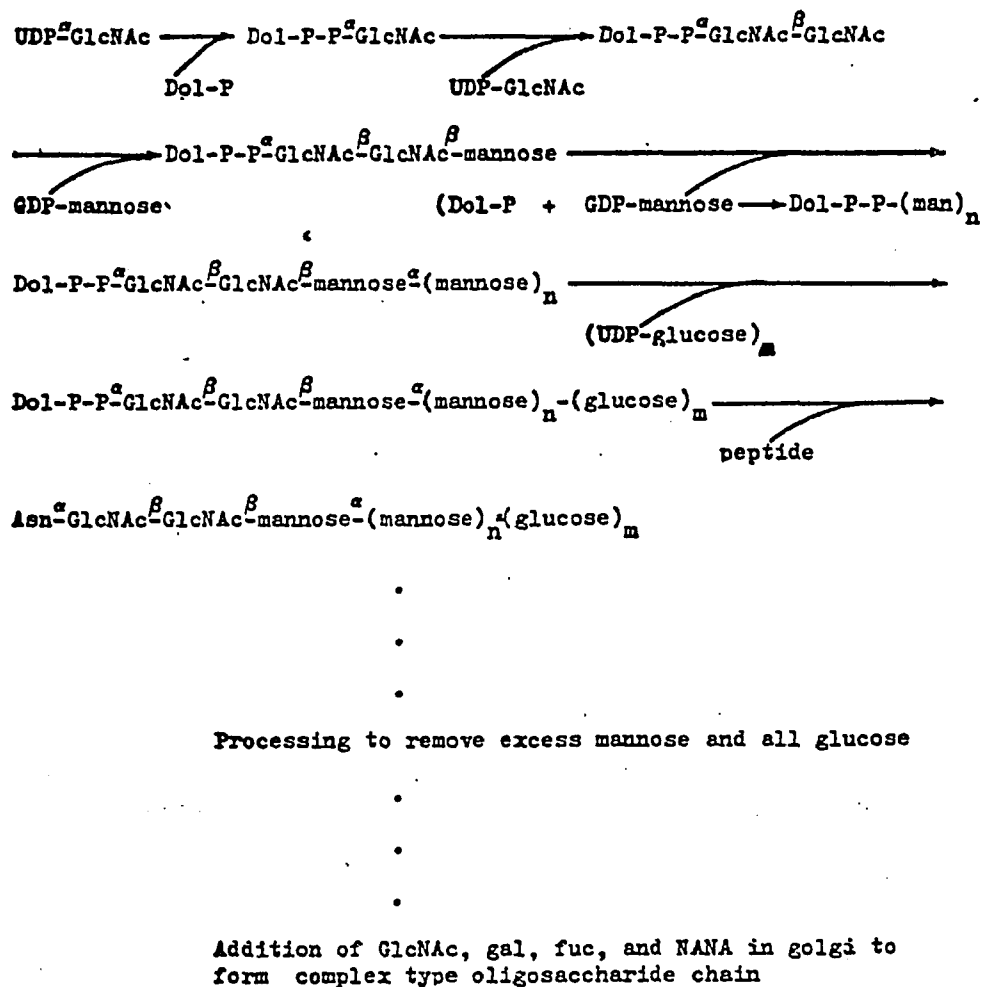


Fig. 4. Biosynthesis of N-linked glycopeptide via lipid-linked intermediate.

sylated bovine submaxillary gland mucin as the glycoprotein acceptor. Neither the hydroxyamino acids, serine and threonine, nor small peptides composed of these amino acids could act as acceptors. The strict specificity of this enzyme would be expected since the attachment of the first sugar determines the location of the oligosaccharide on the protein. The amino acid sequence of the recognition site on the protein backbone contains a number of prolines in the immediate vicinity of the serine or threonine to which the carbohydrate chain is attached (23,24). The most recent evidence indicates that polypeptididyl-galNAc transferase is localized in membranes derived from smooth endoplasmic reticulum and golgi apparatus (25). This is in distinct contrast to the site of N-glycosylation which has been shown to be in the rough endoplasmic reticulum.

C. Role of the Golgi Apparatus in Glycosylation

The golgi apparatus has been shown by many investigators to be the site of incorporation of sialic acid, fucose, galactose, and peripheral N-acetylglucosamine. Schachter and co-workers (26-28) and Fleisher and Fleisher (29) have demonstrated that golgi fractions contain glycosyltransferases capable of transferring each of these sugars to glycoprotein acceptors. Ehrenrieck et al. (30) report a 70% recovery of N-acetylglucosamine; galactosyltransferase from golgi. The subcellular location of these enzymes is now so well accepted that galactosyl- and sialyltransferases have been used as marker enzymes for golgi membranes (31).

III. Function of Glycosylation

The functions of the oligosaccharide moieties are quite diverse. They appear to play an important role in the movement of secretory and transmembrane proteins from the RER to smooth membrane organelles.

Additionally, they can mediate hormone-cell interactions, uptake of serum proteins, removal of senescent erythrocytes by the liver, and formation of hematopoietic spleen colonies.

A. Role of the Core Region of N-Linked Oligosaccharides

It has been postulated that glycosylation plays a role in the secretory process, but until recently there was little evidence for this. However, recent reports indicate that the core region of N-linked oligosaccharides is required for the secretion of many glycoproteins and for the appearance of transmembrane proteins on the cell surface.

Hickman et al. (32) studied the role of core glycosylation in the secretion of IgA and IgE by two mouse plasmacytoma cell lines. Tunicamycin, an antiviral agent that inhibits the first reaction in the formation of the lipid-linked intermediate, was used at a dosage level that inhibited glycosylation of IgA by more than 90% and overall protein synthesis by only 13%; secretion was inhibited by 85%. IgE secretion was also completely inhibited; however, in this case protein synthesis was inhibited by 40%. Experiments using cell-free systems derived from these cell lines indicate that no selective inhibition of immunoglobulin protein synthesis takes place. Additionally, secretion of light chains, which are not glycosylated, takes place normally in the presence of tunicamycin, indicating that it is not a general inhibitor of secretion. It has been demonstrated in other systems, such as yeast and chick embryo fibroblasts (33) that lack of secretion is due to increased intracellular degradation of nonglycosylated proteins; however, this does not appear to be the case in these cell lines. When the tunicamycin-treated cells are studied by electron microscopy, marked dilations of the RER are seen. Direct immunofluorescence studies indicate that these dilated cisternae

contain IgA. It would appear then that the lack of secretion is due solely to the inability of nonglycosylated IgA to leave the RER.

When cells infected with enveloped viruses such as VSV are grown in the presence of tunicamycin, viral replication is inhibited. All viral proteins appear to be made in normal amounts; however as would be expected from the known mechanism of tunicamycin, the glycoproteins are devoid of carbohydrate. Leavitt et al. (34) grew cultures infected with VSV or Sindbis virus in the presence of tunicamycin; using a variety of techniques they could not detect nonglycosylated proteins on the cell surface, indicating that the lack of carbohydrate inhibits the migration of transmembrane proteins to the plasma membrane. Large amounts of these nonglycosylated proteins were found in the interior of the cells and it was noted that their solubility properties were greatly altered.

Ciccimarra et al. (35) describe a defect in IgG glycosylation in patients with common, variable agammaglobulinemia. The patients' secretory block was associated with failure to glycosylate the IgG heavy chain although other proteins incorporated normal amounts of mannose and glucosamine. Waring (36) also has reported finding five nonsecreting variant myeloma lines. In each case the heavy chain was synthesized but was devoid of carbohydrate.

Hercz et al. (34) have reported an important finding in patients with the ZZ variant of α_1 -antitrypsin. In these patients, who have severe liver and pulmonary dysfunction, α_1 -antitrypsin accumulates in the RER of the liver and only 10-20% of normal levels are found in the serum. Normal α_1 -antitrypsin contains four oligosaccharide chains, all of the N-linked type, while the ZZ variant has been found to have only three. The work of this group indicates that there is an amino acid substitution

for one of the asparagines, thereby allowing for the attachment of only three chains. The oligosaccharide chains exhibit an amount of mannose and glucosamine consistent with normal core glycosylation and processing (H. Schachter, private communication, 1978), but there is an absence of all distal sugars. The lack of the fourth chain appears to prevent the transfer of ZZ α_1 -antitrypsin to the SER and golgi, where these sugars would normally be attached. That all aspects of the transfer mechanism are most likely fully functional is indicated by the fact that no other glycoproteins appear to accumulate in the livers of these patients.

The recognition site for the attachment of the completed core appears to be the amino acid sequence Asn-X-Ser (Thr), where the X can be any amino acid (23,38). The importance of this sequence in N-glycosylation is revealed when a statistical analysis of its occurrence in sequenced proteins is undertaken (39). In prokaryotes, which have very few N-linked carbohydrates, the frequency of this sequence is essentially random. This is true as well in eukaryotic intracellular, soluble proteins, which also are rarely, if ever, glycosylated. However, in extracellular proteins, the frequency of such sequences is considerably lower than would be expected from amino acid composition. This would indicate evolutionary pressure toward conservation of the sites of glycosylation.

From these studies, it may tentatively be concluded that the oligosaccharide moieties play a crucial role in transferring newly made glycoproteins from RER down the biosynthetic pathway to their final destination. Exactly why this is so is the subject of a great deal of speculation. There is some indication from the experiments of Kornfeld's group that altered solubility and aggregation of the proteins make transfer of these proteins very difficult. Recent crystallographic evidence from

Huber et al. (40) indicates that the carbohydrate units cover apolar amino acid residues, which may be necessary for stabilization of the proper conformation of a soluble protein and for prevention of adverse protein-protein and membrane-protein interactions. Another indication for the correctness of this hypothesis is the finding by Kornfeld of a VSV mutant that replicates in the presence of tunicamycin. When the mutant G protein was isolated, it was found to be larger than authentic G protein and to contain more hydrophilic residues, and it was totally unglycosylated (41).

B. Role of Terminal Sugars

The terminal sugars of the oligosaccharide moiety appear to play a role in directing various glycoproteins and cells to target organs. Several laboratories have studied carbohydrate recognition systems that mediate turnover of glycoproteins (42) and erythrocytes (43).

The best-studied system is the clearance of asialoglycoproteins from the plasma by hepatocytes (42). This system was first described by Morell et al. in 1971 and appears to be a general phenomenon in many mammalian species. When asialoglycoproteins, such as asialoorosomucoid or asialoceruloplasm, are injected into a rat, they are cleared in a matter of minutes, whereas the intact glycoprotein survives for several days. The asialoglycoprotein is recognized and bound by a receptor on the hepatocyte plasma membrane and is then phagocytized and catabolized by hepatic lysosomes.

The penultimate galactose residue, exposed by removal of the neuraminic acid, is the specific recognition signal for binding. Galactosidase treatment of the ASGP abolishes binding by the hepatic receptor. Neuraminidase treatment of the receptor protein, which has now been purified

to homogeneity, also abolishes binding (44). This provides a very strong indication that this phenomenon is mediated by carbohydrate-carbohydrate interactions.

The level of circulating ASGP's in normal individuals is quite low, whereas in patients with hepatic cirrhosis or hepatitis, the values are two to three times higher, although they are unchanged in other nonhepatic pathologies (45). This indicates that hepatic binding and uptake is a normal physiological mechanism that regulates circulating plasma glycoprotein levels.

Several laboratories (46-48) have been studying another clearance system which depends on the removal of both sialic acid and galactose from lysosomal hydrolases. These enzymes, almost all of which are glycoproteins, are found intracellularly, with small amounts found in plasma and intercellular fluids. Highly purified glycosidases from tissue sources are rapidly cleared from the circulation after injection. This clearance is inhibited when asialo, agalactoorosomucoid is injected along with the glycosidases, indicating that terminal N-acetylglucosamine acts as the recognition site for uptake of lysosomal hydrolases from plasma.

Uptake of lysosomal hydrolases by fibroblasts was first reported by Neufeld's laboratory (49). She reported that when fibroblasts derived from patients with two different mucopolysaccharide storage diseases are cultured together, correction of both defects is seen. The uptake of these enzymes by fibroblasts displays a selectivity and saturability expected for a receptor-mediated process.

Kaplan et al. (50) studied this phenomenon further, using platelet beta-glucuronidase as their enzyme source. They were able to distinguish two forms of the enzyme: a high-uptake form which was highly acidic and

whose uptake appeared to be receptor mediated and a low-uptake form which appeared to be taken up by normal fluid pinocytotic mechanisms only. Once the high-uptake form entered the cell, it was converted into a low uptake form. Uptake activity was inhibited by mannose-6-phosphate, fructose-1-phosphate, phosphorylated yeast mannans, and other platelet glycoproteins (which are predominantly high-uptake forms). Of the commonly occurring monosaccharides, only mannose, fucose, and alpha-methylmannoside inhibited; however, concentrations 100 times those of the phosphorylated compounds were required for inhibition. When the high-uptake form of the beta-glucuronidase was treated with alkaline phosphatase, 85% of the high uptake activity was destroyed; enzyme activity was unaffected. Thus it is suggested that high uptake is specifically mediated by a phosphohexose, which is probably mannose-6-phosphate. The inhibition of receptor-mediated uptake by mannose-6-phosphate and yeast mannans points to a possible role for the high-mannose chains.

The physiologic significance of fucose residues on glycoprotein chains is largely unexplored. Discussion of this subject is postponed until Sec. VI. B so that the reader can become better acquainted with the biosynthetic mechanisms of fucosylation.

IV. Glycosyltransferases

The sequences of sugar residues in glycoproteins, oligosaccharides, glycolipids, and mucopolysaccharides are for the most part genetically determined. They are quite reproducible and characteristic of the specific macromolecule.

Biosynthesis proceeds in a stepwise manner, each sugar residue being transferred sequentially from a sugar nucleotide donor to a suitable acceptor. The sequence of these transfers is determined by a series of

glycosyltransferases that have a very high specificity for both the sugar donor and for an amino acid, lipid, or sugar residue that serves as the attachment point for the donor sugar.

Each time another sugar residue is added, the macromolecule then becomes a specific acceptor for the next glycosyltransferase. This process continues until there is no enzyme capable of transferring a sugar residue to the newly synthesized chain. In this way the sequence of sugars in a carbohydrate chain elaborated by a cell is a consequence of the genetic information within that cell, i.e., the presence or absence of glycosyltransferases of differing acceptor and donor sugar specificities.

A. Subcellular Location

Cellular glycosyltransferases are found in a number of subcellular fractions: RER and golgi, as discussed in Sec. II, and there are reports that they are associated with mitochondria (51,52) and the cell surface (53). In the organelles, they are integral membrane proteins, requiring detergent treatment for their solubilization. The existence of cell surface glycosyltransferases is questionable, however, since very few experiments have been done in such a way that all the necessary criteria are satisfied. Studies using intact cells are often open to question because cell viability, plasma membrane integrity and intactness of the sugar donor have not been unequivocally determined. Subcellular fractionation studies have always been in doubt because of the lack of purity of the various fractions.

Many glycosyltransferases are also found in body fluids such as milk, seminal fluid, saliva, plasma, cervical mucus, etc. These enzymes differ from the cellular enzymes in that they are water soluble.

There are indications that both forms, membrane-bound and soluble, of a given transferase are products of the same gene. Firstly, the enzymes responsible for the synthesis of ABH antigens are always present in concert. For example, in Type A donors, the enzyme responsible for synthesis of A antigen, α -D-N-acetylgalactosaminyl transferase is always present in tissue, plasma, and secretions (if the donor is a secretor), but is never found in type B and O donors. Population studies have demonstrated unequivocally that the gene associated with synthesis of A antigen on the erythrocyte resides at a single locus; if the soluble forms are synthesized from a gene at another locus, donors of other blood types would be expected to have this enzyme, and the resulting antigen, in their sera and secretions. Secondly, there is evidence that the soluble form of the enzyme is simply a proteolytic fragment of the membrane bound form. Several laboratories report finding sialyltransferases in two forms, differing in either molecular weight or glycosylation, but having very similar kinetic properties and substrate specificities (54,55). Rearick et al. (55) have purified β -D-galactoside $\alpha 2 \rightarrow 3$ sialyltransferase from porcine submaxillary gland and found two molecular weight forms after gel filtration on Sephadex G-200. The enzymatic properties of both enzymes are very similar and the acceptor specificities are also the same. However, the higher molecular weight form binds 1.18 grams of Triton per gram of protein, while the smaller form binds none. Thus it seems that these enzymes are identical with respect to catalytic properties, but not detergent binding. These findings support the conclusion that the larger form may represent the native, membrane-bound enzyme containing the intact membrane binding site, while the smaller form represents a large proteolytic fragment of the native enzyme.

B. Regulation of Glycosyltransferases

All cells will at some time in their life cycle elaborate glycoproteins and glycolipids; the structure of the carbohydrate moieties will be determined by (a) which enzymes are present (genotype), (b) their absolute amounts, and (c) their relative activities. The acceptor molecules elaborated by a given cell also influences the structure of the oligosaccharide chain.

Four major factors determine the kinds and absolute amounts of glycosyltransferases in a given cell: (a) genetic variation, (b) developmental changes, (c) hormone effects, and (d) transformation and possibly other pathological states. In vivo, activity is also regulated by the absolute levels of nucleotide sugars within the cell.

1. Genetic Variation

The biosynthesis of the blood group substances provides the best example of how genetic variation influences the kind of transferases elaborated by a cell.

The blood group substances are water-soluble glycoproteins found in saliva, intestinal juices, seminal plasma, ovarian cyst fluid, vaginal secretions, milk, tracheobronchial secretions, and urine of individuals of the appropriate blood type and secretor status. They are immunologically cross reactive with the blood group specific antigens found on erythrocytes and a wide variety of tissues. They are 80 to 90% carbohydrate and 10-20% amino acid, have a molecular mass of between 3×10^5 and 1×10^6 daltons, and contain a large number of oligosaccharide chains per protein backbone. Their amino acid composition is similar to mucins from porcine submaxillary gland and bovine cervix, i.e., 66% proline, serine, threonine, and alanine. The carbohydrate portions are O-linked

and contain five sugars--fucose, galactose, N-acetylgalactosamine, N-acetylglucosamine, and sialic acid. A composite "negaliosaccharide" structure for these oligosaccharides, which are similar in structure and composition to cellular ABH and Lewis antigens, is shown in Fig. 5.

The antigenic specificity of the blood group substances and of erythrocytes is a reflection of the composition and structure of the non-reducing end of the carbohydrate chain. The blood group antigens are inherited in a Mendelian fashion; however, it is important to note that they are the secondary products of functional genes at three loci--ABO, H, and Le (Table 3), the primary product of each gene being a glycosyltransferase. As is described, antigenically active structures are a result of the sequential action of these enzymes, which transfer single sugar residues to the growing carbohydrate chain. The immunologic activity of the blood group substances is controlled by a gene at the Se locus; this subject is discussed more extensively in Sec. IV. B. 1. b.

a. ABH Antigens

It is possible to classify the population into four serologically determined blood groups, A, B, O, and AB, that depend on the presence or absence of erythrocyte antigens. In Europeans, 47% of the population is Group O, 42% Group A, 8% Group B, and 3% Group AB. These types are inherited and are determined by three major alleles at the ABO locus (located at the distal end of the long arm of chromosome 9). I^A and I^B are the two functional alleles at this locus; the primary gene products are α -N-acetylgalactosaminyltransferase and α -galactosyltransferase, respectively. As can be seen from Table 3, A-active structures terminate with α -N-acetylgalactosamine and B-active structures with α -galactose; it is these sugars in $\alpha 1 \rightarrow 3$ linkage to galactose that determine the antigenic

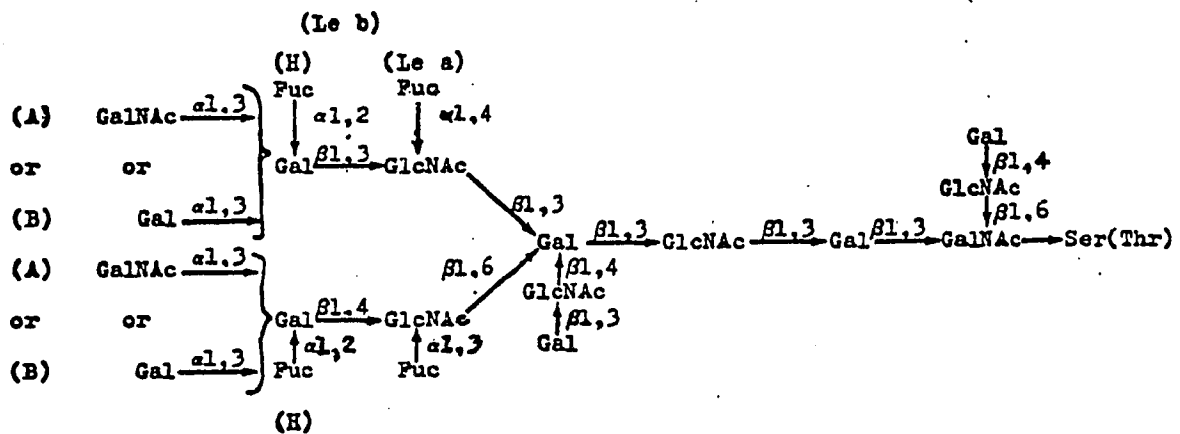


Fig. 5. Composite "megalosaccharide" structure proposed for the blood group substances. From Ref. 56.

Table 3

Alleles, Gene Products, and Resulting Carbohydrate Structures of the H, ABO, and Lewis Systems

Blood Type	Antigen	Allele	Primary gene product	Structure at reducing end
H system				
O	H	HH or Hh	$\alpha 1 \rightarrow 2$ Fucosyltransferase	fuc $\alpha 1 \rightarrow 2$ gal $\beta 1 \rightarrow 3(4)$ glcNac...
O _h (Bombay)	-	hh	none ^a	gal $\beta 1 \rightarrow 3(4)$ glcNac
ABO system				
A	A	I ^A I ^A or I ^A I ^O	α -N-acetylgalactosaminyl transferase	galNac $\alpha 1 \rightarrow 3$ gal $\beta 1 \rightarrow 3(4)$ glcNac... 2 1 α fuc
B	B	I ^B I ^B or I ^B I ^O	α -galactosyltransferase	gal $\alpha 1 \rightarrow 3$ gal $\beta 1 \rightarrow 3(4)$ glcNac... 2 1 α fuc
AB	A and B	I ^A I ^B	α -N-acetylgalactosaminyl transferase and α -galactosaminyl transferase	Composite of A and B structures above α fuc 1 4
Lewis system				
Le a	Le a	Le and sese	$\alpha 1 \rightarrow 4$ fucosyltransferase	gal $\beta 1 \rightarrow 3$ glcNac
Le b	Le b	Le, Se, and H	$\alpha 1 \rightarrow 2$ fucosyltransferase and $\alpha 1 \rightarrow 4$ fucosyltransferase	gal $\beta 1 \rightarrow 3$ glcNac... 1 1 2 4 α fuc α fuc
Le (a-b-)	-	lele	none ^b	

^aSee discussion on page 24.^bSee discussion on 25ff.

specificity of the oligosaccharide units. I^0 is a silent allele and has no antigen associated with it. Type O individuals can only be I^0I^0 ; type A, I^AI^A or I^AI^0 ; and type B, I^BI^B or I^BI^0 . Individuals whose genetic makeup is $I^A I^B$ type AB. The carbohydrate chains of these individuals end in one α -D-N-acetylgalactosamine and one α -D-galactose (see Table 3).

Although I^0 is silent, there is an antigen associated with group O cells and secretions, H antigen (Table 3). It is the secondary product of the H gene, which is found at a locus separate from that of ABO . The primary product of the H gene is $\alpha 1 \rightarrow 2$ fucosyltransferase, which transfers α -L-fucose to C-2 of a terminal galactose residue linked $\alpha 1 \rightarrow 3$ (Type I chain) or $\beta 1 \rightarrow 4$ (Type II chain) to an N-acetylglucosamine residue, and in so doing converts the precursor oligosaccharide to H antigen. This reaction is a key control point in the synthesis of the A and B antigens because this structure, fucal $\rightarrow 2$ gal, is absolutely required for the enzymatic action of the A and B specified enzymes.

The other allele at the H locus is h . Individuals homozygous for this allele are characterized by a lack of A, B, and H antigens on their tissues and erythrocytes and in their secretions; their serum has anti-H activity in addition to anti-A and B. This phenotype is referred to as O_h or Bombay, for the city where it was first discovered. A and B enzymatic activities can often be found in the serum and tissues of Bombay individuals; however, the corresponding antigens have not been detected since lack of the H structure prevents their formation.

Since $\alpha 1 \rightarrow 2$ fucosyltransferase activity is absent in all tissues and body fluids of Bombay donors (63), it is most likely that h is a silent gene. While this is the most accepted explanation, other evidence indicates that there is a suppressor substance, whose gene is at a different

locus than that of H, that inhibits enzyme activity (57). Resolution of this problem awaits the finding of families with more informative genetic constitutions.

b. Secretor Status

About 80% of the population secretes blood group substances with A, B, and H antigenic specificities that correspond to the individual's blood type. The other 20% are known as nonsecretors. In order for an individual to be recognized as a secretor, he or she must carry at least one Se gene in the genome of their secretory cells and at least one H gene. The Se locus is separate from that of H and ABO; it has two alleles associated with it -- Se and se. Secretors may be SeSe or Sese while non-secretors are sese.

The Se gene, when present in single or double dose allows the expression of the H gene in secretory tissue; expression of this gene in erythroid and other nonsecretory tissue is unaffected. It is unclear how this control is exerted since the primary gene product of Se is unknown.

Many investigators have shown that the secretory tissues and secretions of secretors have $\alpha 1 \rightarrow 2$ fucosyltransferase activity while it is absent in nonsecretors (58, 59). However, secretors and nonsecretors of the appropriate blood types have the A and/or B specific transferases (60,61). Thus, even though nonsecretors of Types A, B, and AB have the transferases that form the corresponding antigens, the lack of $\alpha 1 \rightarrow 2$ fucosyltransferase activity, and thus H substance, prevents their synthesis.

c. The Lewis Group

There are two alleles at the Lewis locus (which is independent of the three systems discussed previously). Le is a functional allele whose primary product is $\alpha 1 \rightarrow 4$ fucosyltransferase. Individuals whose red cells

carry a Lewis antigen have a soluble form of the enzyme in milk and most likely other secretions and a particle-bound form in submaxillary glands and gastric mucosa. Unlike the other glycosyltransferases involved in blood group formation, it is not found in plasma (62). le is the other allele found at this locus, and it is believed to be a silent allele because no primary gene product has been detected.

Two antigens are associated with this system. Le a is the secondary product of Le and its antigenic determinant is α -L-fucose linked 1 $\rightarrow$ 4 to N-acetylglucosamine. If H antigen is present on the same carbohydrate chain as Le a, then a new antigen is formed, Le b (see Table 3). Thus, Le b does not represent the product of another gene at this locus. Even though the Le and Se loci are totally independent, the Le antigen carried by an individual is dependent on secretor status. Secretions of nonsecretors that carry at least one Le gene have high Le a reactivity; those of secretors that carry at least one H gene show Le b activity. This would be expected since only secretors can synthesize H structure in their secretory cells.

Erythrocyte Lewis antigens are glycosphingolipids that differ from erythrocyte ABH antigens in that they are not an integral part of the red cell membrane. They are acquired from the plasma in which the cells circulate. Le(a-b-) red cells suspended in plasma from Le positive individuals passively acquire the plasma antigen. Because the same antigen-secretor status relationships hold for erythrocyte antigens as for secretions, it is believed that erythrocyte Lewis antigens are not synthesized by erythroid tissue but by secretory cells. If Lewis antigens were synthesized in erythroid tissue, Le b antigen would be found on the erythrocytes of all individuals carrying a Le gene except for Bombay donors.

d. Synthesis of Blood Group Substances

The basic acceptor molecule for all the blood group substances is shown in Fig. 6. Two types of chain endings exist--Type I, where the terminal galactose is linked $\beta 1 \rightarrow 3$ to N-acetylglucosamine and Type II, where the two are linked $\beta 1 \rightarrow 4$. It is clear that only Type I chains can be converted to Le a antigens since this specificity is determined by a fucose linked to C-4 of the penultimate N-acetylglucosamine. A novel Lewis antigen may be found on Type II chains where fucose is linked $\alpha 1 \rightarrow 3$ to the penultimate N-acetylglucosamine. Le a and Le b immunologic activity is weak in this case.

Because of the marked heterogeneity of the carbohydrate chains of the blood group substances, they can carry multiple antigenic specificities, e.g., secretions of donors carrying A, B, H, Le, and Se genes may have five antigenic specificities in their secretions--A, B, H, Le a, and Le b. Antiserum to any one of these antigens will precipitate the other four.

Once the core region is formed, there are two possible biosynthetic pathways. In vitro studies (64) indicate that if the Le a structure is formed before the H structure, all further synthesis is blocked since this structure is not an acceptor for the other transferases. If, however, H antigen synthesis takes place first, then there are three possible alternatives: (a) synthesis may stop there, (b) the Le b structure may be formed, or (c) the A and/or B structure(s) may be formed. In vitro studies indicate that the Le b structure is not an acceptor for the A and B transferases. However, A structure is a substrate for the Le transferase. Thus, all the reactions involved in the synthesis of the blood group terminal structures are accounted for. Experiments using specific glycosidases to study these carbohydrate structures confirm this order of

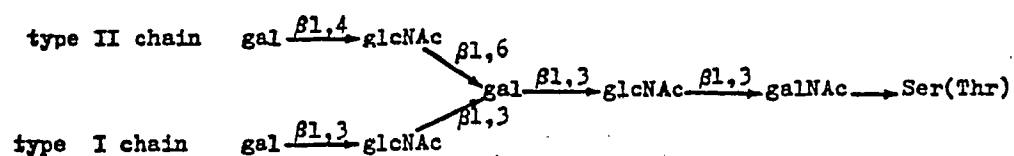


Fig. 6. Basic acceptor molecule for blood group substance formation. In erythrocyte glycolipids, both chains are Type II. Thus no Lewis antigens are found on integral membrane glycolipids.

addition.

2. Developmental Effects

Many studies have shown that the relative and absolute activities of glycosyltransferases vary according to stage of development and that these patterns are tissue specific. Jato-Rodriguez and Mookerjee (65) found that galactosyltransferase and sialyltransferase activities in the organs of the developing rat tend to decrease with increasing age. Carlson et al. (66) studied the specific activities of two galactosyltransferases in the developing rat fetus from 11 days of gestation to the neonatal period. Again the developmental patterns in different tissues varied enormously during gestation, but activity patterns changed during the postnatal period as well, with enzyme activity in some tissues becoming undetectable.

Watanabe and Hakamori (67) have studied the structures of blood group glycolipids during ontogenesis and have provided indirect evidence of developmental control of glycosyltransferase activity. They report that the carbohydrate chains of glycolipids from erythrocytes of fetuses (from 80-150 days of gestation) and newborns lack glycolipids with complex branching patterns. The simple chains that were present showed no significant changes after birth. The authors did not determine if the lack of branching was due to the absence of the appropriate branching enzymes or to the presence of a regulatory factor suppressing enzyme activity. In either case, it is clear that there is a growth-dependent regulation of carbohydrate structure.

Since the function of most carbohydrate structures is still unknown, the significance of these changes in enzyme activity and structure is unclear.

3. Hormone Effects

The enzyme that has been studied most extensively in relation to hormone effects is galactosyltransferase. This enzyme is found in soluble form in many body fluids, especially milk and colostrum, and in particulate form in a large number of tissues. The enzymes from all sources are remarkably similar, particularly with respect to acceptor activity with and without α -lactalbumin.

In the absence of α -lactalbumin, galactose is transferred to beta anomers of N-acetylglucosaminides, including monosaccharides. Galactose is also transferred to glucose but with a much higher K_m than for N-acetylglucosaminides. However, in the presence of α -lactalbumin, the K_m for glucose is lowered and synthesis of lactose is markedly facilitated. Insulin, hydrocortisone, and prolactin induce the synthesis of both galactosyltransferase and α -lactalbumin, while progesterone inhibits induction of α -lactalbumin. In this way the levels of α -lactalbumin, and hence the acceptor specificity of galactosyltransferase are under strict hormonal control (68).

Glycosyltransferase activities in the reproductive tract appear to be under hormonal control. Nelson et al. (69) have shown that 17 β -estradiol causes substantial increases in the activities of several glycosyltransferases in endometrium. Reddy et al. (70), using orchidectomized rats to study the effect of hormones on the levels of glycosyltransferases in male reproductive tissues, report that specific activities of both galactosyl- and fucosyltransferases diminished in both the ventral prostate and the bulbourethral glands over the initial one-week period following castration. After two weeks, the animals were given daily injections of testosterone and enzyme activity was gradually restored in both glands. When hypophysectomized rats were administered testosterone, enzyme levels

were increased two to three times. The administration of prolactin was without effect in the presence and absence of testosterone.

4. Changes Associated with Transformation

Of the many changes accompanying cell transformation, alteration in cell surface carbohydrate antigens are among the most striking. The disappearance of the A and B blood group antigens from the surface of many human carcinomas and transformed cell lines (71-74), with a concomitant decrease in the enzymes responsible for their synthesis is a well-documented finding (72, 75). The carbohydrate content of glycoproteins from malignant cells is also consistent with the serological and enzymatic changes (72). The activity of enzymes responsible for synthesis of serum-type glycoproteins appear to be unaffected. Virally transformed cells also show differences in carbohydrate composition along with changes in transferase activity (76,77).

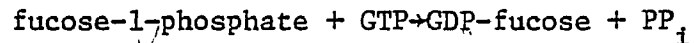
Watanabe and Hakamori (67) have reported that glycolipids with simple (unbranched) chains appear in increased quantities in human colonic adenocarcinomas when compared with adjacent, nonmalignant tissue. These simple chains are characteristically found on fetal and newborn erythrocytes. They suggest that there is a genetic or epigenetic program for synthesis of blood group determinants and their core regions that develops step-by-step during ontogenesis, and that this program is blocked at some point in ontogenesis.

Davidsohn et al. (71) have proposed that the disappearance of blood group antigens from the surface of the malignant cell may be a signal that dedifferentiation of these cells has occurred. They have studied the relationship of malignant transformation of gastrointestinal epithelial cells to the appearance and disappearance of cell surface blood group antigens. Using conjugated antibodies and lectins, they have shown that

once these tissues lose their ability to secrete mucins, the blood group antigens could no longer be detected. In line with these findings, Pann and Kuhns (78) several years later proposed that the appearance of H antigen on the surface of HeLa cells is a signal of differentiation. They noted that H⁻ HeLa cells grown in monolayer culture become H⁺ as time in culture increased and that detached cells, i.e., terminal forms, are entirely H⁺.

5. Nucleotide Sugars

It is now well established that the carbohydrate portions of mammalian glycoproteins and proteoglycans are incorporated by way of nucleotide sugar precursors. In vivo, monosaccharides are normally synthesized from glucose-6-phosphate via the metabolic pathways shown in Fig. 7; however, other monosaccharides may serve as precursors for nucleotide sugars. For example, fucose may be converted to fucose-1-phosphate which then undergoes the reaction



The enzymes responsible for these syntheses are cytosolic (79) except for CMP-NANA synthetase, which is apparently a nuclear enzyme (80).

In vivo, glycoconjugate biosynthesis is controlled, in part, by the levels of nucleotide sugars; therefore a brief look at the factors controlling the levels of these precursors is in order.

Mendicino and Rao (79) studied the regulation of the biosynthetic enzymes in bovine reticuloendothelial tissues; they found that the rate-limiting reactions were those where the commitment to the final product is made, i.e., for UDP-galactose, UDP-galactose 4-epimerase; for UDP-hexosamines and CMP-sialic acid, D-fructose-6-phosphate amidotransferase (FPAT); and GDP-mannose and GDP-fucose, GDP-mannosepyrophosphorylase were

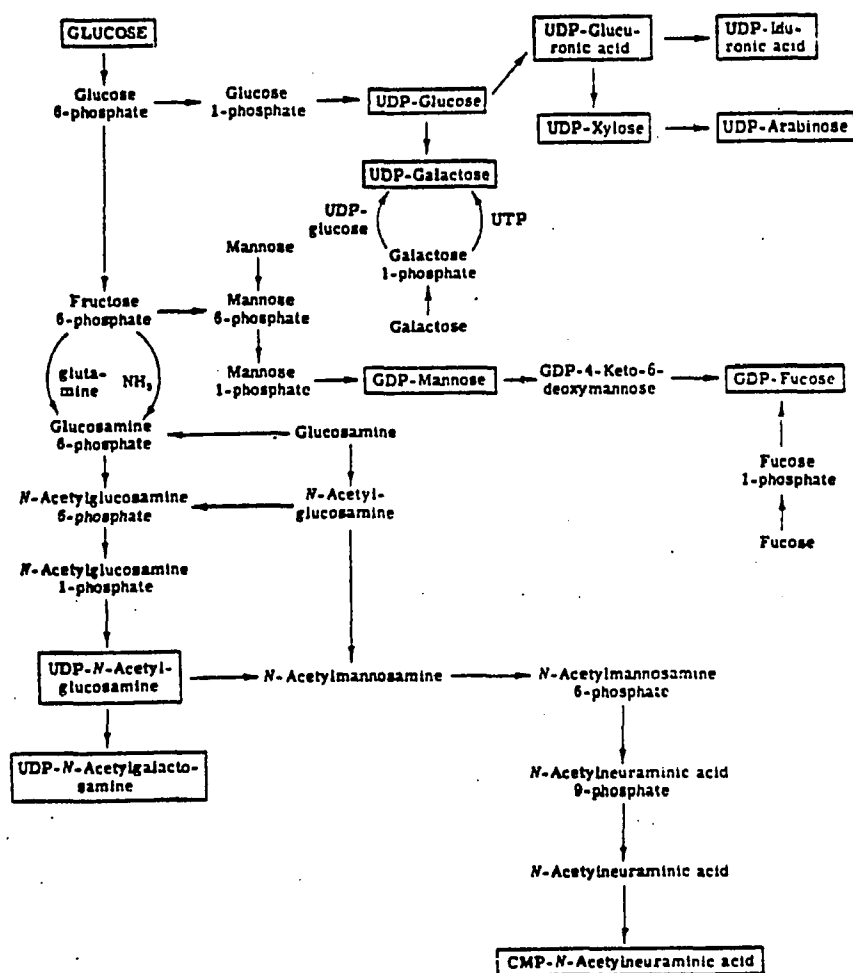


Fig. 7. Pathways of sugar nucleotide biosynthesis.
From Ref. 81.

purified from mesenteric lymph nodes and their regulatory properties examined. In agreement with workers who studied other tissue sources for these enzymes, they found that both these enzymes were subject to cumulative feedback inhibition by the nucleotide sugars that are the final products of the pathways.

FPAT was found in highest concentrations in liver and small intestine and the lowest in spleen and thyroid. The enzyme is inhibited in a competitive manner, relative to fructose-6-phosphate, by UDP-glucose and UDP-glucosamine. The K_m for fructose-6-phosphate is 0.3 mM, while the K_i 's for UDP-glucose and UDP glucosamine are 0.4 and 0.045 mM, respectively. The concentrations of these metabolites in the lymph nodes were found to be very close to these K_m and K_i values.

The series of reactions leading to the formation of GDP-mannose and GDP-fucose are limited by the activities of GMPP and GDP-mannose oxidoreductase. GMPP was found to be ten times less active than the other enzymes on the GDP-mannose pathway. Its concentrations are highest in liver and small intestine and lowest in spleen and thymus. The activity of GDP-mannose oxidoreductase is one-hundredth that of GMPP, which is what would be expected from their relative concentrations in glycoproteins.

GMPP is competitively inhibited, with respect to GTP, by both mannose and GDP-fucose (82). The apparent K_m for GTP is 0.06 mM and the K_i for GDP-mannose is 0.03 mM. The levels of these metabolites were also found to be very close to the K values.

Clearly, formation of nucleotide sugars is very closely regulated, depending to a great degree on the energy charge of the cell, i.e., the levels of nucleotide triphosphates and the extent of glycogen synthesis, as well as the rate of glycoprotein biosynthesis.

Bauer et al. (83) compared the levels of GDP-fucose and CMP-sialic acid with the activity of fucosyl- and sialyltransferases in Morris hepatomas and in the livers of the host animal. In all cases the levels of fucosyltransferase in the hepatomas, as compared with the host or normal control liver were elevated. The levels of GDP-fucose were correspondingly high--up to 21.5 nmoles/gram wet weight in the hepatomas as compared with about one-third that in host liver or control. The activity of sialyltransferase was usually decreased or unchanged in the hepatomas and in this case no significant change in CMP-sialic acid was noted--the level of CMP-sialic acid in the hepatomas ranged from 55.3 ± 6.0 to 61.9 ± 5.9 as compared with 52.5 ± 7.0 in host liver.

These findings are puzzling in view of the well-documented findings on feedback inhibition of the nucleotide sugar biocynthetic pathways. High levels of GDP-fucose should inhibit its further synthesis and yet the levels in the hepatomas are three times normal. It is possible that the high levels of fucosyltransferase are responsible for these findings; for example, the transferase might sequester GDP-fucose in the golgi cisternae where it would be used for glycoprotein and glycolipid synthesis. Thus the effective concentration of the sugar precursor in the cytosol would be decreased.

V. Fucosyl- and Sialyltransferases

The fucosyl- and sialyltransferases catalyze the transfer of fucose and sialic acid to appropriate glycoprotein acceptors. These two sugars are found at the nonreducing terminals of oligosaccharide chains, and there are strong indications that these two sugars play an important physiological role in the homing of cells to target organs and in regulating levels of serum proteins. Studies of the anabolic and catabolic

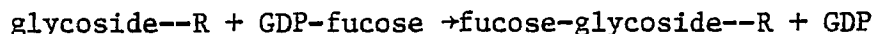
enzymes responsible for their presence on the oligosaccharide chains of glycoproteins and glycolipids and the interactions among these enzymes have potential importance in understanding the physiology of these events.

In the following discussion, each group of enzymes is discussed separately, followed by a comparison of their properties. There is also a discussion of how these enzymes are altered in various disease states.

A. Fucosyltransferases

Fucosyltransferases catalyze the transfer of fucose (6-deoxygalactose) from GDP-fucose to an appropriate receptor, i.e., an oligosaccharide, disaccharide, a glycolipid, or a glycoprotein, forming one of the four linkages that fucose is known to participate in: $\alpha 1 \rightarrow 2$ to galactose, $\alpha 1 \rightarrow 3$, $\alpha 1 \rightarrow 4$, and $\alpha 1 \rightarrow 6$ to N-acetylglucosamine (the beta anomer has not been found in any glycoconjugate of mammalian origin).

Figure 8 shows fucose-containing oligosaccharide structures and the glycoproteins from which they are derived. Each of these linkages is formed by a separate enzyme that has stringent acceptor requirements, the carbohydrate residue at the nonreducing terminal of the oligosaccharide moiety being the primary recognition site. Their general reaction is



The systematic name for this group of enzymes is GDP-fucose glycoprotein fucosyltransferase (E. C. 2.4.1.68). However, there are no systematic names for the different enzymes that catalyze the transfer of fucose into each of its linkages. Additionally, the systematic name is a misnomer since there is no experimental evidence that these enzymes show any specificity for the type of backbone to which the oligosaccharide is attached. Therefore, for the sake of convenience, each enzyme will be referred to by the linkage it forms, e.g., $\alpha 1 \rightarrow 2$ fucosyltransferase.

It should also be noted that the following discussion is restricted almost entirely to those enzymes that transfer fucose to glycoprotein acceptors.

The fucosyltransferases that catalyze the transfer of fucose into 1→2 and 1→6 linkages on glycoproteins acceptors are the most studied, and they are the ones investigated in this project. These two enzymes were chosen specifically because they are found most commonly in glycoproteins derived from human sources, the 1→2 linkage being found most commonly in mucin type glycoproteins and the 1→6 in serum proteins.

Both these transferases can be found in two forms--soluble and membrane bound. When soluble, they require no detergent for activation (62) and are found in the supernatant fraction following high speed centrifugation (59). The membrane bound forms require the use of detergents for maximum solubility. In the absence of detergent treatment, all activity is associated with the membrane pellet. In glandular and secretory tissue, such as submaxillary glands and stomach mucosa, both forms are found (59).

The general subcellular location of these enzymes has been shown by Sturgess et al. (31) to be the golgi apparatus. Tritiated fucose was injected into rats and fucose incorporation into hepatocytes was followed by radioautographic and biochemical techniques. After 2 min, silver grains were localized to the golgi cisternae, at 10 min, 73% were localized to the tubules, and at 20 min, 84% were found in the secretory vesicles. Grains were also found in the plasma membrane. A few grains were found in the SER and none in the nucleus or cytoplasm. Biochemical analysis confirmed the kinetics of fucosylation.

As is true for other enzymes where nucleotide binding is involved,

divalent metal cations are required for maximal activity. Magnesium and manganese, depending on the species, are the usual co-factors, but cobalt, calcium, and several other divalent metal ions can often be substituted, but their enhancement of activity is not as great.

1. $\alpha 1 \rightarrow 2$ Fucosyltransferase

The $\alpha 1 \rightarrow 2$ fucosyltransferase catalyzes the transfer of fucose to C-2 of galactose at the terminal, nonreducing end of an oligosaccharide chain.

This enzyme has been reported to be present in pork liver (89), mouse and rat testis (27,28), HeLa cells (90), human fibroblasts (91), human lung (92), Morris hepatomas and mouse liver (83,93), saliva (94), rat pancreas (95), and rat and human intestine (59,96), serum (59,63,71, 97), human milk (58), and porcine submaxillary gland (98,99). Munro et al. (93) report it to be absent in rat liver. As discussed in Sec. IV. B.1, in humans, this enzyme is the product of the H gene and is thus the control point in the biosynthesis of the blood group substances. In secretory tissues, it is under control of the Se gene.

As expected, this enzyme has been localized to the golgi fraction in liver by Munro et al. (93), where it copurifies with galactosyltransferase, a known golgi marker (100). However, comparatively low recoveries of the enzyme were obtained after subcellular fractionation; they argue that this may point to other subcellular locations for this enzyme. Unfortunately, no data for the other fractions such as plasma membrane or smooth microsomes are provided.

Bayer et al. (98) have solubilized this enzyme with Triton X-100 (TX-100) and purified it 180,000 fold from porcine submaxillary gland by affinity chromatography on GDP-hexanolamine-agarose. In line with other

studies, they report that the $\alpha 1 \rightarrow 2$ enzyme has no strict requirement for a metal cofactor, but is stimulated by divalent metal ions. However, the K_m for both substrates and the V_{max} increase in the presence of Mn^{2+} (99). Prieels et al. (101) also purified this enzyme from human milk; however, this work is reported only in abstract form. Their purification and separation from other fucosyltransferases was reportedly effected using SP-Sephadex C-50 and a salt gradient elution. No kinetic characterization of the enzyme was given.

a. Acceptors

Beyer and Hill (99) report that this enzyme will transfer fucose to galactose with little specificity for the nature of the penultimate residue. In addition, the fuc $\alpha 1 \rightarrow 2$ gal linkage was formed regardless of whether the acceptor was an oligosaccharide, a glycoprotein, or a glycolipid (see Fig. 9 for examples of acceptor molecules used in this and other studies). They confirmed the linkage to galactose by paper chromatography (64) and by enzymatic digestion with a specific $\alpha 1 \rightarrow 2$ fucosidase from Clostridium perfringens (98).

Another report from this group (64) examines the specificity of this enzyme using prototype O- and N-linked glycoproteins. The two glycoproteins used as models were human asialotransferrin, which contains the non-reducing, terminal oligosaccharide sequence gal $\beta 1 \rightarrow 4$ GlcNAc $\beta 1 \rightarrow 2$ Man, and antifreeze glycoprotein, which contains the oligosaccharide gal $\beta 1 \rightarrow 3$ galNAc $\alpha 1 \rightarrow 0$ Thr. These oligosaccharide structures were modified using sialyltransferases whose purification and properties are discussed under Sialyltransferases.

Both freezing point depression glycoprotein and asialotransferrin served as acceptors, as did the following structure:

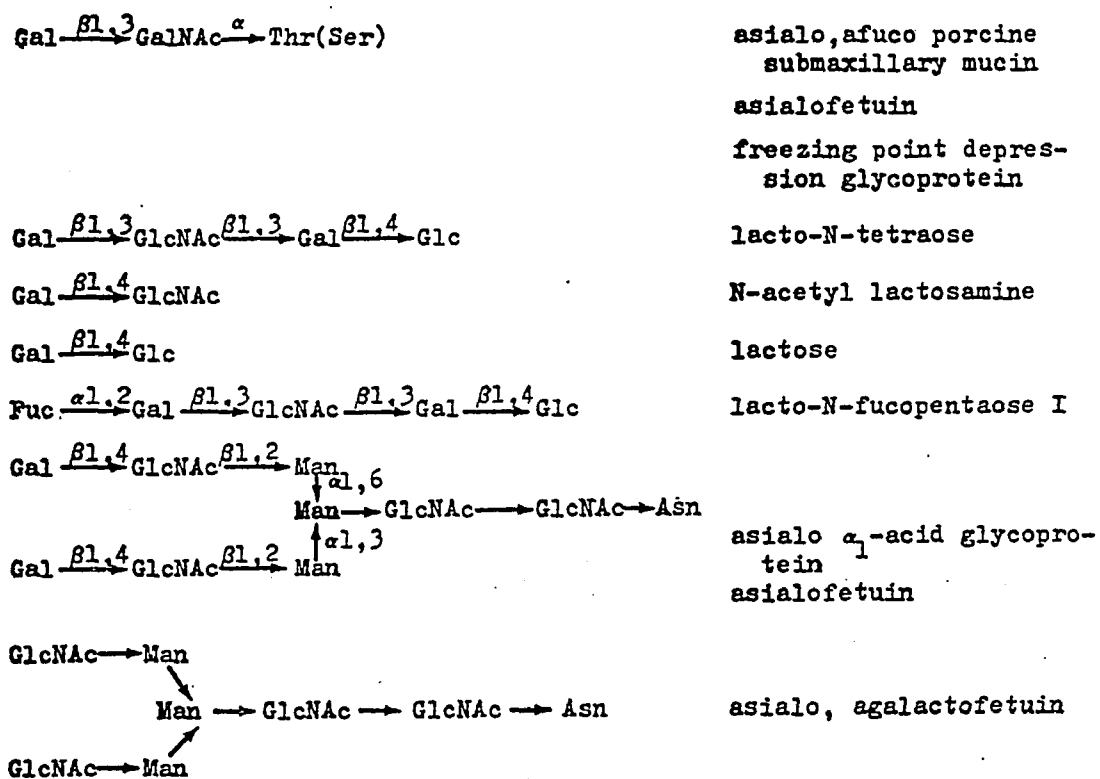
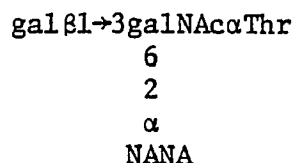
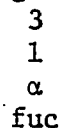
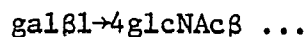
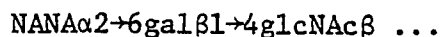
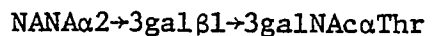


Fig. 9. Oligosaccharide structures used as acceptors in fucosyltransferase assays.



These structures could not act as acceptors:



The finding that sialylated galactose residues do not serve as acceptors is potentially important. A more extensive discussion is found in Sec. VI.

Bella and Kim (96) have studied the acceptor requirements of rat intestinal enzyme using milk sugars (whose structures are well defined and are similar to those of the blood group substances), desialylized fetuin, desialylized, defucosylated porcine submaxillary mucin, and desialylized, defucosylated intestinal protein. The PSM acceptor showed the highest incorporation; however, the K_m 's of all three glycoproteins were similar to each other and to that of lactose. In this study, the most effective acceptors had a galactosyl residue linked $\beta 1\rightarrow 3$ to a penultimate N-acetylglucosamine, e.g., PSM, lacto-N-tetraose. Less incorporation was seen with $\beta 1\rightarrow 4$ isomers, e.g., lactose, fetuin. The α anomers exhibited no activity.

Pork liver enzyme incorporates fucose into sialidase-treated $\alpha 1$ -acid glycoprotein, acid hydrolyzed fetuin, and the disaccharide $\text{gal}\beta 1\rightarrow 4\text{glcNAc}$. Less incorporation is seen with acid-treated porcine submaxillary mucin. The enzyme does not transfer fucose to lactose, $\text{gal}\beta 1\rightarrow 3\text{glcNAc}$, or α or

β methyl-D-galactopyranosides.

Chester et al. (59) have developed a specific assay for the 1 $\rightarrow$ 2 transferase that uses phenyl- β -D-galactopyranoside as acceptor. The enzymatic reaction products were characterized by specific fucosidases and by alkaline hydrolysis. The data obtained could be explained only by the presence of a α 1 $\rightarrow$ 2 fucosyl-galactoside bond. Enzyme derived from human submaxillary gland and stomach mucosa of secretors showed high activity, while little or none was found when the donor was a nonsecretor. Thus the distribution of this enzyme is very similar to that determined by other investigators. Serum from a Bombay donor and a B_h donor did not transfer fucose to the phenylgalactoside acceptor. HeLa cells also showed some activity with this acceptor; however, the incubation times had to be increased considerably.

b. Kinetics and Inhibitors

The reaction catalyzed by the α 1 $\rightarrow$ 2 fucosyltransferase has two substrates--GDP-fucose and the glycoprotein acceptor--and two products--GDP and the fucosylated acceptor. Louisot et al. (102) have studied kinetics of this reaction and found that the mechanism can best be described as "Bi-Bi" ordered. The enzyme has an affinity for GDP-fucose that is independent of acceptor concentration, but is dependent on the concentration of GDP, which acts as a competitive inhibitor. On the other hand, the affinity of the acceptor is directly related to the concentrations of GDP-fucose and GDP. This would tend to rule out both a "Bi-Bi" ping pong or a "Bi-Bi" ambiguous mechanism. This mechanism is confirmed by the work of Beyer and Hill (99) and by the findings of Bella and Kim (96) that the K_m for GDP-fucose is unaffected by the type of acceptor used.

However, the reaction mechanism is further complicated by the finding

that the enzyme exhibits positive cooperativity. At low concentrations of GDP-fucose and high concentrations of acceptor, the Hill coefficient is 2-3; when the substrate concentrations are both high or are both low then the Hill coefficient is 1.

Because GDP presumably binds in the GDP-fucose-binding site, it is of interest to know something of the nature of the nucleotide site. Bella and Kim (103) made an extensive study of the inhibitory properties of nucleotides and found that guanosine nucleotides, as expected, were the most effective inhibitors with GDP having the highest activity. A phosphate at the C-5' position is required for inhibition since guanosine, guanosine plus P_i , and 2 3' cGMP are only slightly inhibitory. dGTP also is an inhibitor, indicating that the ribose moiety is not necessary for inhibition. The most effective inhibitors are purine nucleotides with an OH group on C-6, an amino group on C-2, and a covalently linked phosphate on C-5'. GDP-mannose (96) also acts as an inhibitor of fucosyltransferase activity, presumably through the GDP moiety.

2. α 1 $\rightarrow$ 6 Fucosyltransferase

This enzyme has been found in pork and rat livers (89,93,104), rat small intestine (96), serum (59,62,97), rat ventral prostate (70), mouse and rat testis (39,31), pancreas (95), and HeLa cells (90). However, only the serum enzyme has been studied extensively (62). These results are described in the section on the serum enzymes. Wilson et al. (104) have studied the acceptor specificity of the pork liver enzyme, and Jabbal and Schachter (89) performed mixed acceptor competition studies that showed that the 1 $\rightarrow$ 6 enzyme is different from the 1 $\rightarrow$ 2 enzyme.

Several studies from Schachter's laboratory have localized this enzyme to the golgi apparatus of rat and mouse testis (27,28) and rat and

pork liver (93). Enrichments of 40- and 35-fold were obtained in rat and pork liver, respectively (93), 15- and 24-fold in rat and mouse testis (27).

There have been no studies on the kinetic properties of this enzyme from tissue except for the one by Jabbal and Schachter (89), who report a K_m of 17.0 mM for the acceptor. It is unknown if GDP or other guanosine nucleotides are inhibitory, although presumably they are.

a. Acceptor Requirements

The acceptor for the 1 $\rightarrow$ 6 must have at least one glcNAc-man terminal. Originally it was believed that this enzyme transferred fucose in a 1 $\rightarrow$ 3 or 1 $\rightarrow$ 4 linkage to this terminal glcNAc; however, such a linkage was never reported when an acceptor with a GlcNAc terminal was used. However fucose is commonly found as a branched residue linked 1 $\rightarrow$ 6 to the proximal glcNAc of the chitobiose core in glycoproteins such as H-2 alloantigen (105) and IgG, IgA, IgM, and IgE (8,11,84)(see Fig. 9a).

Wilson et al. (104) have shown that the terminal glcNAc is part of the recognition site for the 1 $\rightarrow$ 6 enzyme. Glycopeptides from pronase-treated α_1 -acid glycoprotein were treated to remove sialic acid and galactose, thus exposing a glcNAc terminal (see Fig. 10 for structures). This substrate has been shown to be a good fucose acceptor. The asparagine residues of the treated glycopeptides were labeled with ^{14}C -acetic anhydride. Another aliquot of glycopeptides was acetylated with cold acetic anhydride and incubated with ^{14}C -GDP-fucose and enzyme extract. Both preparations were then subjected to endoglycosidase treatment and the products analyzed by paper electrophoresis. The mobility of the fucosylated product was identical to that of the acetylated product, showing that fucose remains with the glcNAc--Asn moiety and not with the neutral oligosaccharide

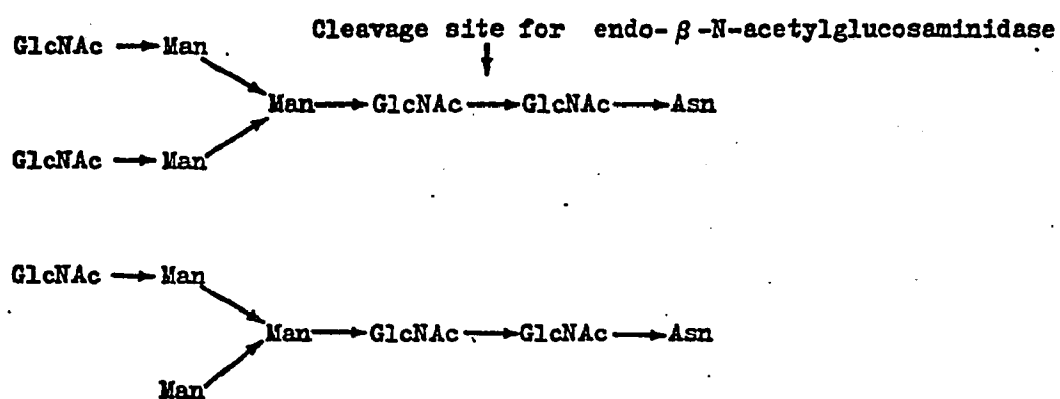


Fig. 10. Oligosaccharide structures of acceptor molecules for 1 $\rightarrow$ 6 fucosyltransferase. Note site of endo- β -N-glucosaminidase cleavage.

portion, which remains at the origin. Additionally, mild acid hydrolysis or α -fucosidase treatment of the fucosylated product resulted in the release of about 60% of the radioactive label as fucose. These workers have not proven that the actual linkage of the fucose is $\alpha 1 \rightarrow 6$; analysis by other workers has occasionally shown fucose linked $\alpha 1 \rightarrow 3$ in the chitobiose core.

The acceptor glycopeptide has no activity as a fucose acceptor after treatment with hexosaminidase; at least one glucosamine must be present for fucose transfer to take place. In confirmation of this, they point out that there is no fucose incorporation into glycoproteins of a mutant line of Chinese hamster ovary cells that lack glucosaminyltransferase I, which transfers the first GlcNAc onto the mannose core. This provides additional evidence that this enzymatic step occurs late in the biosynthetic process, compatible with the location of this enzyme in the golgi apparatus.

3. Serum Fucosyltransferases

Only two fucosyltransferases have been found in human serum (59,62,97). One is the product of the H gene, which transfers fucose to substrates with galactose at the nonreducing terminal, such as lactose, lactosamine, asialofetuin, and asialo α_1 -acid glycoprotein, and phenyl-D- β -galactoside. The other is the $\alpha 1 \rightarrow 6$ enzyme, which transfers fucose to N-acetylglucosamine in the chitobiose core. These enzymes are discussed separately since it is not certain if the findings from these soluble enzymes are applicable to the properties of membrane-bound proteins.

Sera from nonsecretors exhibit $\alpha 1 \rightarrow 2$ activity (59,62); however, as expected, it is absent from the sera of Bombay donors (59,62,97). The $\alpha 1 \rightarrow 6$ enzyme is present in all sera regardless of donor type (59,62,97).

Monro and Schachter (62) performed mixing experiments with the acceptors lactose, lactosamine, asialo α_1 -acid glycoprotein, and asialo, agalacto α_1 -acid, glycoprotein. The first three acceptors appear to compete for the same enzyme site(s) on the $\alpha 1 \rightarrow 2$ enzyme. The asialo, agalacto α_1 -acid glycoprotein is probably acting as an acceptor for the $1 \rightarrow 6$ enzyme. When the reaction products of lactosamine and lactose were characterized by base hydrolysis, they were shown to contain only the $\alpha 1 \rightarrow 2$ linkage. Chester et al. (59) also confirmed the specificity of this enzyme reaction by using phenyl-D- β -galactoside, which they have shown acts as a specific acceptor for the $1 \rightarrow 2$ enzyme.

Neither serum enzyme is affected by the presence of Triton X-100 (59). The $1 \rightarrow 2$ enzyme appears to have no divalent metal ion requirement and is unaffected by the inclusion of EDTA in the incubation mixture but is totally inhibited by 13 mM N-ethylmaleimide (97). Monro and Schachter (62) report that the $1 \rightarrow 6$ enzyme does not require divalent metal ions for maximum activity; however, Chou et al. (97) report that Mg^{++} is needed. The discrepancy in the findings may be explained by the following: Monro and Schachter purified their sera by ammonium sulfate fractionation, whereas the others used straight serum as enzyme source and added EGTA to overcome the inhibition of the high calcium levels found in serum.

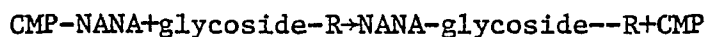
The presence in sera of the Le dependent transferase(s) ($1 \rightarrow 3$ or $1 \rightarrow 4$) has been ruled out by the work of Schenkel-Brunner et al. (63) and Monro and Schachter (62) who used gal $\beta 1 \rightarrow 3$ N-acetylglucosamine and lacto-N-fucopentaose I as acceptors, respectively. Monro and Schachter were unable to demonstrate the presence of a $1 \rightarrow 3$ fucosyltransferase when short incubation times were used. Schenkel-Brunner et al. (63), however, were able to

detect this enzyme with incubation times of 48-64 h and high substrate concentrations. Chester et al. (59) were unable to detect the presence of any serum enzyme capable of transferring fucose to any position of galactose except C-2.

There is very little information as to the tissue of origin of the serum enzymes. However, the presence of $\alpha 1 \rightarrow 2$ fucosyltransferase in the sera of nonsecretors and the absence of $\alpha 1 \rightarrow 3$ or of $\alpha 1 \rightarrow 4$ fucosyltransferases in all sera regardless of the secretor status of the donor point to a nonsecretory tissue of origin. There is speculation in the literature that the liver may be the source of these enzymes.

B. Sialyltransferases

The sialyltransferases catalyze the transfer of sialic acid (N-acetylneuraminic acid) from CMP-sialic acid with the general reaction



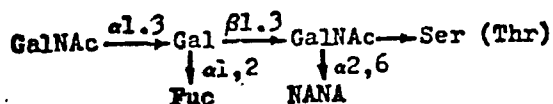
where R can be an oligosaccharide, a disaccharide, a glycolipid, or a glycoprotein.

Mammalian glycoproteins contain three main types of sialic acid linkages-- $\text{NANA} \alpha 2 \rightarrow 6 \text{gal} \beta$, $\text{NANA} \alpha 2 \rightarrow 3 \text{gal} \beta$, and $\text{NANA} \alpha 2 \rightarrow 6 \text{galNac} \alpha \text{Thr (Ser)}$, as shown in Fig. 11. Studies with purified enzymes indicate that at least four sialyltransferases are responsible for the formation of these structures (106).

The glycoprotein sialyltransferases that attach sialic acid to galactose residues have been collectively assigned the name CMP-neuraminate D-galactosyl glycoprotein N-acetylneuraminyl transferase (E. C. 2.4.99.1). There is not yet an official name for the enzyme that transfers sialic acid onto the N-acetyl galactosaminy residue involved in the O-glycosidic peptide bond.



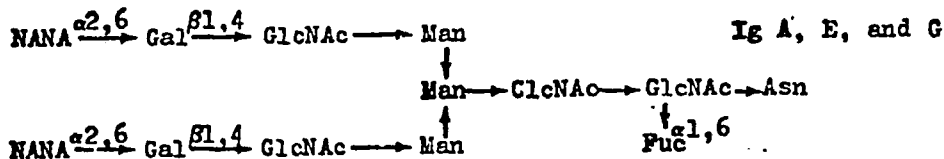
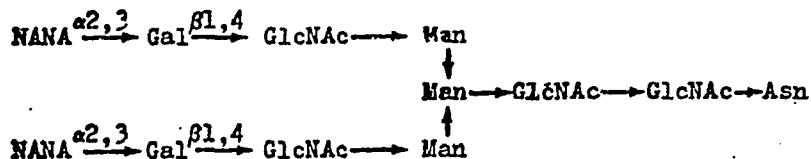
Ovine submaxillary
mucin
Bovine submaxillary
mucin



Porcine submaxillary
mucin



Petuin



Ig A, E, and G

Fig. 11. Representative examples of sialic acid-containing oligosaccharides derived from prototype glycoproteins.

These enzymes have been found in a variety of tissues including rat brain (107), human placenta (54), and liver (108,109), porcine (109), human (110) and rat (111) serum, rat (111,112) and porcine (109) liver, rat and mouse testis (27,93), calf kidney (113), porcine submaxillary gland (59,114) and bovine colostrum (115).

Its subcellular location in rat and pork liver has been reported by Bretz et al. (116) and by Munro et al. (93) to be the golgi apparatus. Similar findings have been reported by Letts et al. (27) in mouse and rat testis.

Like the fucosyltransferases, these enzymes have been found in both soluble and membrane bound forms. The membrane bound forms are reported to be solubilized and activated to various degrees by Triton X-100 (54, 108, 113, 148) and Cutscum (117). Detergents appear to be inhibitory to the soluble enzyme in serum (110).

1. Enzyme Characterization

Hill's laboratory has reported the purification by affinity chromatography using CDP-hexanolamine agarose as adsorbent, of three sialyltransferases: β -D-galactoside α 2 $\rightarrow$ 6 sialyltransferase (106,115), β -D-galactoside α 2 $\rightarrow$ 3 sialyltransferase (55,114), and α N-acetylgalactosaminide α 2 $\rightarrow$ 6 sialyltransferase (114). These are the only studies that unequivocally demonstrate the acceptor characteristics of all three enzymes by using acceptors with rigorously defined structures. These acceptors were synthesized by both chemical and enzymatic procedures. The results are reported here and in Sec. VI in some detail. Although other studies relating to the acceptor specificities and characterization of the sialyltransferases have been carried out, it is clear from the work of Hill's group that the acceptors used in earlier studies did not allow each of the sialyltrans-

ferases to be distinguished from one another (114).

The β -D-galactoside α 2 $\rightarrow$ 6 sialyltransferase has been purified 400,000 fold from bovine colostrum (115). Two molecular weight forms (as determined by SDS-PAGE)--56,000 and 43,000 daltons--were found; they appear to have similar catalytic properties. N-acetyllactosamine (gal β 1 $\rightarrow$ 4glcNAc) and asialoglycoproteins containing the sequence gal β 1 $\rightarrow$ 4glcNAc at the nonreducing end of their oligosaccharide groups are the best acceptors for this enzyme. The 1 $\rightarrow$ 3 and 1 $\rightarrow$ 6 isomers exhibit 1% of the activity of the 1 $\rightarrow$ 4 isomer. Lactose is also a poor acceptor, indicating the importance of the N-acetyl group at C-2 of the penultimate residue. Submaxillary mucins that contain the terminal sequence gal β 1 $\rightarrow$ 3galNAc do not serve as acceptors for this purified enzyme. This is not surprising since sialic acid in 2 $\rightarrow$ 6 linkage has never been reported on glycoproteins having gal β 1 $\rightarrow$ 3glcNAc or gal β 1 $\rightarrow$ 3 galNAc terminals.

This enzyme has also been reported in rat liver (117). Asialo α ₁-acid glycoprotein was used as acceptor and the galactose terminal was labeled using galactose oxidase/ NaB^3H_4 . Permethylation and acid hydrolysis yielded 1,2,3,4 trimethylgalactose, demonstrating transfer to just the C-6 position of galactose. No further characterization of this enzyme has been reported by these workers.

The β -D-galactoside α 2 $\rightarrow$ 3 sialyltransferase and the α -N-galactosamine α 2 $\rightarrow$ 6 sialyltransferase were isolated from porcine submaxillary gland. The α 2 $\rightarrow$ 3 enzyme was extracted with TX-100, purified over 90,000 fold to homogeneity, and characterized as to acceptor specificity (55).

The best acceptors for the α 2 $\rightarrow$ 3 transferase contain the sequence gal β 1 $\rightarrow$ 3galNAc, while β 1 $\rightarrow$ 4 and β 1 $\rightarrow$ 6 isomers have high K_m and low V_{max} values. With glycoprotein acceptors this specificity was even more strict,

with incorporation into $\beta 1 \rightarrow 3$ linkages only. Thus enzyme is not responsible for the synthesis of $\text{NANA}\alpha 2 \rightarrow 3 \text{gal} \beta 1 \rightarrow 4 \text{glcNAc} \dots$ structures seen in serum type glycoproteins such as fetuin and α_1 acid glycoprotein.

This enzyme appears to be a single polypeptide chain of 50,000 daltons as determined by SDS-PAGE. However, when the purified enzyme is subjected to Sephadex G-200 chromatography in the presence of TX-100, two noninterconverting forms of 106,000 and 44,000 daltons are found whose Stokes radii are calculated to be 51 and 31 Å, respectively. Sucrose density gradient centrifugation reveals partial specific volumes of 3.1S and 3.5S, respectively. Assuming that the smaller form has a partial specific volume close to the standards used, these data are consistent with a single globular protein of 50,000 daltons, as determined by SDS-PAGE. The large Stokes radius and low sedimentation coefficient of the larger form indicates that this form is also a globular protein of 50,000 daltons which binds a large amount of low density lipid or detergent. Data from these experiments and sucrose density gradient centrifugation in D_2O allowed the authors to calculate that this form of the enzyme binds 1.18 grams of TX-100 per gram of protein while the smaller form binds none. Because both forms of the enzyme have very similar acceptor specificities and kinetic properties, the authors propose that the larger form represents the native enzyme, containing a small hydrophobic region extracted intact from the membrane, while the smaller form is a large proteolytic fragment of the native form.

α -N-Acetylgalactosaminide $\alpha 2 \rightarrow 6$ sialyltransferase, which is the major species found in porcine submaxillary gland, was also purified to the extent that the preparation contained no other sialyltransferase activity. This enzyme transfers sialic acid to the following structures (64):
I $\text{galNAc}\alpha \rightarrow \text{Thr}$, II $\text{gal} \beta 1 \rightarrow 3 \text{galNAc}\alpha \text{Thr}$, and III $\text{NANA}\alpha 2 \rightarrow 3 \text{gal} \beta 1 \rightarrow 3 \text{galNAc}\alpha \text{Thr}$.

Once sialic acid is transferred to structure I galactose can no longer be added (118). All further synthesis of the carbohydrate chain is terminated. Therefore, formation of structure II is dependent on the activity of galactosyltransferase being higher than that of the sialyltransferase. As expected, in ovine submaxillary glands, where structure I is most predominant, sialyltransferase activity is higher than that of galactosyltransferase while the reverse is true for porcine submaxillary glands (81), where structure II and its derivatives are the predominant species.

Physical characterization of sialyltransferases active against asialofetuin has been carried out by other workers. Since asialofetuin has both $\text{gal}\beta 1 \rightarrow 4 \text{glcNAc}$ and $\text{gal}\beta 1 \rightarrow 3 \text{galNAc}$ structures, it is unclear which enzyme(s) is being characterized. However the findings are still informative.

Liu et al. (54) found two noninterconverting forms of sialyltransferase in human placenta using Sephadex G-200 and concanavalin-A Sepharose column chromatography. Two forms were found, 200,000 and 80,000 daltons; the smaller form bound to ConA-Sepharose and could be eluted with 5% methylmannoside, indicating that this form is probably a glycoprotein of the N-linked type. The K_m 's for CMP-sialic acid are slightly different for the two forms, but the acceptor K_m 's are identical. It is not clear if these two forms represent enzymes of the same or different linkage specificities.

Human liver sialyltransferase active against asialofetuin exhibits multiple isoelectric forms with pI values ranging from 5 to 8.6 (108). While these multiple forms may represent several different sialyltransferases, it is possible that they are due to heterogeneity of sialylation, as has been found with many other enzymes.

2. Kinetics

Rat liver sialyltransferases are competitively inhibited by the reaction product, CMP, and by its analogs CDP and CTP (112). This inhibition was taken advantage of by Hill and co-workers for purifying three sialyltransferases, as discussed in the preceding section. On the other hand, UMP, UDP, and UTP stimulate enzyme activity up to twofold. It is unclear if this effect is due to protection of the substrate CMP-sialic acid from CMP-hydrolase activity (119) or if there is an allosteric site for UDP. Bernacki (112) argues for the latter because (a) CMP-NANA hydrolase, which is inhibited by low concentrations of UDP, has a pH optima of 9 while sialyltransferase assays are routinely carried out near neutrality and (b) UDP is the product of the preceding step in glycosylation--transfer of galactose from UDP-galactose to glcNAc or galNAc.

Paulson et al. (106) and Rearick et al. (55) characterized the kinetic properties of bovine colostrum β -D-galactoside α 2 $\rightarrow$ 6 sialyltransferase and porcine submaxillary gland β -D-galactoside α 2 $\rightarrow$ 3 sialyltransferase, respectively. Initial rate data were obtained by varying the concentrations of both CMP-sialic acid and acceptor, and then analyzed by the method of Dalziel (120). These studies suggest that both enzymes have an equilibrium random order mechanism.

3. Effect of Divalent Metal Ions and Chelating Agents

The effects of divalent metal ions and chelating agents on sialyltransferase activity has been investigated by a number of laboratories.

Generally, sialyltransferases from rat liver (111,112), calf kidney cortex (113), human liver (108) and placenta (54) exhibit some stimulation of activity in the presence of low concentrations of Mg^{2+} . In the presence of detergent, rat liver sialyltransferase exhibits an absolute

requirement for divalent metal ions (112), while calf kidney cortex enzyme behaves quite differently--in the absence of detergent it is stimulated by concentrations of divalent metal ions that are inhibitory in the presence of detergent.

In view of these results it is surprising that human liver and placental enzymes are stimulated by EDTA (54,108), while calf kidney cortex (113) and pork liver enzymes (109) are unaffected.

4. Serum Sialyltransferases

Serum sialyltransferases are usually assayed using desialyzed fetuin as acceptor (109-111,121,122). It is a soluble enzyme that is inhibited by detergents such as TX-100, sodium deoxycholate, and SDS, and by the sulfhydryl reagents N-ethylmaleimide and p-chloromecuro sulfonic acid (110). The enzyme is inhibited by divalent metal ions and stimulated by EDTA (111). This effect may be due to the high calcium concentrations in serum, which also inhibit the serum fucosyltransferases (97).

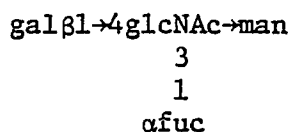
Klohs et al. (110) have shown that all the cytidine nucleotides act as competitive inhibitors with respect to CMP-sialic acid. Analogs such as 5'-fluorocytidine monophosphoric acid, cytosine- β -D-arabinofuranoside-5'-triphosphate, cytosine- β -D-arabinofuranoside-5'-monophosphoric acid, and cytidine 3':5'-cyclic monophosphoric acid are also competitive inhibitors for CMP-sialic acid. Shah and Ragupathy (111) report that all the other nucleotides inhibit enzyme activity and that the inhibition is directly related to the number of phosphates on the nucleotide. Klohs et al. (110) confirm these results and have shown that the inhibition is noncompetitive with respect to CMP-NANA.

VI. Sialylation vs Fucosylation

A. Synthetic Mechanisms

Bayer et al. (64) have examined some of the biosynthetic pathways

involved in the formation of nonreducing terminal sequences of O- and N-linked oligosaccharides by using two prototype glycoproteins--asialotransferrin, which contains the terminal sequence $\text{gal}\beta 1 \rightarrow 4 \text{glcNAc}\beta 1 \rightarrow 2(6) \text{man}$ and freezing point depression glycoprotein, which contains the terminal sequence $\text{gal}\beta 1 \rightarrow 3 \text{galNAc-O-Thr}$ --and their derivatives [see Fig. 12(a)] as substrates for six purified glycosyltransferases. In addition to the three sialyltransferases discussed in Sec. V. B and $\alpha 1 \rightarrow 2$ fucosyltransferase (discussed in Sec. V. A), they also purified the A-gene dependent N-acetylgalactosaminyltransferase from porcine submaxillary gland and human plasma and $\alpha 1 \rightarrow 3$ fucosyltransferase from human milk, which forms the structure



found in transferrin, lactoferrin, and α_1 -acid glycoprotein. These are the only N-linked proteins reported to contain this linkage. The authors hypothesize that this is the Le-dependent enzyme which forms both $1 \rightarrow 3$ and $1 \rightarrow 4$ linkages of the Lewis antigens since it has very similar properties to the $1 \rightarrow 4$ enzyme studied by Jarkovsky et al. (123).

The results of these studies are shown in Fig. 12(b). It is clear that fucosylation and sialylation are generally mutually exclusive events. Of the nine cases where a sialylated substrate was tested for fucosyltransferase acceptor activity or a fucosylated product was tested for sialyltransferase activity, six reactions were completely blocked and two others were strongly inhibited. Thus the observation by Dische (124) on the reciprocal nature of fucose and sialic acid content of glycoproteins now has an enzymatic basis.

There are three exceptions to this exclusivity. One, structure VII, which contains a sialic acid linked to N-acetylgalactosamine, is a good

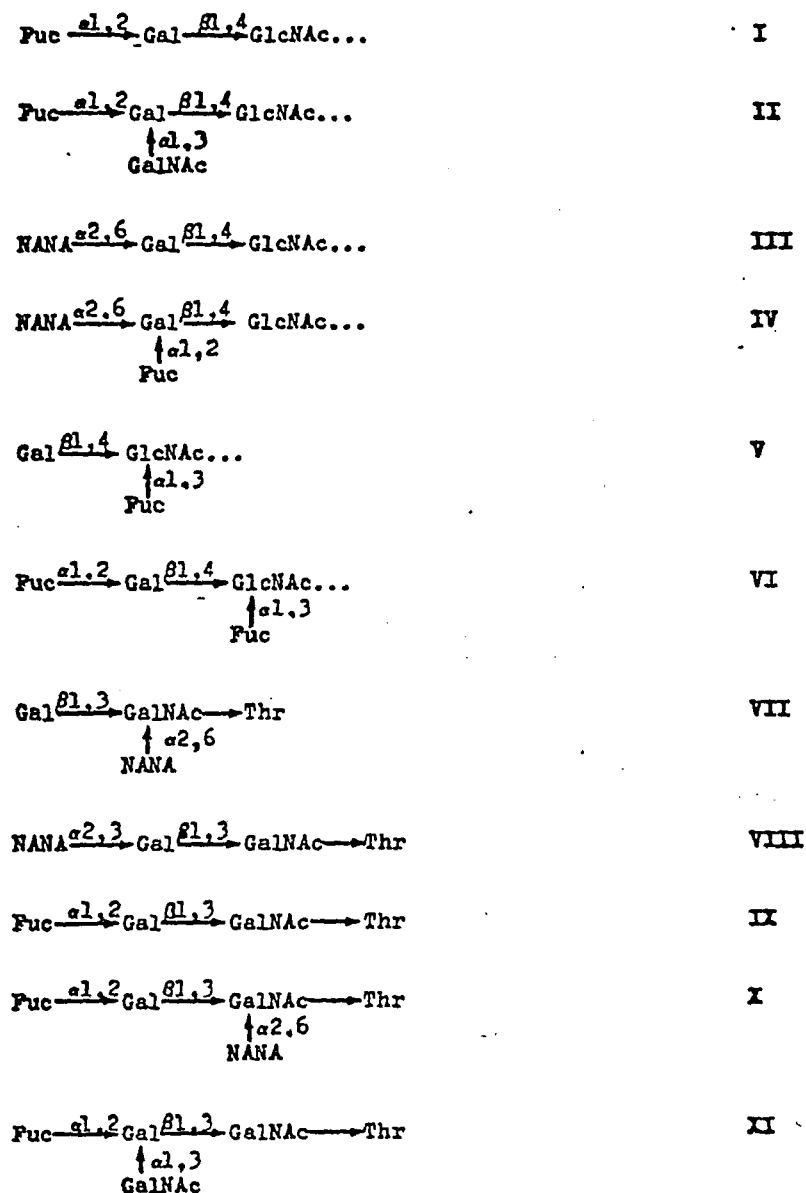


Fig. 12(a). Structures used in the study of glycosylation pathways by Beyer et al. (64).

Enzyme	Structure												
	AT	FP	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
$\alpha 1 \rightarrow 3$ FT	+	-	+	+	-	-	X	X	-	-	-	-	-
gal $\alpha 2 \rightarrow 6$ ST	+	-	$\pm$	-	X	X	-	-	-	-	-	-	-
$\alpha 1 \rightarrow 2$ FT	$\pm$	+	X	X	-	X	-	X	+	-	X	X	X
gal $\alpha 2 \rightarrow 6$ ST	-	+	+	-	-	-	-	-	X	+	$\pm$	X	-
gal $\alpha 2 \rightarrow 3$ ST	-	+	-	-	-	-	-	-	+	X	-	-	-
galNAcT	-	-	+	X	-	-	-	-	-	-	+	$\pm$	X

Symbols refers to moles of sugar incorporated per mole of galactose:
 + = >80; - = <10; $\pm$ = 50; X = product of reaction already present.

Abbreviations: AT, asialotransferrin; FP, freezing point depression glycoprotein; $\alpha 1 \rightarrow 3$ FT, N-acetylglucosaminide $\alpha 1 \rightarrow 3$ fucosyltransferase; gal $\alpha 2 \rightarrow 6$ ST, β -D-galactoside $\alpha 2 \rightarrow 6$ sialyltransferase; $\alpha 1 \rightarrow 2$ FT, β -D-galactoside $\alpha 1 \rightarrow 2$ fucosyltransferase; galNAc $\alpha 2 \rightarrow 6$ ST, β -N-acetyl-galactosaminide $\alpha 2 \rightarrow 6$ sialyltransferase; gal $\alpha 2 \rightarrow 3$ ST, β -D-galactoside $\alpha 2 \rightarrow 3$ sialyltransferase; galNAcT, β -D-galactoside $\alpha 1 \rightarrow 3$ N-acetyl-galactosaminyltransferase.

Fig. 12(b). Substrate specificities of six glycosyltransferases
 From Ref. 64.

surfaces has been shown to mediate the clearance of circulating plasma proteins by hepatocytes (see Sec. III.B.), homing of cells to target organs (125), clearance of senescent erythrocytes by the liver (43), binding of myxoviruses to erythrocyte membranes (126) and to salivary mucins (127), and binding of cholera toxin (128).

The function of fucosylation is almost totally unknown. Bombay donors, Le- individuals, and nonsecretors have no known clinical abnormalities, an indication that fucose, at least in 1→2, 1→4, and 1→3 linkages, is not essential for normal functioning. However, the vast majority of the population has H and Le a antigens and their derivatives, A, B, and Le b antigens, on their cells and secretions. Other mammals, pig, dog, and cat, among others, also exhibit blood group activity. Thus the question arises as to why the enzymes responsible for the synthesis of these antigens have been maintained in our genetic repertoire for so many generations of humans as well as through speciation.

The answers to these questions await further study but some speculations can be made. It can be assumed that fucose in 1→2, 1→3, and 1→4 linkage plays some important but auxiliary role. For example, the solubility characteristics of a glycoprotein can be altered by the nature of its glycosylation. Fucose, with a methyl group rather than a hydroxymethylene group at C-6 is relatively more hydrophobic than galactose, which in turn is more hydrophobic than sialic acid with its carboxyl group. Fucose may also play a role similar to that of sialic acid, i.e., preventing the uptake of glycoproteins by blocking the galactose terminal.

The function of the chitobiose-core fucose is unknown at this time and is ripe for study. This linkage is found in both soluble and membrane bound glycoproteins of many species. A large number of studies have been carried out to examine the levels of α 1→6 fucosyltransferase in the serum

acceptor for $\alpha 1 \rightarrow 2$ fucosyltransferase, and the product of this reaction has been reported in the literature from a variety of sources (13). However, structure IX, which is fucosylated, is a poor substrate for the N-acetylgalactosaminide $\alpha 2 \rightarrow 6$ sialyltransferase. This indicates the importance of the order of addition of the terminal sugars in forming the various structures found on glycoproteins. Two, from the work of Wilson et al. (104) it is clear that the presence of fucose on the chitobiose core does not prevent sialylation. This is because the fucose is added before the galactose is transferred. Three, even though structure I, which is only 50% fucosylated, is a substrate for β -D-galactoside $\alpha 2 \rightarrow 6$ sialyltransferase, the reaction requires 24 hr to reach saturation and is monophasic. This is particularly interesting because sialylation of asialotransferrin, which has a biantennary structure, is biphasic--50% of the sialic acid incorporation takes place within 10 min, while 24 hr is required to reach saturation. This may indicate that fucose may be present on the same arm of the biantenna that is subject to rapid sialylation. This would indicate that the substrate specificity of both enzymes may extend to the mannose-mannose bond, since all the linkages in the biantennary structure are identical except at the branch mannose. Alternatively, the slow sialyltransferase and fucosyltransferase reactions may simply be due to the presence of a sugar residue on the opposite arm.

It is now clear that in addition to the substrate specificities of the glycosyltransferases, the order in which they act determines the carbohydrate structures found in glycoproteins. The mechanisms that control these sequences are unknown.

B. Function of Sialylation and Fucosylation

The presence of sialic acid on glycoproteins, glycolipids, and cell

of patients with malignancies (see Sec. VII); no deficiency of this enzyme has been reported. It is possible that this linkage is involved in receptor-mediated events. For example, Remold (129) has reported that the presence of fucose in the culture medium blocks the activity of migration inhibition factor. Fucosidase treatment of the macrophages abolishes the response as well. This is the only documented evidence that fucose may play a physiologic role.

VII. Fucosyl- and Sialyltransferases in Health and Disease

A. Malignant Disease States

Alterations in serum fucosyl- and sialyltransferases have been noted in patients with malignant diseases along with concomitant rises in fucose (130) and sialic acid (131) content of serum glycoproteins.

Khianini et al. (132) and Kessel et al. (133) have reported that in patients with acute myelogenous leukemia the activity of serum $\alpha 1 \rightarrow 2$ fucosyltransferase is directly correlated with percent myeloblasts in bone marrow. Patients in remission have increased levels of $1 \rightarrow 6$ fucosyltransferase; this increase corresponds to periods of production of normal bone marrow. On the other hand, Bauer et al. (134,135) have reported increases in the levels of both fucosyltransferases in patients suffering from a wide variety of malignant diseases. The levels of enzyme activity were related to the extent of dissemination of the disease, and favorable response to chemotherapy and/or successful removal of the tumor resulted in decreased serum enzyme activity. In patients where surgical removal was incomplete or the metastatic process was extensive and refractory to chemotherapy, there was an initial decrease of activity after treatment followed by a rise to pre-therapy values.

Serum sialyltransferase activity against asialofetuin was also increased in patients with malignant diseases (136), including cancer of the

lung, breast, colon, and prostate as well as leukemias and melanomas. Here again, enzyme levels were proportional to the tumor load, with enzyme levels decreasing after successful therapies. Bernacki and Kim (131) have provided evidence that levels of sialyltransferase found in serum of rats injected with tumor lines correlated with the metastatic potential of each line and with sialyltransferase activity in the tumors themselves.

While the changes seen in patients with malignancies appear to be secondary in nature, all authors expressed the belief that they have clinical importance in following therapeutic effectiveness (130,132-136). It would also be of interest to determine if increased fucosylation and sialylation of serum proteins has any effect on the severity and course of the disease process, e.g., by augmenting metastatic invasion or by masking of the antigenic sites recognized by immune surveillance mechanisms.

The source of these serum transferases is a question that has invited a great deal of speculation. Most authors agree that the concordance between the degree of tumor burden and serum transferase levels point to the malignant tissue as the point of origin. Bauer et al. (134) propose that active secretion by rapidly growing tissue will result in increased plasma levels of the transferases, while Kim and Bernacki (131) propose that increased shedding of the cell surface is the cause of the increase. However, the data presented by these groups could be explained by either mechanism. Further research on this question is clearly required.

A. Cystic Fibrosis

Cystic fibrosis (CF) is the most common genetic disease among Caucasians in the United States, with an incidence of 1 in every 2000 live births. The disease is transmitted as an autosomal recessive trait,

but the basic defect is unknown. No clinical abnormalities are found in obligate heterozygotes. The disease is characterized by defects in exocrine gland function and by progressive pulmonary disease. Patients characteristically present with high sweat chlorides (>60 mEq/liter being diagnostic when other signs are present), failure to thrive, retarded onset of puberty, and pancreatic insufficiency. The most striking characteristics of the pulmonary disease are abnormally thick tracheobronchial secretions that block the airways and chronic staphylococcal and pseudomonas infections. Patients usually succumb to respiratory exhaustion in the second or third decade of life. It is unclear if the disease process involves hypersecretion of mucus (normal or abnormal) or if patients are unable to clear the thick mucus at normal rates.

Because of the striking nature of CF mucus, many workers have postulated that the basic defect in CF relates to defective glycoprotein biosynthetic and degradative mechanisms. Much of the data concerning this disease is highly contradictory, and this area of work has not proved to be an exception.

Early in these studies, several laboratories reported alterations in fucose content and fucose/sialic acid ratios of tracheobronchial secretions (137) and duodenal fluid (138,139). However, Boat et al. (140) studied the carbohydrate composition of mucous glycoproteins secreted by cultured nasal polyps that had been surgically removed from a patient with cystic fibrosis and from a patient with allergic rhinitis. While no striking differences were seen in carbohydrate composition, CF mucous glycoproteins were more heavily sulfated. Boat et al. (141) also studied the composition of purified CF tracheobronchial tract mucin and again the only difference found was in the degree of sulfation.

There are indications that glycosylation of serum proteins may be altered in CF but again the work of different laboratories is contradictory. Pearson and Lubin (142) have recently reported that cystic fibrosis serum precipitates more heparin from solution than does normal sera. Analysis of the precipitated protein reveals that glycoproteins from patients with CF contained, on average, 27% more fucose, 27% less sialic acid, and 31% less hexose; the carbohydrate composition of total serum for both normal individuals and patients with cystic fibrosis was the same. The degree of abnormality in carbohydrate content was related to the severity of disease, indicating that these alterations may be secondary. There are reports from other laboratories (92,143-146) that indicate altered glycosylation of serum-type glycoproteins; however, these data are soft, and often, one report directly contradicts another. Hunt and Summers (147) have shown that when vesicular stomatitis virus is grown in CF fibroblasts, G protein is normally glycosylated. This would be presumptive evidence that glycosylation mechanisms themselves are normal. However, in vivo there may be effector mechanisms that alter glycosylation patterns.

Glycosidases have also been the target of scrutiny by a number of groups. Glycosidase activities in CF tissue and serum have been variously reported to be altered (148), decreased (143,149), and normal (150,151). There is also a report that they are abnormal with respect to cell uptake --these results are directly contradicted by findings of normal tissue levels. There is at present no explanation for the disparities in these results.

Louisot et al. (92) have determined that galactosyl-, mannosyl-, and fucosyltransferase activities toward endogenous acceptors in surgical lung

specimens from nine patients with cystic fibrosis are at least twice those of normals. Levels of protein synthesis were normal, based on incorporation of labeled amino acids. This does not rule out the possibility that in tissue from patients with cystic fibrosis there is increased synthesis of a particular protein that acts as a glycosyltransferase acceptor with a compensatory decrease in others. However, galactosyltransferase and mannosyltransferase activity toward exogenous acceptors are also increased, indicating that there is a real alteration in enzyme activity. The authors have proposed that increased glycosyltransferase activity is the basis of this disease. There are no indications at this time that serum fucosyl- or sialyltransferases are significantly altered in patients with cystic fibrosis (152, 153).

In summary, no definitive statement can be made concerning glycosylation mechanisms in cystic fibrosis. The contradictory results reported by investigators in this area lead one to speculate that any alterations seen are secondary in nature and are probably related to variations in the genetic backgrounds and environments of the patients rather than to the disease process itself. It would probably be most useful for investigators to exchange specimens, cell lines, etc., in an attempt to clarify the various reports that muddy the water of CF literature.

VIII. Objectives

The objective of this project was to characterize the α 1 $\rightarrow$ 2 and α 1 $\rightarrow$ 6 fucosyltransferases in lymphoid lines and to compare their biochemical properties with those of sialyltransferases in this tissue. A study of the enzyme activities on cells derived from donors with various metabolic defects, particularly cystic fibrosis, was also carried out.

Little attention has been paid to characterization of these enzymes in human tissue, particularly the membrane bound forms. There is to this date no extensive study of human α 1 $\rightarrow$ 6 fucosyltransferase from any source other than serum. α 1 $\rightarrow$ 2 fucosyltransferase has been studied mainly in secretory tissue, i.e., tissue that secretes blood group substances. However, H antigen is present on the surface of almost all cells, muscle and connective tissue being the major exceptions; thus a study of this enzyme in nonsecretory tissue is in order.

A comparison of fucosyl- and sialyltransferase activities is particularly relevant in light of Dische's and Hill's observations concerning the reciprocal nature of fucosylation and sialylation. This is especially true since no study has been undertaken comparing the properties of these enzymes in the same human tissue. Therefore the relative activities, differential response to divalent metal ions, effect of sulfhydryl reagents, and kinetic properties of these enzymes were examined.

A. Experimental Rationale

1. Tissue

Lymphoid lines were chosen as the tissue source for a number of reasons. Firstly, fresh tissue from a single donor, grown under controlled conditions would be available for the length of the project. Lymphoid cells grow quickly, doubling in number every 24-48 hr, to a density of 10^6 cells/ ml of culture media. Thus sufficient tissue would be available at all times. Secondly, there is good evidence that the genetic constitution of the donor is maintained in lymphoid lines grown in continuous culture (154). Thus these cells would be suitable for studying glycosyltransferases from patients with a variety of metabolic diseases. Thirdly, the presence of fucose in α 1 $\rightarrow$ 6 linkage to that chitobiose core of immunoglobulins, a major secretory protein of lymphoid cells, made it likely that these cells would be a good source of α 1 $\rightarrow$ 6 fucosyltransferase. The other enzymes under study would also be present in sufficient quantities.

2. Choice of Acceptors

Fetuin treated to remove sialic acid and/or galactose is the most suitable acceptor for these studies. In addition to its commercial availability at reasonable cost, fetuin derivatives have been used by many investigators to study these enzymes and thus their work could be extended and confirmed.

Fetuin has both O- and N-linked oligosaccharide chains that terminate

with sialic acid linked to galactose. These structures, which are almost identical to those found in the immunoglobulin molecule, are shown in Fig. 11. Beyer et al. (64) have shown that once sialic acid is removed, the resultant structures can act as acceptors for the $\alpha 1 \rightarrow 2$ fucosyltransferase and for the β -D-galactoside sialyltransferases. Although resolution of individual sialyltransferases is not possible with asialofetuin, total enzyme activity could be determined. For our purposes, this was deemed adequate.

Asialofetuin that has been subjected to one round of Smith degradation, which results in destruction of the terminal galactose, is a specific acceptor for the $\alpha 1 \rightarrow 6$ fucosyltransferase (104).

Porcine submaxillary gland mucin treated to remove sialic acid and fucose was used occasionally as acceptor for the $\alpha 1 \rightarrow 2$ fucosyltransferase because it was shown by Kim et al. (96) to be absolutely specific for the formation of the $\alpha 1 \rightarrow 2$ linkage.

3. Assay Procedure

Glycosyltransferases are assayed by using as substrate a nucleotide sugar radioactively labeled in the sugar moiety. The amount of radioactivity incorporated into an exogenous glycoprotein acceptor is considered a measure of enzyme activity. Endogenous acceptor levels can be determined by measuring the amount of radioactive label incorporated in the absence of exogenous acceptor. A number of investigators have used incorporation of a particular sugar into endogenous acceptors as a measure of transferase activity. This is a most unsatisfactory method of measuring a particular transferase activity in view of the multiplicity of enzymes and acceptors involved. This procedure can also result in erroneous activity levels since the amount of endogenous acceptor may be

a limiting factor.

One of the major stumbling blocks to the study of these enzymes has been the lack of a reliable and rapid method for separating the labeled acceptor molecule from the unreacted nucleotide sugar. Many investigators have used paper electrophoresis and ion exchange chromatography. Others have precipitated the acceptor glycoprotein with trichloroacetic acid (TCA) and counted the resulting pellet. This method involves numerous centrifugations and presents difficulties in freeing the pellet of trapped nucleotide sugar, particularly if the protein concentration is high. All these methods are very time-consuming and cumbersome, so that the processing of large numbers of samples is very difficult.

An assay method that is generally applicable for assaying glycoprotein transferase activity was developed. TCA is added to the reaction mixture and the precipitate applied to glass fiber filters, which are thoroughly washed and then counted. The radioactivity remaining on the filter represents the transfer of radioactive sugar to glycoprotein acceptor. The assay is rapid--each reaction tube taking about two minutes to process completely, thus a large number of samples can be handled at one time.

Once the problem was solved, optimal assay conditions could be determined. In addition to investigating the usual factors of optimal substrate and enzyme concentration, incubation time and pH, there are several others that are peculiar to glycosyltransferases. In common with other enzymes that have nucleotide substrates and cofactors, most glycosyltransferases require a divalent metal ion for optimal activity. Thus it is necessary to determine which ion is required, its optimal concentration, and the effect of other divalent metal ions.

In order to detect activity of cellular glycosyltransferases in

vitro, the presence of detergent is required. These enzymes are located on the inner face of the golgi membrane (155), and detergent is required to disrupt the membrane sufficiently to allow the exposure of the catalytic sites to substrates. A major drawback of the studies reported in the literature is that isolated microsomes were used as enzyme source and detergent was added only during the reaction period, a procedure which leaves the efficiency of the detergent treatment unknown.

Enzymes that have been solubilized are preferable because the catalytic sites are more available during the entire reaction period and because some partial purification is obtained. A method of enzyme preparation has been developed that allows efficient solubilization and maximal activity of both fucosyltransferases and is discussed in detail on p.100. Unfortunately, the sialyltransferases proved refractory to a large variety of solubilization methods and so they had to be examined in their membrane bound state.

4. Disease States

It is unknown if alterations of fucosyl- and sialyltransferase activity represent a basic defect in any disease state. In light of proposals that cystic fibrosis is the result of a basic defect in glycosylation enzymes, the activities of these enzymes in cells from homozygotes and obligate heterozygotes for cystic fibrosis were examined. The multiplicity of symptoms found in this disease make it imperative that as many factors as possible be controlled for when trying to distinguish between primary and secondary effects. The use of cultured cells, as opposed to tissue obtained directly from patients, presents certain advantages in this regard. All cells can be grown under identical conditions, eliminating differences due to humoral factors, e.g., hormones, which have been shown to affect fucosyltransferase activity, severity of disease,

and altered nutritional status. The last-mentioned is particularly relevant in studies of cystic fibrosis, where one of the major symptoms is malabsorption. Since the fucosyl- and sialyltransferases are expressed in lymphoid lines, alterations in their activities should be detectable in these cells.

MATERIALS AND METHODS

I. Materials

GDP-[$^{14}\text{C}(\text{U})$]-fucose (specific activity 112-120 mCi/mmol), CMP-[4,5 6,7,8,9- ^{14}C]sialic acid (s.a. 218 mCi/mmol) and OCS scintillation fluid were obtained from Amersham. Fetuin (Type III) and deoxycholate was obtained from Sigma. Porcine submaxillary glands were from Pel-Freeze. Whatman GF/A 2.4 cm glass fiber filters were used throughout. RPMI 1640, fetal calf serum, penicillin, streptomycin, glutamine, and trypsin were all supplied by GIBCO. Plasticware for tissue culture was obtained from Falcon. All other reagents were of analytic grade and supplied by commercial sources.

II. Methods

A. Standard Preparation of Cellular Homogenates

The cells were suspended in 20 mM HEPES, pH 7, 0.13 M NaCl, 0.5 mM MgCl_2 (10 ml of buffer per milliliter of packed lymphoid cells or 2.5 ml of buffer per 75 cm^2 flask of fibroblasts). An equal volume of 0.5 M sucrose was then added. This was essential since the lymphoid cells would clump and become gelatinous if sucrose was added first.

The cell suspension was homogenized using 20 strokes of the tight-fitting pestle in a Dounce homogenizer. The homogenate was spun at 17,500 rpm at 4°C for 30 min (37,000 x g) in a Sorvall Model RC-5. The resulting supernatant fraction (S_1), having no fucosyltransferase activity, was routinely discarded.

The membrane pellet (P_1) was resuspended in the appropriate detergent (5 ml detergent per milliliter of packed lymphoid cells or 0.5 ml per 75 cm² flask of confluent fibroblasts) using 20 strokes of a ground glass homogenizer.

For fucosyltransferase studies, P_1 was resuspended in 0.3% deoxycholate (early studies) or 0.3% deoxycholate plus 0.1% TX-100, and then centrifuged again under the conditions outlined above. The supernatant fraction, S_2 , was found to contain almost all the fucosyltransferase activity; this fraction was used for all studies unless otherwise stated. If the pellet, P_2 , was to be assayed as well, an amount of detergent equivalent in volume to S_2 was added and the pellet resuspended using 20 strokes of a ground glass homogenizer.

Cellular homogenates for sialyltransferase studies were prepared in much the same way with the following exceptions: 0.5% Cutscum was used to resuspend P_1 , and it was this suspension that was used for all assays.

All fractions could be stored for at least 1 month at -20°C with no loss in activity.

B. Preparation of Sera

Whole blood was allowed to clot for 1 hr at room temperature, centrifuged at low speed, and the serum removed and stored at -80°C. No loss of any enzyme activity could be detected upon repeated freezings and thawings.

C. Preparation of Acceptors

1. Asialofetuin (AS)

Fetuin was desialylated by mild acid hydrolysis, following the method of Spiro (156). Fetuin was dissolved in 0.05 N H₂SO₄ (4 mg/ml) and incubated at 80°C for 1 hr. The solution was then neutralized with

1 N NaOH, dialyzed extensively against distilled water, and lyophilized.

2. Asialo,agalactofetuin (ASAG)

Asialofetuin was prepared as described above. The terminal galactose residue was removed by the method of Spiro as modified by Kim et al. (157). Oxidation of the terminal galactose was carried out in a solution of 0.01 M sodium meta-periodate in 0.05 M acetate buffer, pH 4.5. The protein concentration was approximately 5 mg/ml and the periodate to protein ration 100:1. The reaction was allowed to proceed in the dark for 24 hr at 4°C and was terminated by the addition of excess glycerol. The product was dialyzed extensively against distilled water and reduced using sodium borohydride (0.15 M in a 0.15 M sodium tetraborate buffer, pH 8). The molar ratio of protein to borohydride was 2000:1. The reaction was allowed to proceed at 4°C for 13 hr and was terminated by the addition of 1 N acetic acid until the pH was lowered to 5. The reaction mixture was then dialyzed for several days against distilled water. The protein solution was adjusted to 0.05 N H₂SO₄ and incubated at 80°C for 1 hr. The mixture was immediately neutralized with 1 N NaOH, dialyzed against distilled water, and lyophilized.

3. Porcine Submaxillary Gland Mucin (PSM)

Porcine submaxillary gland mucin was prepared by the method of de Salegui and Plonska (158). The glands were shipped frozen in dry ice and stored at -80°C. The glands were trimmed of all gross fat and connective tissue immediately before use.

In the following description, the volume of 1 g of wet tissue equals 1 ml. All procedures were carried out at 4°C unless otherwise stated.

The glands were homogenized (using a Virtis homogenizer) in 3 volumes of ice-cold 0.01 M Tris buffer, pH 7.6. Three more volumes of buffer

were then added, the mixture stirred for 15 min, and centrifuged for 20 min at 20,000 x g.

The supernatant was strained through cheesecloth, the residue re-extracted twice with three volumes of buffer, and the combined supernatants acidified to pH 4.6 by adding 2 M acetate buffer, pH 3.8, with vigorous stirring. The solution was centrifuged at 37,000 x g for 30 min and the pellet discarded. These last steps were carried out quickly to limit lysosomal hydrolase activity. One volume of 10% Cetavalon (Eastman Kodak) was slowly stirred in and a clot formed almost immediately.

After standing overnight, the clot was washed twice with four volumes of distilled water and then dissolved in 40 volumes of 0.154 M NaCl. The solution containing the dissolved clot was then brought to pH 4.8 by adding 0.5 N sodium acetate buffer, pH 5.1. Wet packed CM-25 Sephadex (Pharmacia) swollen in distilled water (0.2 volumes) was added. The mixture was stirred for 1 hr and centrifuged at 10,000 rpm for 20 min. Wet packed CM-50 Sephadex (Pharmacia) was added to the supernatant until the solution was colorless. The mixture was spun again and the supernatant brought to a final concentration of 48% ethanol and left in the cold for 48 hr. The resulting precipitate was removed by centrifugation at 37,000 x g for 20 min and discarded. The supernatant was brought to 54% ethanol and solid NaCl was added until a visible precipitate formed. This mixture was allowed to stand for several hours, and the precipitate was then collected by centrifugation at 15,000 rpm. The pellet was dissolved in distilled water, dialyzed extensively against distilled water, and lyophilized.

4. Desialylated, Defucosylated PSM

About 10 mg of PSM was dissolved by adding distilled water dropwise

until the solution was homogeneous and no gel-like formations could be seen (approximately 6-7 drops). The mixture was brought to 0.05 N H_2SO_4 (1.5 ml/10 mg), incubated at 85°C for 90 min, and immediately neutralized with 0.05 N NaOH. The hydrolyzed mucin was extensively dialyzed against distilled water and lyophilized.

D. Assay Methods

1. Cellular Fucosyltransferase Assay

Enzyme assays were carried out in 12 x 75 mm plastic tubes containing 10 mmoles HEPES, pH 7.2, 2 mmoles MgCl_2 , 0.5 mmoles UTP, 0.75% TX-100, 0.5 mg of acceptor, 0.4 mmoles GDP- $^{14}\text{C}(\text{U})$ -fucose (68,000 cpm), 50 μl of enzyme source in 0.1% TX-100 and 0.3% DC in a total volume of 200 μl . $\alpha 1 \rightarrow 2$ activity was measured using asialofetuin as the fucose acceptor and $\alpha 1 \rightarrow 6$ activity was determined using asialoagalactofetuin.

After incubation at 37°C for 1 hr, the reaction was stopped by plunging the assay tubes into ice water and adding 20 μl of 100% TCA to each tube. The tubes were then allowed to stand on ice for 20 min to ensure complete precipitation. Three milliliters of 10% TCA was then added to each tube; the precipitates were then collected on Whatman glass fiber filters (GF/A, 2.4 cm) under vacuum. Each tube was rinsed three times with 3-ml aliquots of 10% TCA. The filters were then washed thoroughly with additional 10% TCA and with a small amount of methanol. The filters were thoroughly dried, placed in scintillation vials containing 10 ml of OCS (Amersham Searle), and counted in a liquid scintillation counter.

It was determined that endogenous activities in lymphoid lines and fibroblasts were never greater than 5% of total exogenous activity; therefore, this determination was omitted in all assays of these tissues

unless otherwise stated.

Blanks consisted of all assay components except enzyme source, which was added after incubation and immediately prior to addition of 100% TCA.

2. Serum Fucosyltransferase Assays

Serum fucosyltransferase activities were determined by the method of Chou et al. (97) which is similar to the assay for the cellular enzyme but with several modifications. The assay buffer was MES, pH 6, TX-100 was replaced by 2 mmoles EGTA (included because serum calcium levels were inhibitory to these enzymes, at least under the conditions employed), and 50 μ l of serum was used as the enzyme source.

The enzyme reactions were terminated as described previously; however, instead of adding 10% TCA to each tube, 1 ml of acetone was used. The precipitates were spun down and the supernatants discarded. Two milliliters of acetone was added to each pellet, and the precipitate collected on a glass fiber filter as described previously, except that the methanol wash was omitted. This additional step of adding acetone apparently extracts lipid-associated material that would otherwise clog the filters and make it difficult to wash the precipitate properly.

Endogenous activities were determined for each sample by omitting exogenous acceptor during the reaction period. However, in order to keep the protein concentrations equivalent, exogenous acceptor was added to the reaction mixture after addition of the 100% TCA. Blanks consisted of all assay components except serum, which was added immediately prior to the 100% TCA.

3. Cellular Sialyltransferase Assay

Enzyme assays were carried out in 12 x 75 mm plastic tubes in a

final assay volume of 300 μ l containing 10 mm MES, pH 6.0, 2 mmoles MgCl_2 , 1.0 mg of asialofetuin, 0.115 nmoles of CMP-N-acetyl-[4,5,6,7,9- ^{14}C] neuraminic acid ($\sim 23,000$ cpm/tube), and 50 μ l of enzyme source in 0.5% Cutscum. After incubation for 1 hr at 37°C , the reaction was stopped by plunging the tubes into ice water and adding 25 μ l of 100% TCA. The tubes were allowed to stand on ice for 20 min; 3 ml of ice-cold 10% TCA was then added to each tube. The precipitates were collected and counted as described for the fucosyltransferase assays.

Blanks were prepared in the same way as those for the fucosyltransferases assays, and the same comments apply to endogenous activity determinations.

4. Serum Sialyltransferase Assays

Serum sialyltransferase activities were determined in much the same way as for the cellular enzyme with the following modifications: 2 mmoles of EGTA were added to the reaction mixture and 50 μ l of serum was used as the enzyme source.

The enzyme reactions were terminated as described for the cellular enzyme except that 2 ml of acetone was added instead of 10% TCA. The precipitates were spun down, the supernatants discarded, and 2 ml of acetone added to the pellet. The precipitates were then collected on glass fiber filters but the methanol wash was omitted.

Determination of endogenous activities and preparation of blanks were essentially the same as described for the serum fucosyltransferases.

E. Protein Determinations

Protein content was estimated in two ways. At the beginning of this project, the method of Lowry et al. (159) was used. Later, the fluorescamine protein assay, using the method of Udenfriend et al. (160), was

employed because this method is considerably faster, more reproducible, and more economical with respect to use of sample than the Lowry method.

The fluorescamine assay as done in this laboratory used dry acetone-trile as solvent for the Fluram reagent, and a Turner flurometer equipped with a Corning 7-51 (390 nm peak) as the primary filter and a Wratten #4 1 or 10% as secondary filters. Bovine serum albumin was used as standard in all cases.

F. Cell Culture

Both lymphoid lines and normal skin fibroblasts were grown under sterile conditions at 37°C in RPMI 1640 supplemented with 16% fetal calf serum, 1% 200 mM glutamine, and 100 µg/ml each of penicillin and streptomycin. All cultures were checked for microbial contamination, and contaminated flasks discarded immediately. For details on the establishment of lymphoid lines and fibroblast strains, see Refs. 158 and 159.

1. Lymphoid Lines

Lymphoid lines were grown by doubling the cell suspension volume every 2 to 3 days with complete medium. Cells to be harvested were fed and incubated overnight. The cells were spun down and the medium discarded; the pellet was washed three times with normal saline. If the pellet was not used immediately, it was frozen at -80°C. Cells were thawed on ice before use.

It should be noted that unless otherwise stated NB 83 was used for all studies involving lymphoid cells as the enzyme source.

The following is a list of all lymphoid lines used in this project, along with a description of each donor.

<u>Line</u>	<u>Donor</u>
EBV 13	obligate heterozygote for cystic fibrosis, female

<u>Line</u>	<u>Donor</u>
EBV 14	obligate heterozygote for cystic fibrosis, female
EBV 15	cystic fibrosis, male
NB 16	obligate heterozygote for cystic fibrosis, female
EBV 17	cystic fibrosis, male
EBV 19	cystic fibrosis, male
NB 41	fucosidosis, male
NB 49 AD	obligate heterozygote for fucosidosis, female
NB 69	normal sib of patient with B ₆ responsive cystathioninuria
NB 75 AD	hypercholesterolemia Type II
NB 77 D	B ₆ responsive cystathioninuria
NB 78 AD	hypercholesterolemia Type III, female
NB 83	obligate heterozygote for homocystinuria (B ₆ responsive), female
NB 84 A	adult onset acid maltase deficiency, female
NB 94 A	Sanfilippo syndrome, male
NB 95 BD	cystathioninuria, B ₆ responsive
NB 104	hypercholesterolemia, female
NB 118	balanced translocation carrier, 46 XX t(5;7) (p15;p15)

2. Fibroblasts

Fibroblast cultures were grown in 75 cm² Falcon flasks and fed with complete media twice a week. If the cells were to be passaged, they were removed when confluent with trypsin and replated at a ratio of 1:4. Otherwise they were removed using 0.02% Na₂EDTA in normal saline. The

cells were spun down and the cell pellet washed three times with normal saline; the pellet was stored at -80°C if not used immediately.

All fibroblast cultures used in this project were grown as described for each experiment.

The following is a list of all fibroblast strains used in this project along with a description of the donor.

<u>Strain</u>	<u>Donor</u>
GM 5080	Normal female
GM 5832	Normal female
GM 770	CF male
GM 1957	CF male
GM 1959	CF male sibs
GM 1348	CF female

RESULTS

I. Optimization of Fucosyltransferase Assay

A. Determination of pH Optima

1. $\alpha 1 \rightarrow 2$ Fucosyltransferase

Three buffer systems were used to determine the optimal pH for the $\alpha 1 \rightarrow 2$ fucosyltransferase, all at a final concentration of 50 mM: cacodylate HCl, pH 5-7; HEPES, pH 6.5-8.5, and Tris-maleate, pH 5.5-8.5.

The optimal pH was found to be pH 6.5 and 7.0 when HEPES buffer was used and between 6.0 and 7.0 for cacodylate, although the activity at any given pH was not as high as that seen with HEPES. Tris-maleate strongly inhibited at all pH values and gave variable results in terms of an optimum over the pH range used (Fig. 13).

2. $\alpha 1 \rightarrow 6$ Fucosyltransferase

The same buffer systems were used to determine the pH optimum of the $\alpha 1 \rightarrow 6$ fucosyltransferase (Fig. 14). Unlike the $\alpha 1 \rightarrow 2$ fucosyltransferase, the activity of this enzyme did not appear to be significantly affected by the buffer used. Maximal activity was seen at pH 7.0 with HEPES and again Tris-maleate was strongly inhibitory over the entire pH range.

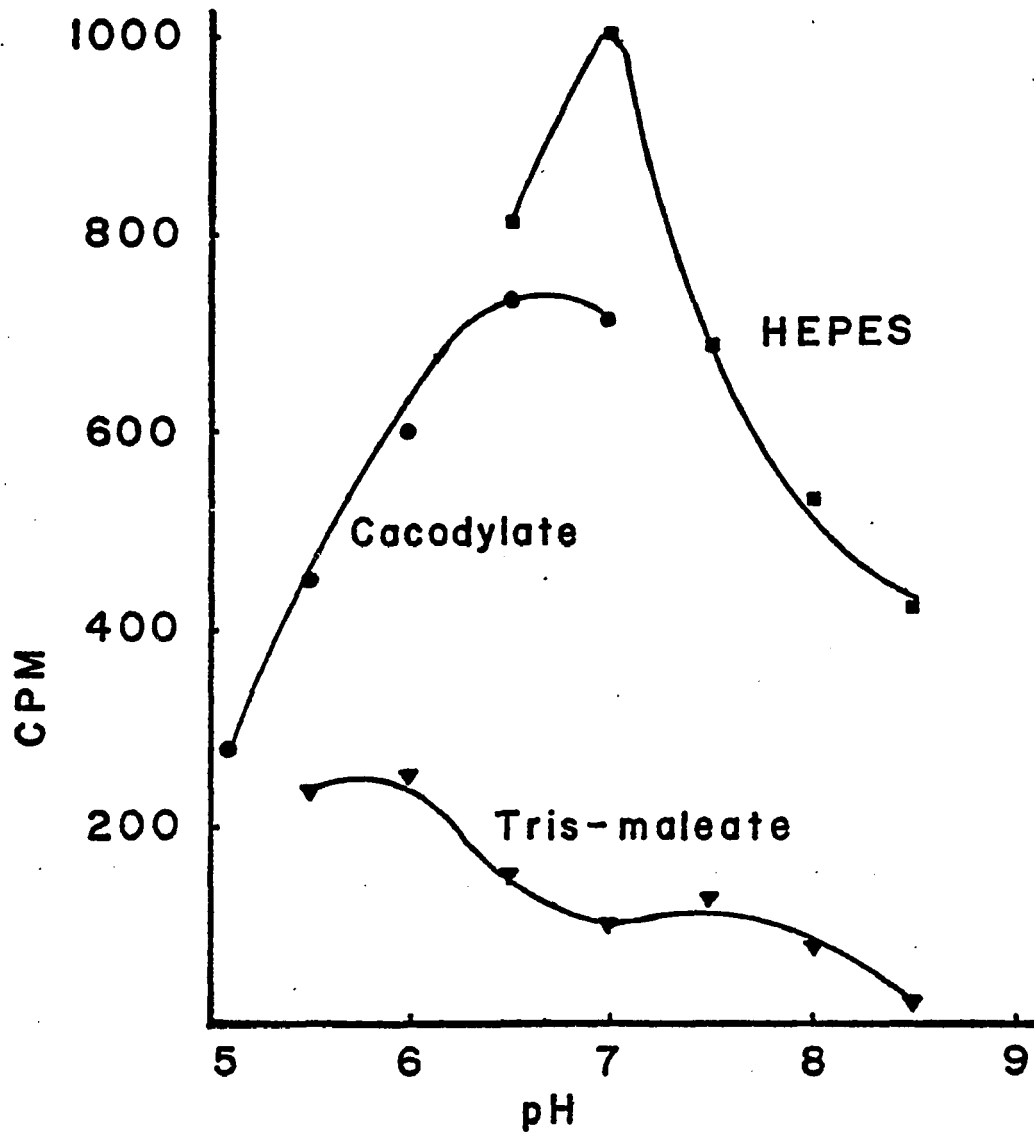


Fig. 13. Effect of pH on $\alpha 1 \rightarrow 2$ fucosyltransferase activity.

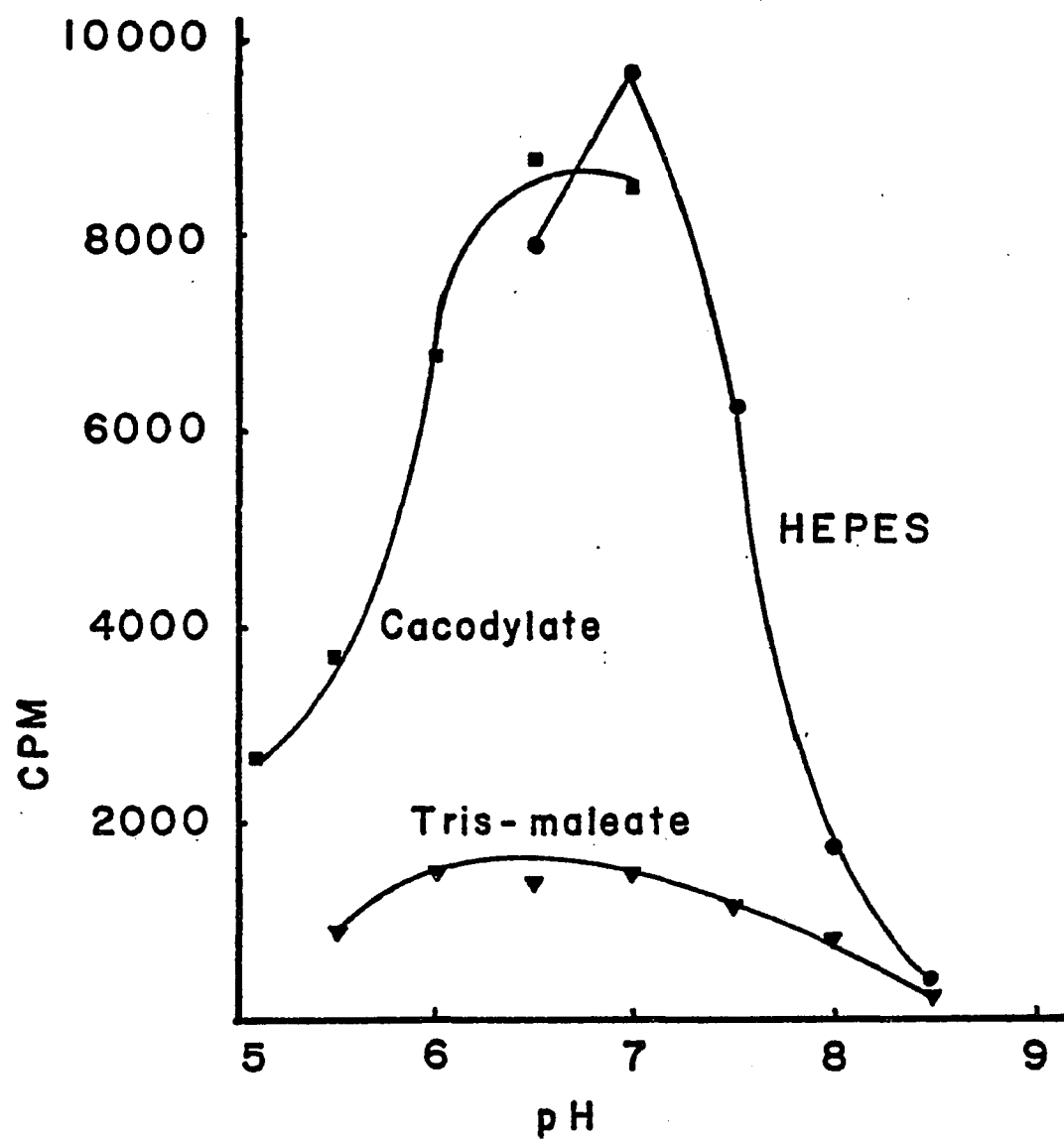


Fig. 14. Effect of pH on 1→6 activity.

As a result of these two experiments, 50 mM HEPES, pH 7, was subsequently used in all assays for these two enzymes.

B. Effects of Divalent Metal Ion

The effect of MgCl_2 , MnCl_2 , and CaCl_2 concentration on $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ enzyme activity is shown in Table 4 and Fig. 15. It should be noted that the $\alpha 1 \rightarrow 2$ enzyme showed little requirement for divalent metal ions, although its activity was stimulated by low concentrations of Mg^{2+} and Ca^{2+} . The $\alpha 1 \rightarrow 6$ enzyme exhibited a dramatic increase in activity in the presence of relatively low concentrations of all three ions. Subsequently, both enzymes were assayed with 10 mM MgCl_2 .

C. Effect of Chelating Agents

In order to determine the nature of the effect of divalent metal ions on enzyme activity, various concentrations of disodium EDTA, pH 7.0, were substituted for divalent metal ion (Table 4).

$\alpha 1 \rightarrow 2$ fucosyltransferase exhibited a small increase in activity at 20 mM EDTA, which was probably not significant. On the other hand, a 33% decrease in activity of the $\alpha 1 \rightarrow 6$ enzyme was seen at 5 mM; this inhibition persisted up to 25 mM. The data were consistent with the response of these enzymes to divalent ions.

D. Effect of Varying Substrate Concentration

1. GDP-Fucose

The effect of varying GDP-fucose concentration on $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ fucosyltransferase activity is shown in Figs. 16 and 17, respectively.

Table 4

Effect of Divalent Metal Ions

Enzyme	Ion	Activation	Inactivation
$\alpha 1 \rightarrow 2$	Mg^{2+}	Slight, 1-10 mM	>10 mM
	Mn^{2+}	None	All conc.
	Ca^{2+}	Slight, 1 mM	>10 mM
	EDTA	Slight, 20 mM	>20 mM
$\alpha 1 \rightarrow 6$	Mg^{2+}	Optimum, 5 mM	>20 mM
	Mn^{2+}	1 mM	> 5 mM
	Ca^{2+}	1-10 mM	>20 mM
	EDTA	None	> 5 mM

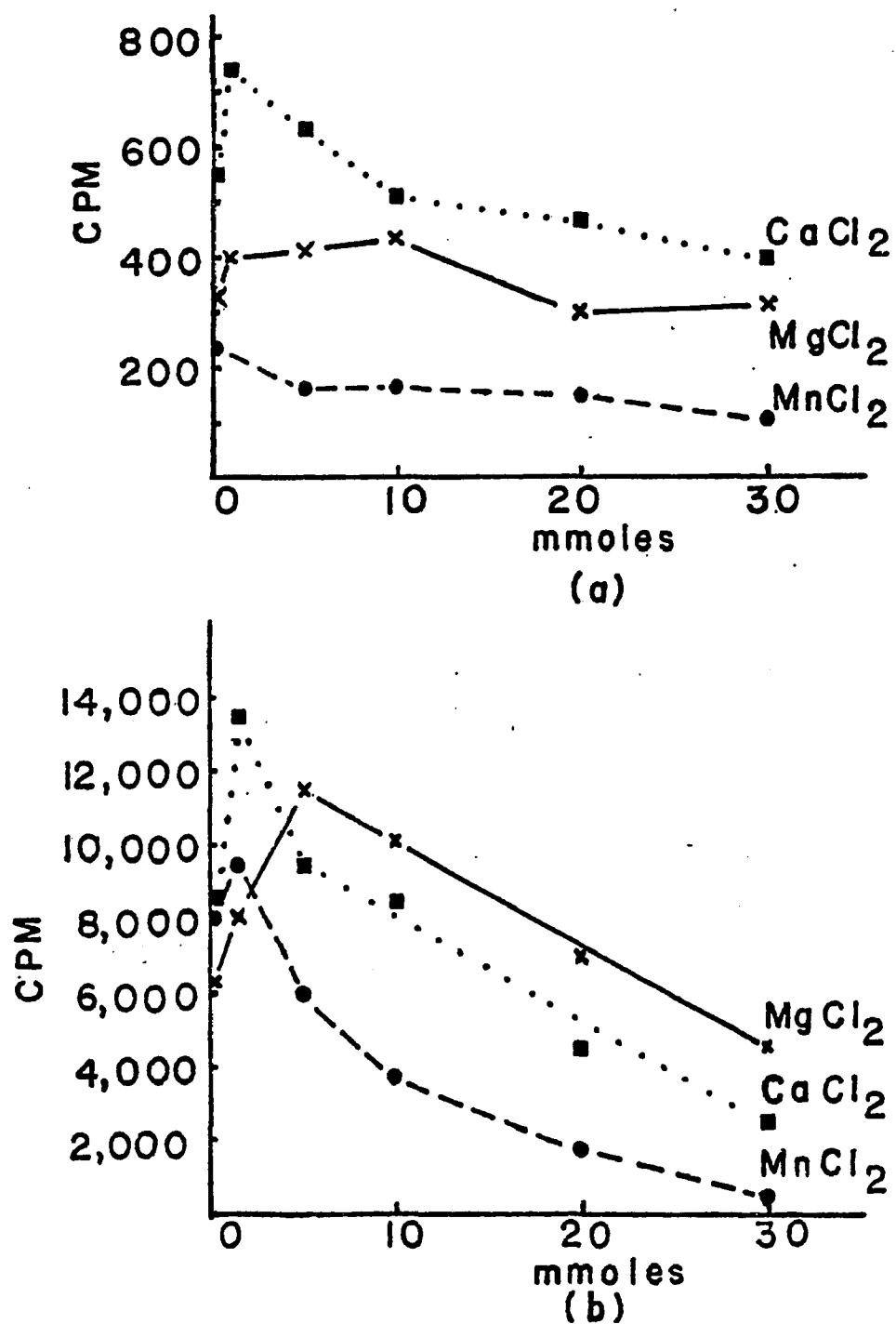


Fig. 15. Effect of divalent metal ions on fucosyltransferase activity. (a) $\alpha 1 \rightarrow 2$ activity. (b) $\alpha 1 \rightarrow 6$ activity.

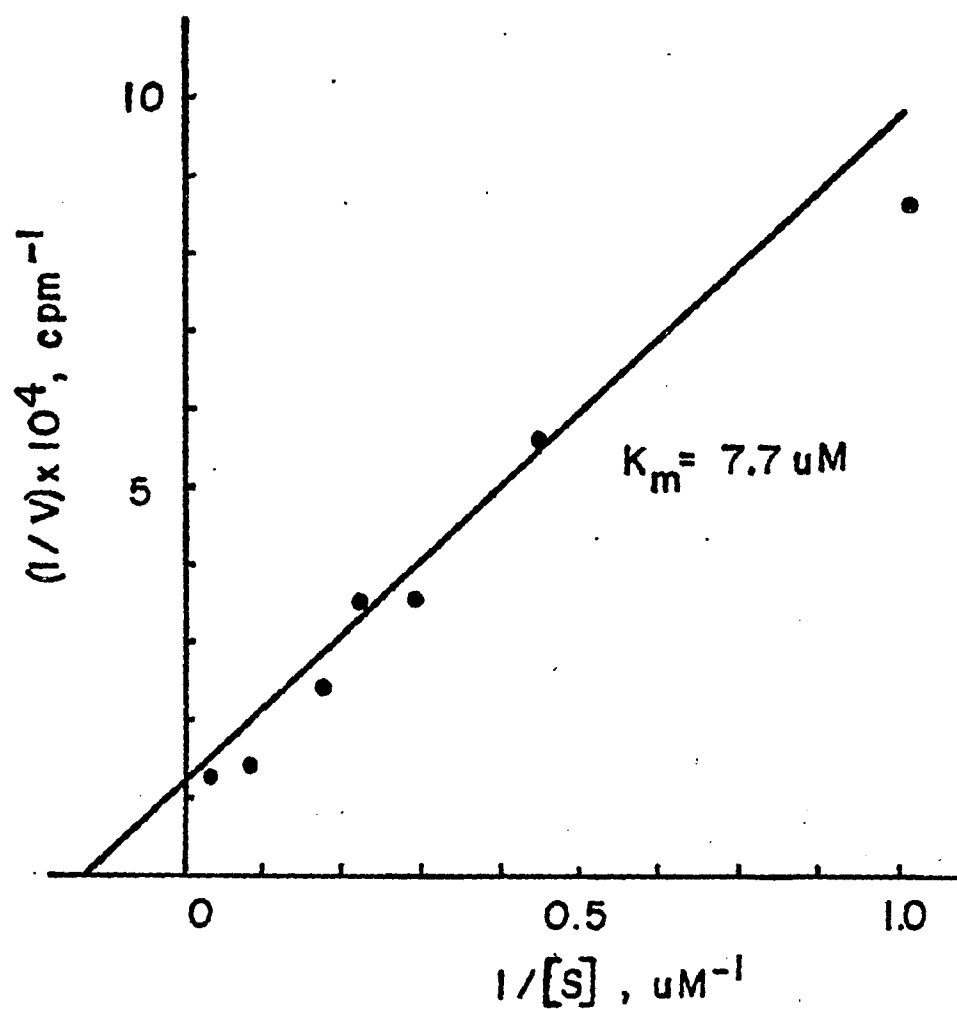


Fig. 16. Lineweaver-Burk plot for $\alpha 1 \rightarrow 2$ fucosyltransferase. The effect of varying concentrations of GDP-fucose was determined under standard conditions. Enzyme concentration was 0.42 mg of protein per assay. The line represents a least-squares fit for the data points.

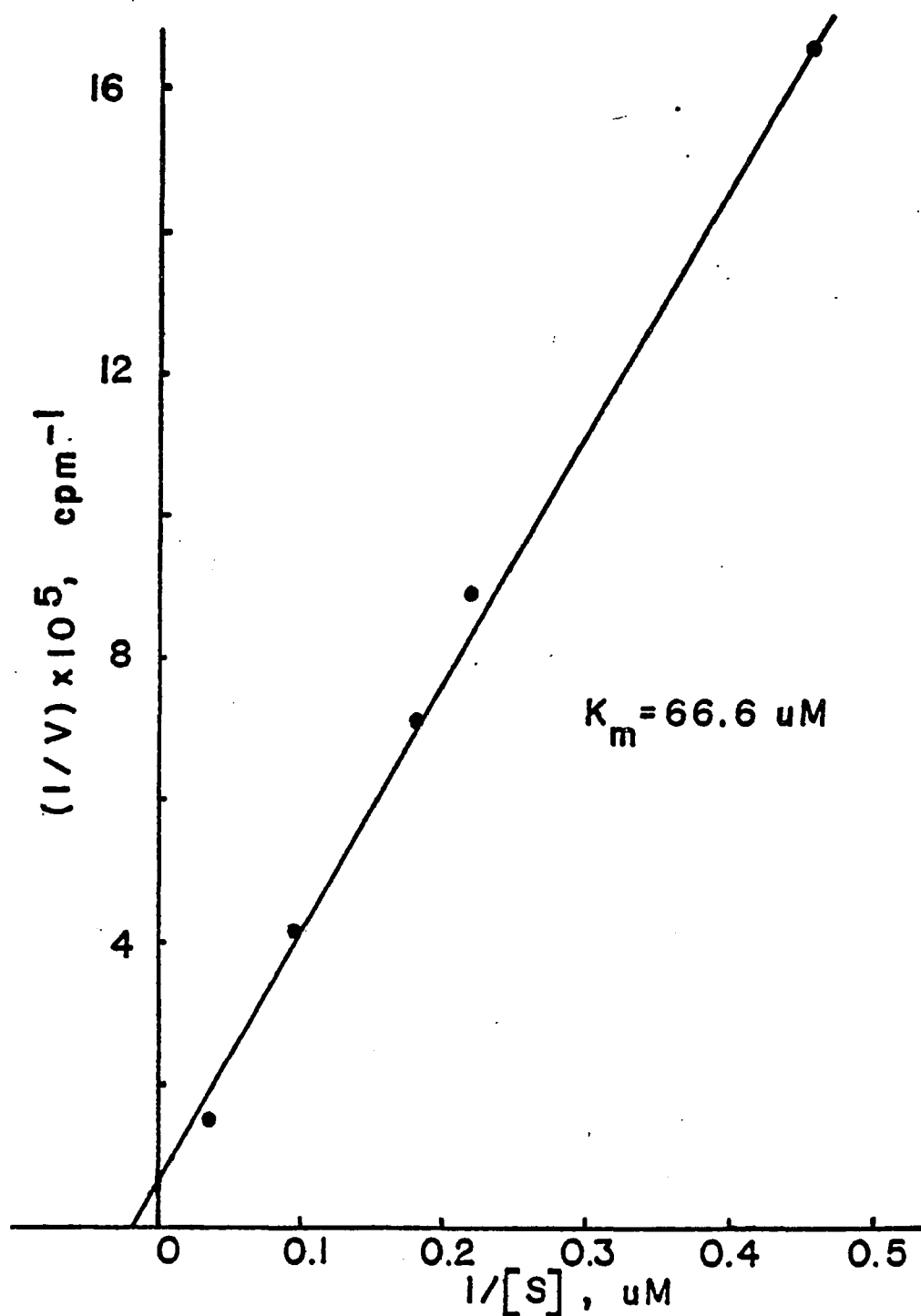


Fig. 17. Lineweaver-Burk plot for $\alpha 1-6$ fucosyltransferase. The effect of varying concentrations of GDP-fucose was determined under standard conditions. Enzyme concentration was 0.42 mg protein per assay. The line is a least-squares fit of the data points.

Both enzymes exhibited Michaelis-Menton kinetics with apparent K_m values of 7.7 μ M for the α 1 $\rightarrow$ 2 fucosyltransferase and 66.6 μ M for the α 1 $\rightarrow$ 6 enzyme. All assays were done under conditions of saturating acceptor concentrations (see Fig. 17a).

2. Glycoprotein Acceptor

The effect of varying the concentrations of asialofetuin and asialo, agalactofetuin is shown in Fig. 17a. Note the substrate inhibition exhibited by the α 1 $\rightarrow$ 2 fucosyltransferase.

Apparent K_m 's for these acceptors are 0.326 mM for asialofetuin and 0.24 mM for asialo, agalactofetuin when the GDP-fucose concentration is 3.65 μ M. A molecular weight of 50,000 (156) was used for calculating these concentrations.

E. Effect of Time and Enzyme Concentration

Under standard assay conditions, enzyme activities for both fucosyltransferases were linear for up to 60 min and with enzyme concentrations of 0.42 mg of enzyme preparation per assay.

F. Effects of Other Factors Affecting Fucosyltransferase Activity

1. NaCl

While attempting initial purification of these enzymes, it was found that they were both extremely sensitive to exposure to high salt, particularly after detergent was added to the enzyme preparation.

A 0.3% DC enzyme preparation was assayed for α 1 $\rightarrow$ 2 fucosyltransferase activity under standard conditions in the presence of a 5 mM phosphate buffer, pH 7.5, containing various concentrations of NaCl (0.025-0.5, final concentration). The enzyme exhibited a linear decrease in activity with increasing salt concentration.

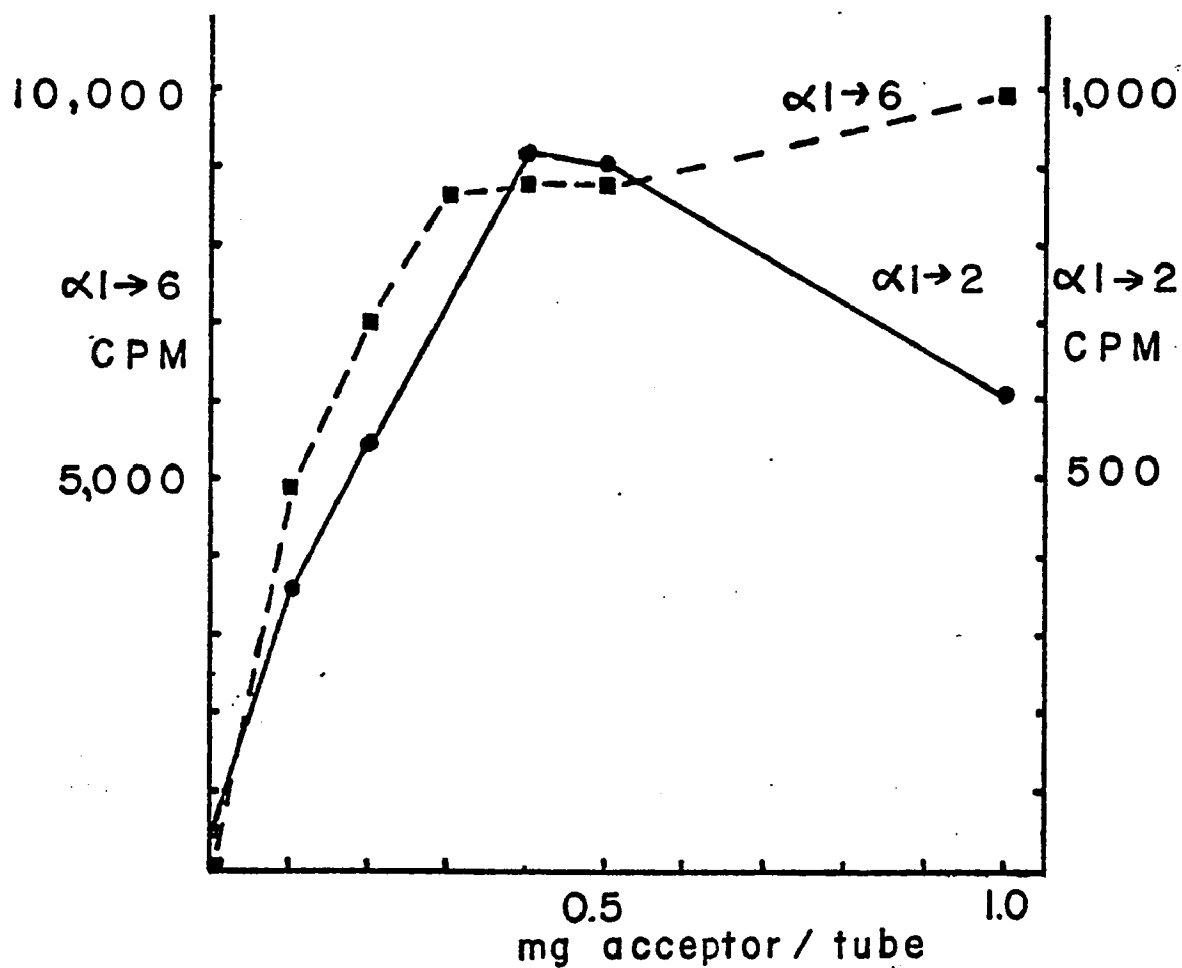


Fig. 17(a). Glycoprotein acceptor concentration curves for $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ fucosyltransferases. Note the substrate inhibition exhibited by the $\alpha 1 \rightarrow 2$ fucosyltransferase at higher acceptor concentrations. The apparent K_m 's were 0.326 mM for the $\alpha 1 \rightarrow 2$ fucosyltransferase and 0.24 mM for the $\alpha 1 \rightarrow 6$ when the GDP-fucose concentration was 3.65 μ M.

2. Fucose and Galactose

A standard enzyme preparation was assayed with various concentrations of fucose and galactose (0.01-0.1 M final concentration). At low concentrations of fucose, inhibition of $\alpha 1 \rightarrow 2$ activity was found, but at higher concentrations this effect was no longer seen. On the other hand, the $\alpha 1 \rightarrow 6$ enzyme was slightly inactivated in a linear manner (80% of control activity was seen at 0.1 M fucose).

The $\alpha 1 \rightarrow 2$ enzyme was inhibited at all concentrations of galactose (maximum, 78% of control activity) and as would be expected, the $\alpha 1 \rightarrow 6$ enzyme was unaffected.

3. Ammonium Sulfate and Butanol

Ammonium sulfate (20-40%) and butanol (20%) almost totally inactivated both fucosyltransferases and activity was not recovered upon dialysis.

II. Effects of Detergents on Solubility and Activity of Fucosyltransferase

As discussed earlier, the cellular glycosyltransferases are integral proteins of the golgi membrane, and the solubilization of these enzymes has been one of the major problems of this project. Solubilization not only provided at least partial purification, but also resulted in a significant increase in activity.

A large number of detergents, both ionic and nonionic, were tested in varying concentrations for their ability to solubilize without inhibiting enzymatic activity. While it is possible to add detergent to the incubation mixture and achieve reasonably good results, it was observed that higher activity and more reliable data were obtained when solubilized enzyme was used for the assay.

The general methodology for these studies already has been described

in the Methods section and is shown in summary in Fig. 18.

Assays of all fractions were carried out so that the detergent concentration in the assay mixture itself was always the same; thus, the effect of a given concentration of detergent in the homogenization mixture could be determined.

Tables 5 and 6 provide a summary of the results with respect to solubilization and recovery.

A. Triton X-100 (TX-100)

Although there are literature reports that Triton X-100 is an efficient solubilizing agent for fucosyl- and sialyltransferases (98,114), it proved unsuitable for the fucosyltransferases under study, since the solubility never exceeded 40% (see Tables 5 and 6 for a summary of these results). However, TX-100 had an activating effect on the 1 $\rightarrow$ 6 enzyme. The various fractions were assayed at a final concentration of 0.1% TX-100. Specific activities are shown in Table 7.

B. Deoxycholate (DC)

In order to determine the effect of deoxycholate on the solubility and activity of fucosyltransferases, varying amounts of deoxycholate were added during the homogenization procedure and S₂ and P₂ fractions obtained. These fractions were assayed at a final concentration of 0.25% deoxycholate and 0.1% TX-100. As shown in Table 5 and Fig. 19, the highest solubility reached was at concentrations of between 0.25 and 0.5% DC, with solubilities of 68 and 66%, respectively. While recovery data were not available from this experiment, later work showed that the recovery at 0.3% deoxycholate was 248%, a sizeable activation effect. The solubility at this concentration increased to 88%. It should be kept in mind, however, that the data from the two experiments reported

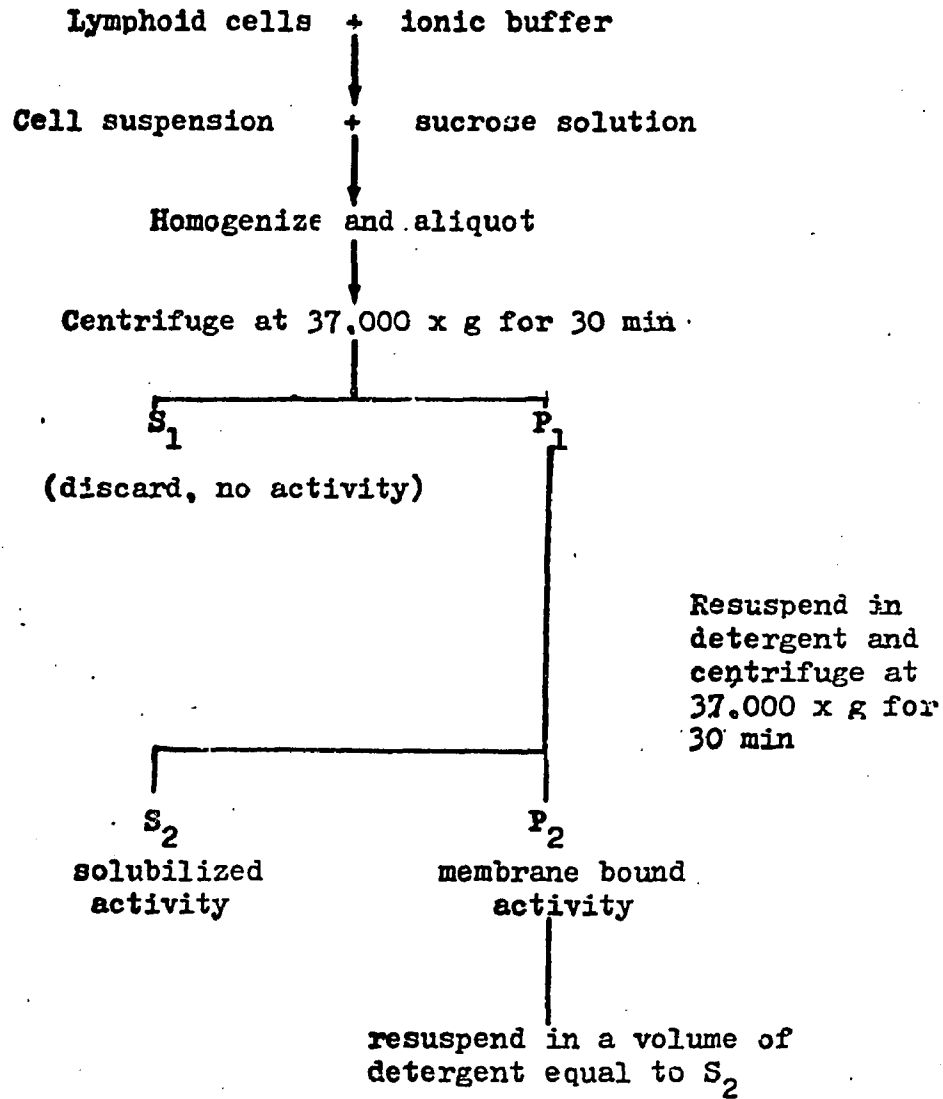


Fig. 18. Preparation of lymphoid line detergent extract.

Table 5

Effect of Detergents on the Solubility and Activity of $\alpha 1 \rightarrow 2$ Fucosyltransferase

Detergent	Conc. %	Total activity, cpm/ml of cell homogenate			Solubility ^a %
		P ₁	P ₂	S ₂	
^b H ₂ O		^c ND	6540	2220	25
TX-100 ^b	0.1	8800	5260	2020	28
	0.2	6600	6660	1100	14
	0.3	8200	3888	2560	40
	0.4	5700	5660	2420	30
DC ^c	0.1	ND	7880	2960	27
	0.25	ND	3800	8040	68
	0.5	ND	3420	6560	66
	0.75	ND	3220	3720	54
	1.00	ND	2920	3680	56
0.3%DC and TX-100 ^d	0	7120	2000	15,000	88
	0.1	10,100	940	13,200	93
	0.2	17,200	1000	16,800	94
	0.3	20,000	1500	23,480	94
	0.4	14,780	2380	13,440	85

^aCalculated as $S_2 / (S_2 + P_2) \times 100$.

^bFinal concentration of detergent in the assay was 0.1% TX-100.

^cND--not done.

^dFinal concentration of detergent in the assay was 0.25% DC and 0.1% TX-100.

^eFinal concentration of detergent in the assay was 0.075% DC and 0.1% TX-100.

Table 6

Effect of Detergents on the Solubility and Activity
of $\alpha 1 \rightarrow 6$ Fucosyltransferase

Detergent	Conc. %	Total activity, cpm/ μ l of cell homogenate			Solubility ^a %
		P ₁	P ₂	S ₂	
Triton b	0.1	564	504	300	37
	0.2	472	456	214	32
	0.3	456	362	238	40
	0.4	412	476	300	39
0.3% DC ^c and TX-100	0	82.8	22.7	115.8	84
	0.1	79.7	16.5	180.0	92
	0.2	101.8	15.5	192.6	93
	0.3	118.7	20.5	177.0	90
	0.4	117.3	31.9	179.7	85

^aCalculated as $S_2 / (S_2 + P_2) \times 100$.

^bFinal concentration of detergent in the assay was 0.1% TX-100.

^cFinal concentration of detergent in the assay was 0.75% DC and 0.1% TX-100.

Table 7
Effect of TX-100 on Specific Activity
of Fucosyltransferases

cpm/mg protein x 10^{-2}

Fraction	Concentration, %			
	0.1	0.2	0.3	0.4
$\alpha 1 \rightarrow 2$ fucosyltransferase				
P ₁	17.9	14.2	22.2	17.8
P ₂	15.9	18.0	21.6	33.3
S ₂	11.9	5.7	16.0	14.2
$\alpha 1 \rightarrow 6$ fucosyltransferase				
P ₁	576	492	608	644
P ₂	762	616	1000	1400
S ₂	881	562	744	880

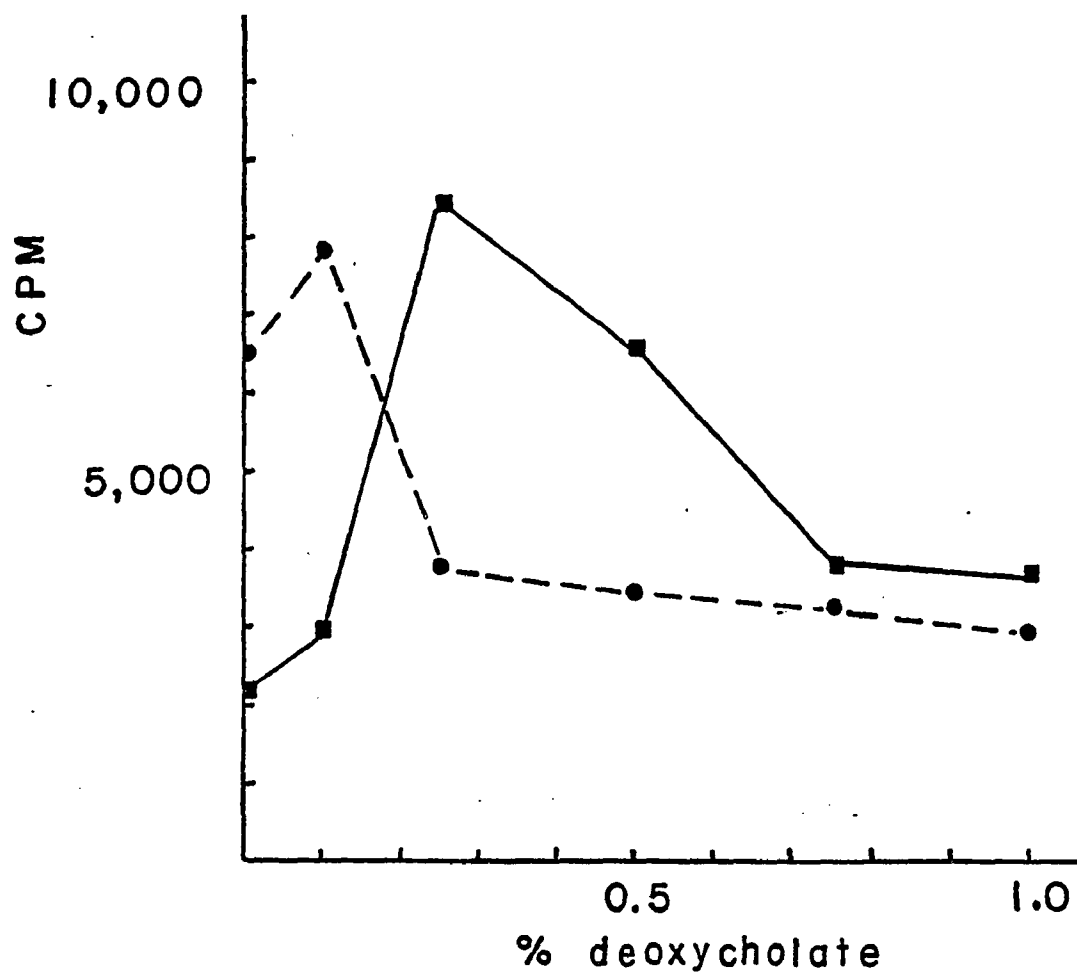


Fig. 19. Effect of deoxycholate on $\alpha 1 \rightarrow 2$ activity and solubility.
-.-.-.-. pellet activity. x _____ x supernatant activity.

in Table 5 are not comparable because the final concentrations of deoxycholate in the assays were different for the two experiments, and only general trends and not absolute numbers can be compared.

While the effect of different concentrations of deoxycholate on the $\alpha 1 \rightarrow 6$ enzyme was not determined, it can be seen from Table 6 that at 0.3% DC, solubility was 84% and the recovery, 168%. A later experiment confirmed that 0.1% TX-100 was required in the assay for optimal activity of the $\alpha 1 \rightarrow 6$ enzyme, even though detergent, in the form of deoxycholate, was already present (see Sec. II.C).

C. Deoxycholate and Triton

Even though it was inefficient as a solubilizing agent, TX-100 was required for optimal activity (see Fig. 20) when the enzyme was prepared in 0.3% DC alone. In order to determine the effect of using a combination of DC and TX-100 during the extraction procedure, membrane pellets were homogenized in detergent solutions containing 0.3% DC and 0-0.4% TX-100. P_1 , P_2 , and S_2 fractions were collected and assayed under standard conditions at a final concentration of 0.075% DC and 0.1% TX-100. Solubilities and total activity are given in Tables 5 and 6.

Addition of 0.1% TX-100 during the homogenization procedure not only provided optimal solubilization and recovery of enzyme activity (particularly for the $\alpha 1 \rightarrow 6$ enzyme), but it also had a strong activating effect. Additionally, as shown in Table 8, use of this detergent combination resulted in a partial purification of the $\alpha 1 \rightarrow 6$ enzyme that was most dramatic when the concentration of TX-100 was 0.1%. Consequently all further characterization of these enzymes was done using an S_2 fraction prepared with 0.3% DC and 0.1% TX-100.

Table 8
Effect of Adding TX-100 and DC
During Enzyme Preparation^a

cpm/mg protein/hr

Fraction	Concentration, %				
	0	0.1	0.2	0.3	0.4
$\alpha 1 \rightarrow 2$ fucosyltransferase					
P ₁	1343	2525	2606	2822	2484
P ₂	851	391	444	1076	952
S ₂	5084	7764	3906	5336	4634
S ₂ /P ₁	3.79	3.08	1.50	1.89	1.87
$\alpha 1 \rightarrow 6$ fucosyltransferase					
P ₁	31,260	39,830	30,836	33,442	39,428
P ₂	19,336	13,766	13,742	28,386	25,488
S ₂	38,494	211,858	89,580	80,472	123,916
S ₂ /P ₁	1.23	5.32	2.90	2.40	3.14

^aFinal concentration in the assay was 0.075% DC and 0.1% TX-100.

D. Tauracholate

Crude sodium tauracholate was used in concentrations of 0 to 1% using the methodology outlined previously. Tauracholate was found to have a strong inhibitory effect on $\alpha 1 \rightarrow 2$ activity in all fractions; consequently, it was impossible to determine if there was any solubilization.

E. Solubility Studies

An 0.3% DC S_2 fraction was subjected to high-speed ultracentrifugation to determine the degree of solubility obtained by the use of this detergent. A Beckman Model L5-75 ultracentrifuge equipped with a Beckman SW 27 rotor was used for this study. The sample was spun at 5°C for 90 min at 25,000 rpm ($\omega^2 t = 3542 \times 10^7$). An aliquot of the original S_2 sample kept on ice for the duration of the run, the supernatant, and the pellet resuspended in 0.3% DC were assayed for $\alpha 1 \rightarrow 2$ activity under standard conditions. The results are shown in Table 9.

An 0.3% DC and 0.1% TX-100 S_2 fraction were subjected to similar conditions of centrifugation (an SW 56 rotor, 37,000 rpm for 1 hr, $\omega^2 t = 4740 \times 10^7$, 5°C). Samples were obtained as described above but the pellet was resuspended in 0.3% DC and 0.1% TX-100 and assayed for both $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ activity under standard conditions. The results are shown in Table 9.

F. Effect of TX-100 on the Response of $\alpha 1 \rightarrow 6$ Fucosyltransferase to Divalent Metal Ion

The response of $\alpha 1 \rightarrow 6$ fucosyltransferase to divalent metal ion was determined using an 0.3% DC S_2 fraction in the presence and absence of 0.1% TX-100. The results of this study are shown in Fig. 20.

III. Optimization of Sialyltransferase Assay

A. pH Optima

Three buffer systems were used to determine the optimal pH of

Table 9

Solubility Studies of Detergent-Extracted
Fucosyltransferases

Fraction	Total activity, cpm/ml of extract		Solubility ^a	
	$\alpha 1 \rightarrow 2$ FT	$\alpha 1 \rightarrow 6$ FT	1 $\rightarrow$ 2 FT	1 $\rightarrow$ 6 FT
0.3% DC				
Original S ₂	20,240		85	
High-speed super.	10,760 (53%) ^b			
High-speed pellet	1,897 (9%)			
0.3% DC and 0.1% TX-100				
Original S ₂	8,740	415,440	75	79
High-speed super.	4,320 (49%)	262,620 (63%)		
High-speed pellet	1,465 (16%)	69,090 (16%)		

^aSupernatant activity/(supernatant + pellet activity).

^bNumbers in parentheses represent the percentage of original activity found in the fraction.

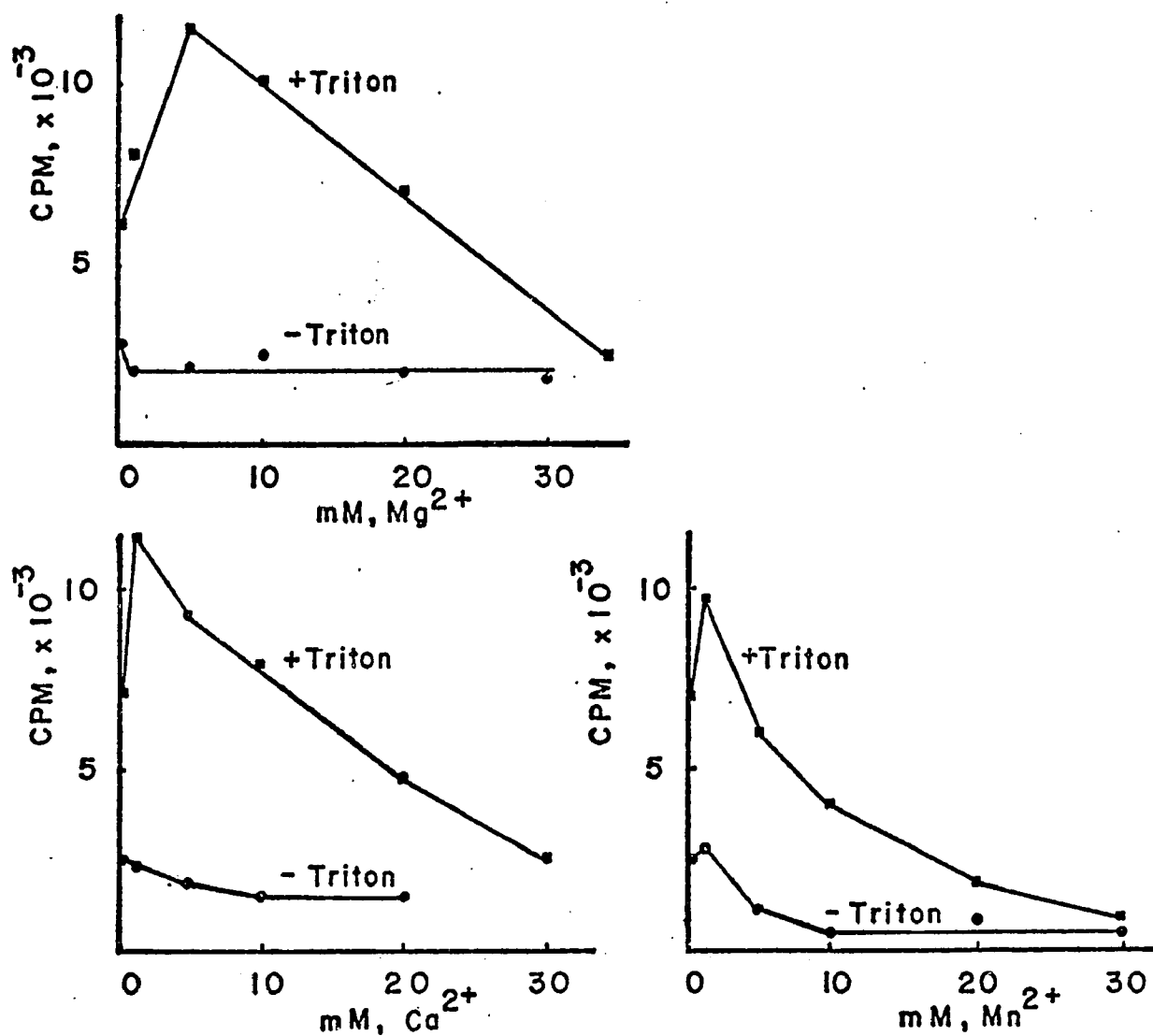


Fig. 20. Effect of TX-100 on $\alpha 1 \rightarrow 6$ enzyme activity and response to divalent metal ions. The enzyme preparation contained 0.3% DC only. The enzyme was assayed with and without 0.1% Triton TX-100.

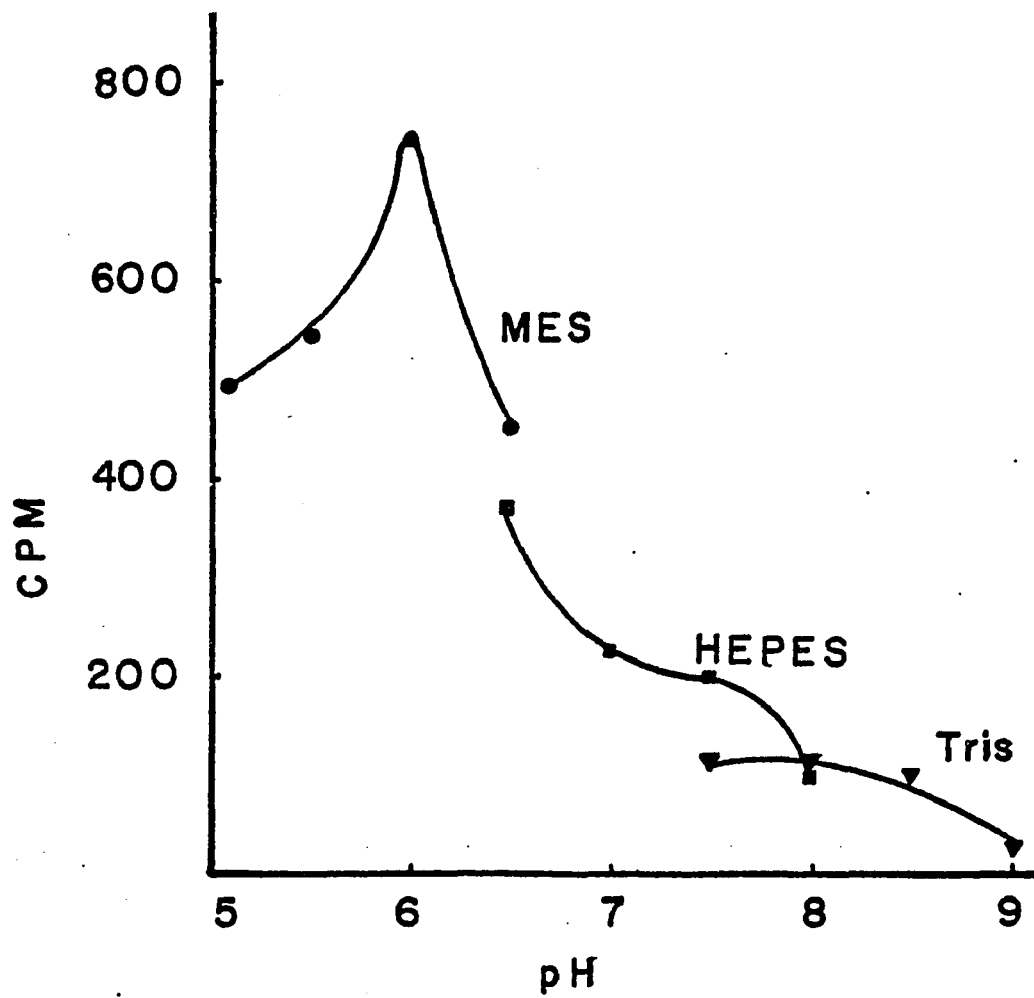


Fig. 21. Effect of pH on sialyltransferase activity.

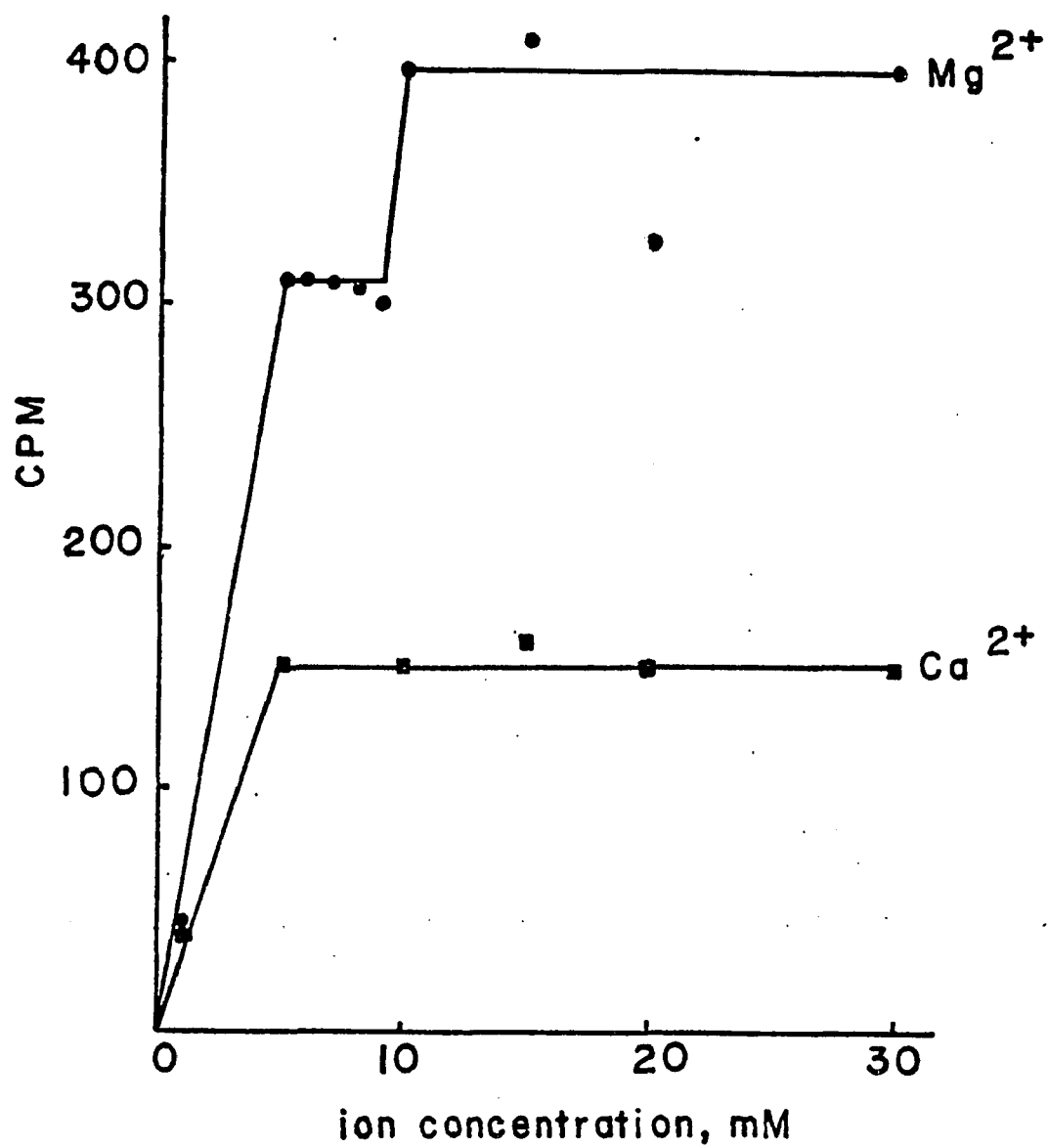


Fig. 22. Effect of divalent metal ion concentration on sialyltransferase activity.

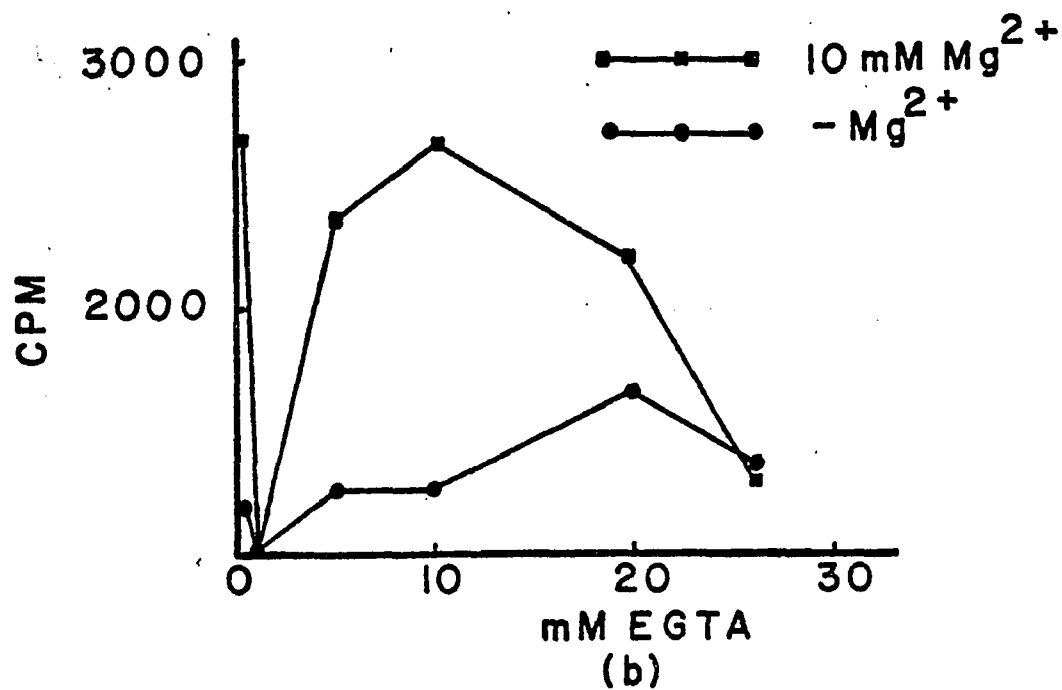
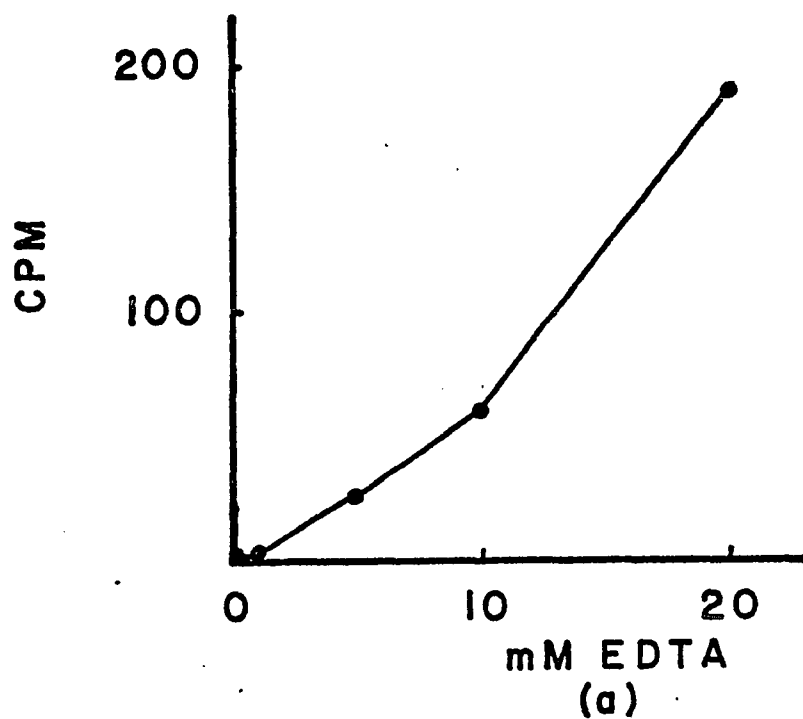


Fig. 23. Effect of chelating agents on sialyltransferase activity. (a) Effect of EDTA. The enzyme preparation for this experiment included Triton X-100. (b) Effect of EGTA. The enzyme preparation for this experiment included Cutscum.

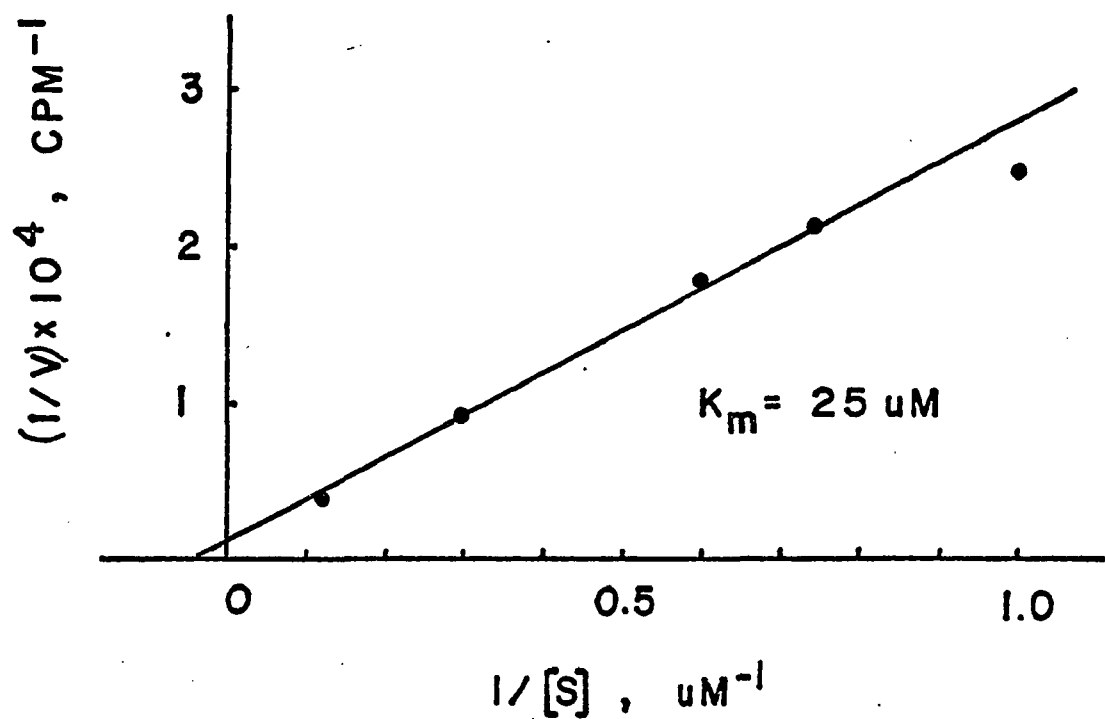


Fig. 24. Effect of varying concentrations of CMP-sialic acid. The data are plotted as a least squares fit for a Lineweaver-Burk plot.

sialyltransferase, all at a final concentration of 50 mM: MES, pH 5.2-6.5; HEPES, pH 6.5-8.0; and Tris-HCl, 7.5-9.0. A membrane pellet resuspended in 0.5% Cutscum was used as enzyme source.

Maximal activity was seen at pH 6, using MES buffer (Fig. 21). Note that the buffer system did not appear to exert a differential effect on enzyme activity.

B. Divalent Metal Ion Requirement

As shown in Fig. 22, lymphoid line asialofetuin sialyltransferase appeared to have a requirement for divalent metal ion and in this respect was unlike the fucosyltransferases. Since this experiment was carried out with TX-100 as detergent, the activity was quite low, thus there was the possibility that the requirement was not absolute. In a later experiment, about 10% of maximum activity was seen when 0.5% Cutscum was present.

C. Effect of Chelating Agents

In the absence of exogenous divalent metal ion, EDTA, in concentrations of up to 20 mM stimulated enzyme activity in a manner that was dose-related (Fig. 23a) and was equivalent to the activity seen with Ca^{2+} . EGTA caused a biphasic response in the presence and absence of 10 mM MgCl_2 (Fig. 23b) when 0.5% Cutscum was present.

D. Effect of CMP-Sialic Acid Concentration

The effect of varying CMP-sialic acid concentration is shown in Fig. 24. The data are shown as a Lineweaver-Burk plot. When 1 mg of desialylated fetuin was used as acceptor, a K_m of 25 μM was found (0.16 mg of enzyme preparation was used in each assay).

E. Effect of Nucleoside Triphosphates

Nucleoside triphosphates are commonly included in glycosyltransferase

(130,132) assays to inhibit the action of pyrophosphatases that hydrolyze the nucleotide sugar substrates (89,96). However, nucleoside triphosphates have been reported to inhibit sialyltransferase activity (110,111), and so their effect was examined in our system. It was found that 2 mM UTP and 2 mM ATP inhibited enzyme activity by 60 and 50%, respectively. Therefore no nucleotides were included in sialyltransferase assays.

F. Effect of N-Acetylneuraminic Acid and Galactose

At 10 mM N-acetylneuraminic acid, sialyltransferase activity was inhibited 80% and at 25 mM all activity was abolished. When 5 mM galactose was present, a 25% increase in enzyme activity was seen.

G. Effect of Incubation Time and Enzyme Concentration

Under standard conditions, sialyltransferase activity was linear for 60 min and 0.48 mg of enzyme protein per assay.

IV. Effect of Detergents on Activity and Solubility of Sialyltransferase

The effects of various detergents on the solubility and activity were studied in much the same way as described in Sec. II for the fucosyltransferases; P_1 , P_2 , and S_2 fractions were obtained and the results summarized in Table 10. Briefly, no detergent used in this study proved to be both an efficient solubilizer and a stimulator of enzyme activity. However, two were particularly strong activators, Cutscum and octylglucoside. Cutscum, 0.5%, was used routinely in all studies unless otherwise stated. Note that the water homogenized cells were assayed with 1% octylglucoside.

A. TX-100

TX-100 was used in our first attempt to solubilize the sialyltransferases since there are numerous literature reports that it is a good solubilizing agent for this enzyme (54,108,113,114). However, as can be

Table 10
Effect of Detergents on the Solubility of Sialyltransferase

Detergent ^c	Concentration %	Total activity, cpm/ml extract			Solubility ^a %
		P ₁	P ₂	S ₂	
H ₂ O ^b		11,900	20,900	2,840	12
TX-100	0.1(v/v)	3,440	ND	980	--
	0.2	8,000	6,440	2,260	26
	0.4	7,680	4,880	1,640	27
	0.6	9,360	ND	4,160	--
	0.8	6,080	3,380	960	22
DC	0.05(w/v)	8,040	ND	320	--
	0.1	7,800	ND	1,380	--
	0.2	4,000	900	1,780	66
	0.4	3,540	280	3,600	93
	0.6	2,080	200	1,640	89
	0.8	1,760	0	1,220	100
	1.0	620	0	0	0
Cutscum	0.1(v/v)	11,920	10,020	3,200	39
	0.5	14,340	11,720	2,720	27
	1.0	6,620	ND	1,220	--
Octylglucoside	0.1(w/v)	17,450	30,360	3,720	11
	0.25	31,760	23,850	3,180	12
	0.5	14,670	14,660	1,440	9
	0.75	19,270	20,980	2,520	11
	1.0	14,080	26,000	4,420	15

^aS₂/(P₂+S₂) x 100.

^b Assayed at a final concentration of 0.17% octylglucoside.

^cND--not done.

seen from Table 10, this detergent was not only a very inefficient solubilizing agent (27% maximum) for this tissue, but it appeared to have an inhibitory effect when compared with other nonionic detergents, such as Cutscum and octylglucoside.

B. Deoxycholate

As can be seen from Table 10, 0.4 and 0.6% deoxycholate efficiently solubilized sialyltransferase. Unfortunately, this detergent inhibited enzyme activity severely, and inclusion of TX-100 in the reaction mixture was unable to reverse this inhibition.

C. Cutscum (iso-octylphenoxy polyoxyethanol)

Although it had limited solubilizing ability, Cutscum, a nonionic detergent, had no inhibitory effect on sialyltransferase. It was used for almost all of the studies reported here because at the time this project was being done it provided the highest enzyme activities of any detergent. Because of the problem of partial solubilization, a 0.5% (v/v) P_1 fraction was routinely used.

It was initially hoped that both $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ fucosyltransferase activities could also be assayed with this detergent. However, both enzyme activities were reduced by 90% and so it was impossible to assay all three enzyme activities in the same preparation.

D. Octylglucoside

Octylglucoside, a nonionic detergent, is reportedly able to solubilize membrane-bound proteins while still retaining enzyme activity (163).

Concentrations ranging from 0-1% (grams/100 ml of 50 mM MES, pH 6) were used to homogenize lymphoid cells, and P_1 , P_2 , and S_2 fractions were collected and all assayed at a final concentration of 0.17%.

Like Cutscum, octylglucoside did not appreciably solubilize; however, it did activate considerably, with recovery at 1.0% octylglucoside being 216% (see Table 10). No other detergent, ionic or nonionic, showed this property.

E. EDTA

EDTA has been used to solubilize proteins from erythrocyte ghosts and bacterial cell walls (164). Because EDTA in concentrations up to 20 mM (final concentration) activated sialyltransferase (see p. 109 and Fig. 23) even in the presence of divalent metal ion, it seemed possible that EDTA had a solubilizing effect on sialyltransferase that resulted in an increase in activity.

Membrane pellets from NB 118 homogenized in (a) distilled water, (b) 20 mM EDTA, (c) 50 mM EDTA, (d) 100 mM EDTA, (e) 100 mM EDTA plus 0.2% TX-100, and (f) 0.2% TX-100 were allowed to stand in the cold for 3 hr to allow chelation to go to completion. P_1 and S_2 fractions were collected and assayed at a final concentration of 10 mM $MgCl_2$, 17 mM EDTA, and 0.03% TX-100. As can be seen in Table 11, no solubilization was effected at any concentration of EDTA alone or in combination with TX-100, although enzyme activity at 100 mM EDTA was, as expected, high.

F. Brij 35

Brij 35, at concentrations of 0.1, 0.5, and 1% was an inefficient solubilizer and no significant enzyme activity resulted from its use.

V. Sulfhydryl Reagents

A. Effect of NEM on the Cellular $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ Fucosyltransferases

As has already been discussed, Chou et al. (97) reported that serum $\alpha 1 \rightarrow 2$ fucosyltransferase was completely inhibited by 3 mM N-ethylmaleimide (NEM) and they used this sensitivity to distinguish the endogenous

Table 11

Effect of EDTA on Sialyltransferase Solubility

Concentration	cpm/mg protein	
	P ₁	S ₂
0	1200	--
20 mM EDTA	1820	--
50 mM EDTA	2430	--
100 mM EDTA	5060	--
100 mM EDTA + 0.2% TX-100	2010	79
0.2% TX-100	2940	--

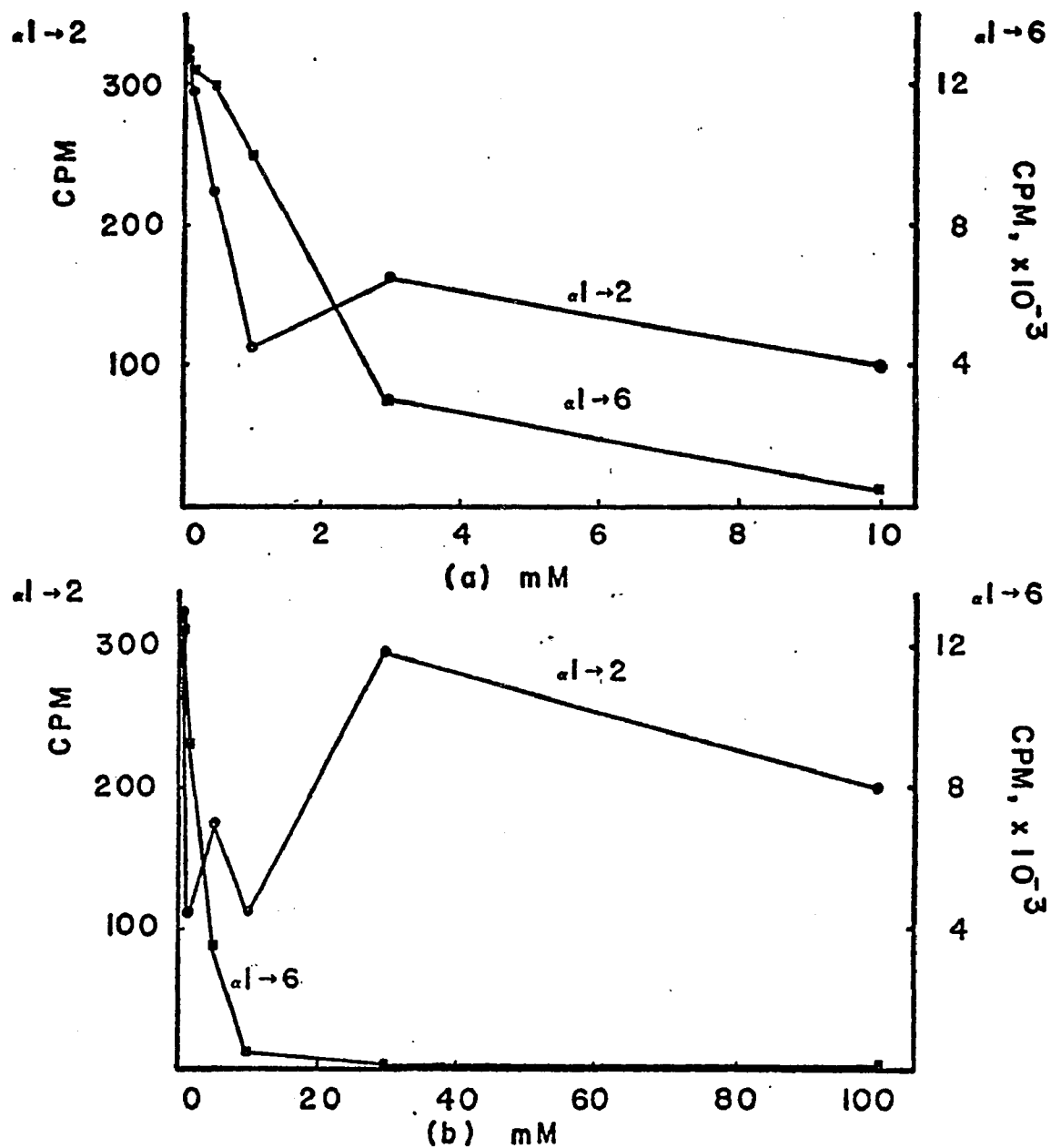


Fig. 25. Response of fucosyltransferases to varying concentrations of DTT. (a) Effect of low concentrations of DTT. (b) Effect of higher concentrations of DTT.

activities of the $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ enzymes in serum.

In order to determine whether cellular enzymes are also inhibited, a 0.3% DC S₂ fraction was assayed for both enzyme activities with and without 3 mM NEM. In addition, desialylated, defucosylated PSM was also used as acceptor to show that the effect of the sulfhydryl reagent was a result of an alteration of the enzyme and not of the glycoprotein acceptor. The results were as follow:

<u>Acceptor</u>	<u>Enzyme</u>	<u>-NEM</u>	<u>+NEM</u>	<u>% Control Activity</u>
PSM	$\alpha 1 \rightarrow 2$	878 cpm	789	90
Asialofetuin	$\alpha 1 \rightarrow 2$	1,246	1,272	102
Asialo, agalacto fetuin	$\alpha 1 \rightarrow 6$	16,114	15,934	99

The results of this and later experiments indicated that NEM did not inhibit either cellular enzyme.

B. Effects of Sulfhydryl Reagents on Fucosyl- and Sialyltransferases

Enzymes from three sources--lymphoid lines, fibroblasts, and serum--were tested for differential response to: NEM, mercaptoethanol (ME), iodoacetate (IO), glutathione (reduced)(gluSH), dithiothreitol (DTT), and para-chlormercuribenzoate (PCMB). All reagents were used at a final concentration of 10 mM, except for PCMB, which had a final concentration of 1.25 mM in the fucosyltransferase assays and 1 mM in the sialyltransferase assays. The PCMB was prepared by dissolving 90 mg in 25 ml of 1 N NaOH. The enzyme preparations were all preincubated with the reagents for 1 hr prior to assay.

As can be seen from Table 12, the $\alpha 1 \rightarrow 2$ enzyme was generally stimulated by sulfhydryl reagents, while the $\alpha 1 \rightarrow 6$ fucosyltransferase and sialyltransferase were generally inhibited. PCMB inhibited all three

Table 12

Effect of Sulfhydryl Reagents on Enzyme Activity ^e

Reagent	% control activity									Mechanism of Action
	α1→2 FT			α1→6 FT			ST			
	L ^a	F ^b	S ^c	L	F	S	L	F	S	
N-ethylmaleimide	110	164	0	80	79	78	120	-- ^d	94	Alkylating agent--adds no charge.
Mercaptoethanol	185	179	114	120	113	82	62	--	81	Reduction--adds no charge.
Iodoacetate	155	129	98	89	72	76	40	--	44	Alkylates--adds a minus charge for each site that is carboxymethylated. Also alkylates methionines and histidines.
Glutathione (red)	113	290	114	56	126	103	92	--	73	Reduction--adds a minus charge at each site that is reduced
Dithiothreitol	193	212	125	7	31	65	7	--	3	Reduction--adds no charge.
para-Chloro-mercuribenzoate	32	86	1	14	17	64	14	--	63	Formation of mercaptide. Adds one minus charge.

^a Lymphoid line--NB 83 used for all determinations.^b Fibroblast--JG, transfer 5, used.^c Serum--normal serum.^d Not done.^e All enzyme preparations were preincubated for 1 hr with the sulfhydryl reagent prior to assay, which was carried out under standard conditions.

enzymes from all sources to a greater or lesser degree.

A most surprising finding was the differential effect caused by DTT on the fucosyltransferases. While this is discussed in greater detail later, it should be noted that this is the only known treatment that allows a marked distinction between the $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ cellular fucosyltransferases.

C. Dose Response of Fucosyltransferases to DTT

Because DTT produced such a dramatic effect on the activities of the two fucosyltransferases, a dose response curve over four orders of magnitude (0.1-100 mM DTT) was determined using a 0.3% DC and 0.1% TX-100 S_2 fraction as enzyme source.

This experiment was originally conceived to test the hypothesis that perhaps DTT was causing a conformer effect, i.e., that a single molecular species was responsible for the formation of both the $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 6$ linkages and that the ratio of the activities of the two enzymes was dependent on the conformation of the molecule. Thus, if this hypothesis were true, then as the $\alpha 1 \rightarrow 6$ enzyme activity decreased, the $\alpha 1 \rightarrow 2$ activity would increase. It would appear from this experiment (see Fig. 25) that this was clearly not the case, especially at low concentrations of DTT where the inhibitory effects were parallel.

It should be noted that while the response of the $\alpha 1 \rightarrow 6$ enzyme at 10 mM was what would be expected on the basis of previous experiments, the response of the $\alpha 1 \rightarrow 2$ was not. However, there was still a significant difference in the response of these two enzymes, particularly at high concentrations where DTT had no inhibitory effect on the $\alpha 1 \rightarrow 2$ enzyme.

VIII. Comparison of Fucosyl- and Sialyltransferase Activities in Lymphoid Lines and in Fibroblasts

A. Lymphoid Lines

A comparison of fucosyl- and sialyltransferase activities was carried out.

Each line was homogenized and divided into two aliquots. The aliquots were then centrifuged, and the pellets rehomogenized in the appropriate detergent. A 0.3% DC and 0.1% TX-100 P_1 fraction was assayed for 1 $\rightarrow$ 2 and 1 $\rightarrow$ 6 fucosyltransferase activities and a 0.5% Cutscum P_1 fraction for sialyltransferase activity, as previously described. In this way, the three enzyme activities were determined from the same cell preparation. The results are shown in Table 13.

The most striking finding was the relatively constant ratio of 1 $\rightarrow$ 2 to 1 $\rightarrow$ 6 activity (0.1-0.7) despite the wide range of specific activities found in the cell lines--1.09-18.3 pmoles/mg protein/hr for α 1 $\rightarrow$ 2 activity and 87-1128 pmoles/mg protein/hr for α 1 $\rightarrow$ 6 activity.

Sialyltransferase activities ranged from 1.35-14.5 pmoles/mg protein/hr. With few exceptions, both fucosyltransferases were more active than the sialyltransferase under the assay conditions described, with the α 1 $\rightarrow$ 2 to sialyltransferase ratio ranging from 0.32 to 13.4 and the α 1 $\rightarrow$ 6 to sialyltransferase ratio ranging from 25 to 462.

B. Lymphoid Lines from Cystic Fibrosis Homozygotes and Obligate Heterozygotes

As discussed in Sec. VI. B of the Introduction, alterations in fucose/sialic acid ratios have been reported in glycoproteins derived from patients with cystic fibrosis. In order to determine if these alterations were related to changes in fucosyl- and sialyltransferase activities, the activities of these enzymes were determined as described above, and compared

Table 13
Fucosyl- and Sialyltransferase Activities
in Lymphoid Lines^a

Line--Syndrome	Activity, pmoles/mg of protein/hr			Ratios of Activities		
	α1→2	α1→6	ST	2FT/6FT	2FT/ST	6FT/ST
NB 41--Fucosidosis	9.33	441	1.52	0.02	6.14	290
NB 49--Obligate hetero- zygote, fucosidosis	8.19	254	4.37	0.03	1.87	58
NB 69--Normal sib of NB 77	34.7	465	3.49	0.07	9.95	133
NB 75--Hypercholesterol- emia, Type II	1.09	87	3.41	0.01	0.32	25
NB 77--Homocystinuria	8.19	199	4.37	0.04	1.87	46
NB 78--Hypercholesterol- emia, Type II	13.78	527	7.50	0.03	1.84	70
NB 83--Obligate hetero- zygote, B ₆ responsive cystathioninuria	18.3	628	1.36	0.03	13.4	462
NB 84--Acid maltase deficiency	2.43	91	4.06	0.03	0.60	22
NB 94--San fillipo syndrome	4.29	98	2.44	0.04	1.76	40
NB 95--B ₆ responsive cytathioninuria	13.9	549	10.91	0.03	1.27	50
NB 104--Hypercholesterol- emia	9.19	221	5.36	0.04	1.71	41
NB 118--Normal balanced translocation carrier, t (5;7)(p 15, p 15)	17.65	1128	14.5	0.02	1.22	78

^aFucosyltransferase activity was determined in a 0.3% deoxycholate and 0.1% TX-100 P₁ fraction. Sialyltransferase activity was determined in a 0.5% Cutscum P₁ fraction. Each point is the result of a single determination done in duplicate.

with those found in lines derived from normal donors (see Table 14 for results). No significant differences were found among any of these groups except that the $\alpha 1 \rightarrow 2 / \alpha 1 \rightarrow 6$ ratio of obligate heterozygotes was significantly different from those of normals and affecteds. However, the importance of this finding is diminished considerably if one considers the small number of samples. Otherwise the activities and the activity ratios were within the ranges seen with other lymphoid lines.

C. Fibroblasts from Donors with Cystic Fibrosis and From Normal Individuals

The findings in lymphoid cells were confirmed by extending this study to the activities of fucosyl- and sialyltransferases in fibroblasts from normal individuals and donors with cystic fibrosis.

After being replated at a 1:3 split ratio, fibroblasts were grown to confluency. Cells from three 150 cm² flasks were harvested and prepared as described above for the lymphoid lines. The results are shown in Table 15. Again no significant differences were found between the two groups. The CF fibroblasts used in this experiment were used at the earliest passage possible since Shapiro et al. (164) have reported that CF fibroblasts exhibit early senescence.

The effect of time in culture on enzyme activity in fibroblasts also was examined to determine when maximal enzyme activity was obtained. As can be seen from Table 16, enzyme activities and the ratio of activities varied greatly with time; however, maximal activity of both the $1 \rightarrow 6$ fucosyltransferase and of the sialyltransferase were maximal at day 5 when the cultures were confluent. Although $1 \rightarrow 2$ activity was not maximal at this point, it was still quite sizable. Thus confluent cultures were used to determine the enzyme activities in fibroblasts from normal individuals and patients with cystic fibrosis.

Table 14

Comparison of Fucosyl- and Sialyltransferase Activities in Lymphoid Lines from Normal Individuals and from Cystic Fibrosis Homozygotes and Obligate Heterozygotes^a

Line	Activity, pmoles/mg of protein/hr			Ratios of Activities		
	$\alpha 1 \rightarrow 2$ FT	$\alpha 1 \rightarrow 6$ FT	ST	2FT/6FT	2FT/ST	6FT/ST
NORMAL						
NB 69	34.7	465	3.49	0.07	9.95	133
NB 83	18.3	628	1.36	0.03	13.4	462
NB 118	17.6	1128	14.5	0.02	1.22	78
Mean $\pm$ S.D.	23.53 $\pm$ 9.68	740 $\pm$ 345	6.45 $\pm$ 7.05	0.04 $\pm$ 0.03	8.19 $\pm$ 6.28	224 $\pm$ 207
CYSTIC FIBROSIS						
EBV 15	41.0	541	2.79	0.08	14.7	194
EBV 17	14.1	842	6.21	0.02	2.27	135
EBV 19	9.46	484	7.00	0.02	1.35	69
Mean $\pm$ S.D.	21.5 $\pm$ 17.03	622 $\pm$ 192	5.33 $\pm$ 2.24	0.04 $\pm$ 0.03	6.11 $\pm$ 7.46	133 $\pm$ 63
CYSTIC FIBROSIS HETEROZYGOTES						
EBV 13	16.3	896	1.01	0.02	16.1	887
EBV 14	18.44	1467	3.79	0.01	4.87	387
NB 16	5.45	676	2.29	0.01	2.36	295
Mean $\pm$ S.D.	13.4 $\pm$ 6.96	1013 $\pm$ 408	2.36 $\pm$ 1.39	0.013 $\pm$ 0.006	7.78 $\pm$ 7.32	523 $\pm$ 319

^a Each point is the result of a single determination done in duplicate.

Table 15

**Fucosyl- and Sialyltransferase Activities in Fibroblasts
from Normal Individuals and Patients with Cystic Fibrosis**

Strain (transfer no.)	Activity, pmoles/mg of protein/hr			Ratios of Activities		
	$\alpha 1 \rightarrow 2\text{FT}$	$\alpha 1 \rightarrow 6\text{FT}$	ST	2FT/6FT	2FT/ST	6FT/ST
CYSTIC FIBROSIS						
770 (9)	16.8	292	4.77	0.06	3.52	61
1348 (10)	17.8	302	5.20	0.06	3.28	58
1959 (11)	18.1	345	5.20	0.05	3.48	66
1957 (14)	18.5	213	1.68	0.09	11.0	126
Mean $\pm$ S.D.	17.8 $\pm$ 0.73	288 $\pm$ 55	4.21 $\pm$ 1.70	0.07 $\pm$ 0.02	5.32 $\pm$ 3.79	78 $\pm$ 32
NORMALS						
5080 (4)	6.4	283	2.11	0.02	3.03	134
5832 (14)	26.2	683	3.59	0.04	7.30	190
JG (9)	11.8	313	5.85	0.04	2.02	53
Mean $\pm$ S.D.	14.8 $\pm$ 10.2	426 $\pm$ 222	3.85 $\pm$ 1.88	0.03 $\pm$ 0.01	4.12 $\pm$ 2.8	125 $\pm$ 69

^aThe fibroblasts used for this experiment were harvested at confluency after replating at a density of 1:3. Fucosyltransferase activity determined after homogenization in 0.3% deoxycholate and 0.1% TX-100. Sialyltransferase activity determined after homogenization in 0.5% Cutscum. Each point is the result of a single determination done in duplicate.

The specific activities seen in the experiment shown in Table 15 were a good deal lower than those shown in Table 16. While there was no ready explanation for this discrepancy, it is possible that differences in the split ratios may be a factor.

Table 17 presents the fucosyltransferase activities found in a variety of human tissues. All tissues had been kept frozen at -70°C for periods of up to 1 year. The tissues were prepared in much the same way as for lymphoid lines except that they were homogenized in distilled water using a Polytron Kinematica homogenizer. All samples were kept at 4° throughout the preparation steps.

The activities shown do not include the endogenous levels of activity, which are presented in the right-hand column. The kidney data were the result of determinations from two different individuals.

Table 16

Effect of Time in Culture on
Fibroblast Fucosyl- and Sialyltransferase Activities

Days in Culture	Activity, pmoles/mg protein/hr			Ratios of activities		
	$\alpha 1 \rightarrow 2\text{FT}$	$\alpha 1 \rightarrow 6\text{FT}$	ST	2FT/6FT	2FT/ST	6FT/ST
1.5	158	767	13	0.21	12	59
3	9	393	6	0.02	1.5	66
4	25	481	4	0.05	6.25	120
5	86	1276	17	0.07	5.06	75

^a

JG at transfer 8 was used for this experiment. Two 150 cm² flasks were used for each determination. Flasks were replanted at a ratio of 1:4 from confluent flasks. At day 1.5 (40 hr) cells were relatively sparse, but many round cells could be seen. At day 5 all cultures were confluent.

Table 17

Fucosyltransferase Activity in Human Tissues^a

Tissue	Activity, cpm/mg protein/hr		
	$\alpha 1 \rightarrow 2\text{FT}^b$	$\alpha 1 \rightarrow 6\text{FT}^b$	Endogenous
Kidney ^c	718	737	2,182
Spleen ^c	2,112	2,318	1,040
Kidney ^d	2,008	5,756	1,672
Adult Intestine ^d	3,481	2,125	181
Fetal Intestine ^d	2,874	20,208	1,505
Liver ^d	8,034	117	2,356

^aTissues were prepared as described for lymphoid lines except that a Polytron Kinematica homogenizer was used.

^bActivities do not include endogenous activities.

^cS₂ fraction used.

^dP₁ fraction used.

Table 18

Summary of the Properties of Fucosyl- and Sialyltransferases Found in Lymphoid Lines

Property	$\alpha 1 \rightarrow 2$ Fucosyltransferase	$\alpha 1 \rightarrow 6$ Fucosyltransferase	Sialyltransferase
pH optima	6.5-7.0	7.0	6.0
Divalent metal optimal concentration	10 mM Mg^{2+} 1 mM Ca^{2+}	5 mM Mg^{2+} 1 mM Ca^{2+}	10 mM Mg^{2+} 5 mM Ca^{2+}
Effects of chelating agents	Slight stimulation by 20 mM EDTA	Inhibition over 5 mM EDTA	Stimulation up to 20 mM EDTA or EGTA
Apparent K_m for nucleotide sugar	7.7 μM	66.6 μM	25 μM
Apparent K_m for acceptor	0.326 mM	0.24 mM	N.D. ^a
Effect of: fucose	Inhibition at low conc., stimulation at high conc.	Slight inactivation at all conc.	N.D.
galactose	Slight inhibition	No effect	Stimulation at 5 mM
sialic acid	N.D.	N.D.	Complete inhibition at 25 mM
Effect of sulphydryl reagents	Generally stimulatory	Generally inhibited	Generally inhibited
Detergent required for maximal solubilization	0.3% DC + 0.2% TX-100	0.3% DC + 0.1% TX-100	0.8% DC
Detergent required for maximal activity	0.3% DC + 0.1% TX-100	0.3% DC + 0.2% TX-100	0.1-0.25% octylglu- coside

^aN.D. - Not Done.

DISCUSSION

I. Enzyme Characterization

A. $\alpha 1 \rightarrow 6$ Fucosyltransferase

The most interesting result of this study is the first report of $\alpha 1 \rightarrow 6$ fucosyltransferase in a tissue of human origin; this enzyme was characterized in human lymphoid lines and detected in human skin fibroblasts, kidney, spleen, adult and fetal intestine, and liver.

Briefly, lymphoid line $\alpha 1 \rightarrow 6$ fucosyltransferase was found to be membrane bound, requiring detergent treatment for solubilization and activation. It had a pH optimum of 7.0 and was stimulated by divalent metal ions. Apparent K_m 's for GDP-fucose and asialo, agalactofetuin were determined to be 66.6 μ M and 0.24 mM, respectively.

There is only one report in the literature of an enzyme similar to $\alpha 1 \rightarrow 6$ fucosyltransferase in a tissue of human origin. Bosmann et al. (90) reported the presence in HeLa cells of an enzyme that used fetuin subjected to mild acid hydrolysis and subsequent treatment with snail β -galactosidase as its acceptor. However, only 50% of the galactose was

removed and the treated glycoprotein did not serve as an acceptor for galactose. The work of Wilson et al. (104) and Jabbal and Schachter (84) showed if galactose was present on the nonreducing terminal of an oligosaccharide, it was no longer a substrate for the $\alpha 1 \rightarrow 6$ fucosyltransferase. Thus, it is unclear which fucosyltransferase was studied by these authors.

The method used in this study to remove terminal galactose, that is, Smith degradation, completely destroys the terminal galactose (156). Thus the specificity of the acceptor used in this study was much more definitive. Additionally, the activity of this enzyme was unaffected by the presence of galactose, while the activity of the $\alpha 1 \rightarrow 2$ fucosyltransferase, which shows an absolute requirement for a galactosyl residue at its nonreducing terminal, was somewhat inhibited.

Since there are no published data concerning this enzyme from human tissue, the following is a brief summary of the characteristics of the human serum enzyme. Munro and Schachter (62) characterized the human serum $\alpha 1 \rightarrow 6$ fucosyltransferase using asialo, agalacto α_1 -acid glycoprotein as acceptor. They reported that this enzyme had a pH optimum of 5.0 and that TX-100 and divalent metal ions had no effect on enzyme activity. The K_m for the acceptor was 1.2 mM. This enzyme was present in the serum of Bombay and lele donors. Mixed substrate experiments showed that this enzyme was distinct from the $\alpha 1 \rightarrow 2$ fucosyltransferase, which was also present in serum.

Other investigators detected this enzyme in tissues of other species; however, none of these workers did any extensive characterization of this

enzyme. Section IV.A.2 of the Introduction contains a discussion of the work now in the literature.

B. $\alpha 1 \rightarrow 2$ Fucosyltransferase

Lymphoid line $\alpha 1 \rightarrow 2$ fucosyltransferase was a membrane-bound enzyme that required both deoxycholate and Triton X-100 for solubilization and activation. It was slightly stimulated by divalent metal ions, had a pH optimum between 6.5 and 7.0 when HEPES buffer was used, and had an apparent K_m of 7.7 M for GDP-fucose and of 0.326 mM for asialofetuin.

The pH optimum found for $\alpha 1 \rightarrow 2$ fucosyltransferase compared favorably with the optimum of 7.2 found by Pacuska and Koscielak (166) for the human bone marrow enzyme. These workers used Tris-HCl as the buffer and 0.6% TX-100 in their determinations. Human milk enzyme was reported to have a pH optimum between 5.9 and 6.2 (101). However, this enzyme was completely soluble and the difference found between this enzyme and the tissue enzymes could possibly be ascribed to the presence of detergent in the assay procedure.

The following tabulation summarizes the pH optima found for this enzyme in tissues of nonhuman origin.

<u>Tissue</u>	<u>pH optima</u>	<u>buffer</u>	<u>Ref.</u>
rat intestine	6.0	cacodylate acetate	96
rat pancreas	5.5-6.0	cacodylate acetate	95
rat gastric mucosa	6.8	Tris-HCl	167
pork liver	6.5-8.0	Tris-HCl	89
porcine submaxillary gland	6.0	cacodylate	98

It has been shown in this study that the optimal pH for this enzyme was apparently dependent on the buffer system used; thus the differences

in the optima found in the reports cited above may be explained by the different buffering systems used.

Both human milk (101) and porcine submaxillary gland (98) $\alpha 1 \rightarrow 2$ fucosyltransferases have no divalent metal ion requirement; however, the porcine submaxillary gland enzyme was reported to be stimulated by a variety of divalent metal ions (98). The findings of other investigators with regard to this enzyme are given in the following tabulation.

<u>Tissue</u>	<u>Ion</u>	<u>Optimum Concentration</u>	<u>Required</u>	<u>Ref.</u>
Rat intestine	Mg ²⁺	20 mM	No	96
	Mn ²⁺	20 mM	No	
Rat pancreas	Mg ²⁺	5 mM	Yes	95
	Mn ²⁺	10 mM		
	Cd ²⁺ , Cu ²⁺ , Pb ²⁺	All inhibitory at 10 mM		
Rat gastric mucosa	Mn ²⁺	5 mM	No	167
Pork liver	Mg ²⁺	20-40 mM	No	89
	Mn ²⁺	20-40 mM		

Apparent K_m 's for GDP-fucose reported by other investigators for $\alpha 1 \rightarrow 2$ fucosyltransferase differ by as much as one order of magnitude from the one found in this study. Rat intestinal enzyme had an apparent K_m of 0.78 μ M (96), pork liver enzyme a value of 1.4 μ M (89), porcine submaxillary gland enzyme had a value of 27 μ M in the presence of 20 mM MnCl₂ (99), and rat brain microsome enzyme had a value of 1 μ M (102). These differences could be accounted for by species differences and by the use of deoxycholate in this study.

The substrate inhibition of the human enzyme that was seen at high asialofetuin concentrations was reported for the pork liver enzyme by Jabbal and Schachter (89).

1. Specificity of Acceptor

Asialofetuin has been used by many investigators to study the $\alpha 1 \rightarrow 2$ fucosyltransferase, which transfers fucose to the galactose terminal of the two types of carbohydrate chains found on asialofetuin (see Fig. 9 for structures). However, Hill's laboratory recently reported the presence of an $\alpha 1 \rightarrow 3$ fucosyltransferase in human milk that used the identical substrate but transferred fucose in $\alpha 1 \rightarrow 3$ linkage to the penultimate glucosamine of asparaginyllinked oligosaccharides. This calls into question the specificity of the acceptor used in this study for the $\alpha 1 \rightarrow 2$ enzyme. However, there is presumptive evidence that this enzyme is not active in lymphoid lines.

a. The transfer of fucose to desialylated, defucosylated porcine submaxillary mucin demonstrated the presence of $\alpha 1 \rightarrow 2$ fucosyltransferase in lymphoid line extracts. Bella and Kim (96) and Paulson et al. (168) showed this acceptor to be specific for this enzyme. Thus, the presence of $\alpha 1 \rightarrow 3$ fucosyltransferase would be revealed if there were evidence of a second enzyme, e.g., a broad or biphasic pH curve, or nonlinear kinetics for either time or enzyme concentration, when asialofetuin was used as acceptor. Beyer et al. (64) showed that the reaction product of the $\alpha 1 \rightarrow 2$ enzyme was an even better substrate for the $\alpha 1 \rightarrow 3$ enzyme; thus breaks in the curves would be expected. No evidence of this sort was found.

b. Human $\alpha 1 \rightarrow 3$ fucosyltransferase from milk had an absolute requirement for divalent metal ion. While some stimulation of fucosyltransferase activity was seen with increasing ion concentration, chelating agents had a stimulatory effect when asialofetuin was used as acceptor. This result would not be expected if even part of the activity was the result of $\alpha 1 \rightarrow 3$ activity.

C. Sialyltransferase

Lymphoid line sialyltransferase had a pH optimum of 6.0; appeared to have an absolute requirement for divalent metal ion, although it was stimulated by the presence of the chelating agents, EDTA and EGTA; and had an apparent K_m of 25 μ M for CMP-sialic acid. This enzyme was strongly membrane bound, requiring 0.8% deoxycholate for solubilization. However, this concentration inhibited enzyme activity to a great degree.

The pH optimum found in this study differed somewhat from those reported in the literature, which ranged from 6-7.2. The following is a summary of the pH optima found. The reasons for the discrepancies among these studies are unclear.

<u>Tissue</u>	<u>pH optima</u>	<u>Ref.</u>
Calf kidney cortex	6-6.5	113
Porcine submaxillary gland $\alpha 2 \rightarrow 3$	6.5	55
Bovine colostrum $\alpha 2 \rightarrow 6$	6.8	106
Pork liver	7.0	109
Human liver	7.2	108

As shown in Fig. 22, asialofetuin sialyltransferase had an absolute requirement for divalent metal ions in the presence of TX-100. However, much higher activities were seen overall when Cutscum was used and in this case there was some activity found in the absence of divalent metal ions (13% of that seen with 10 mM $MgCl_2$), indicating that even though divalent metal ions were not absolutely required under optimal conditions, they were absolutely necessary for maximal activity.

Reports in the literature vary with regard to this question; however,

as can be seen from the tabulation below, many authors reported that there was a stimulation of activity, although none of them reported an absolute requirement for divalent metal ion.

<u>Tissue</u>	<u>Ion</u>	<u>Conc., mM</u>	<u>Effect</u>	<u>Req.</u>	<u>Ref.</u>
Rat liver	Mg	10	Stim.	No	111
	Mn	10	Stim.	No	
Human liver	Mg	10-35	Stim.	No	108
	Mn	5	Stim.	No	
		25-100	Inhib.	No	
	Ca	5	None	No	
		100	Inhib.		
Human placenta	Mg	10	None	No	54
Calf kidney cortex	Mg	<25	None	No	113
		>25	Inhib.		
	Mg + TX-100	10-25	Stim.		
	Ca + TX-100	10-25	Stim.		
	Mn + TX-100	25	Stim.		

Although lymphoid line sialyltransferase was greatly stimulated by divalent metal ions, it was also stimulated by chelating agents, and as can be seen in Fig. 23b, this stimulation was found in the presence or absence of divalent ions. Other laboratories reported similar effects. Human placental enzyme, which was unaffected by exogenously added Mg^{2+} or Ca^{2+} , was stimulated two- to threefold by 10 mM EDTA (54). On the other hand, human liver asialofetuin sialyltransferase, which showed a 60% increase in activity with Mg^{2+} , was also stimulated 50% with 1 mM EDTA (108). Calf kidney cortex sialyltransferase (113), which exhibited no stimulation in the presence of TX-100 and divalent metal ion, was

unaffected by 10 mM EDTA. In the absence of TX-100, a condition under which Mg^{2+} and Ca^{2+} did stimulate, EDTA decreased residual activity by 18%. Thus mechanism(s) by which chelating agents affect sialyltransferase activity are unclear.

Low concentrations of both Zn^{2+} and Hg^{2+} have been reported to inhibit enzyme activity (108,113) and the EDTA and EGTA may simply be chelating these and other inhibitory heavy metal ions, thus causing an increase in residual activity.

EDTA was reported to solubilize bacterial membrane proteins (164) and since such solubilization would account for the increase in activity seen in the presence of chelating agents, EDTA was examined for its ability to remove sialyltransferase from the golgi membrane. The results of these experiments, shown in Sec. IV.E., Results, show that even at concentrations as high as 100 mM, no solubilization was effected, although the presence of increasing concentrations of EDTA was associated with increased enzyme activity.

Another possible explanation for the increase in activity might be the inhibition of CMP-sialic acid hydrolase. Kean and Bighouse (119) reported that as little as 0.2 mM EDTA inhibited the reaction of this enzyme by 50% and 0.2 M EDTA provided complete inhibition. However, these authors report that the activity of this enzyme was almost negligible at pH 6, the pH of the sialyltransferase assay, and that this enzyme was undetectable in human tonsillar lymphocytes. Thus it is unlikely that the effect of EDTA can be explained by the inhibition of this enzyme.

Other laboratories have reported apparent K_m values for CMP-sialic acid that range from 0.13 mM to 3 μ M. However, the findings of Rearick

et al. (96) and Paulson et al. (106) that β -D-galactoside $\alpha 2 \rightarrow 6$ and $\alpha 2 \rightarrow 3$ sialyltransferases have random order kinetic indicate that the K_m values for CMP-sialic acid will vary according to the concentration of acceptor used. It is thus very difficult to compare the K_m values obtained in these studies (see below).

<u>Tissue</u>	<u>Acceptor</u>	<u>K_m, CMP-sialic acid</u>	<u>Ref.</u>
Calf kidney cortex	asialo α_1 acid glycoprotein	0.13 mM	113
Porcine submaxillary gland	asialo, afuco porcine submaxillary mucin	0.169 mM	96
Human liver	asialofetuin	80 μ M	108
Bovine colostrum	asialo α_1 acid glycoprotein	3 μ M	106
Pork liver	asialofetuin	0.13 mM	107
Human placenta	asialofetuin	0.27 mM	54

II. Effect of Detergents

The approach of this project was to study the cellular fucosyl- and sialyltransferases in soluble form. Toward this end, studies were undertaken to determine which detergents were able to do this while still maintaining maximal enzyme activity. The results of these studies are shown in Tables 5, 6, and 10.

It should be noted that the data in these tables have been expressed as total activity per fraction. The reason for this is shown in Table 7; the specific activities of fucosyltransferases in the P_2 fraction increased with increasing TX-100 concentration and were equal to or greater than the specific activities of the corresponding P_1 fraction. However, the specific activities of the S_2 fractions showed no relation to TX-100 concentration or to P_2 activity. This was probably the result

of the solubilization of other membrane proteins into S_2 , increasing the specific activities in the P_2 fraction and decreasing those of S_2 even though the amount of fucosyltransferases in S_2 most likely remained the same. Clearly comparison of specific activities of enzymes in detergent solubilized preparations can only be made when the detergent concentrations are the same.

In our initial studies, solubility was defined as the proportion of recovered enzyme activity found in the supernatant fraction following detergent treatment and centrifugation at 37,000 x g for 30 min. As shown in Tables 5 and 6, maximal solubilization of the fucosyltransferases was attained using a combination of deoxycholate and TX-100; 0.3% deoxycholate plus 0.1% TX-100 was chosen as optimal since these concentrations provided high activity of both enzymes along with maximal solubilization.

In order to determine if the fucosyltransferases were actually soluble or just small membrane particles that did not sediment at 37,000 x g for 30 min, high-speed ultracentrifugation was carried out and the results of these experiments are shown in Table 9. While there was some difference in the solubilities of the two detergent preparations, it was unclear if this difference was significant since the recoveries from the two preparations were never greater than 80%. However, even if it is assumed that the loss of activity was only associated with the pelleted material, at least half of the original activity was seen in the supernatant fraction. However, since there is no reason to believe that the enzyme in the pelleted fraction was more labile under these conditions, the solubilities were probably closer to what was seen in the third column of Table 9.

Although deoxycholate alone clearly solubilized significant amounts of $\alpha 1 \rightarrow 6$ fucosyltransferase activity, the presence of TX-100 was still required in the assay for maximal enzyme activity, as shown in Table 20. In addition, the response to divalent metal ion was virtually abolished. The reason for the stimulation of enzyme activity seen upon the addition of TX-100 may be that the detergent bound to the hydrophobic portion of the enzyme, as described by Sadler et al. (114) for sialyltransferase. How this binding affects enzyme activity is unknown but it may help stabilize enzyme conformation, thereby increasing enzyme activity and/or making it more resistant to protease activity.

The stimulation of the $\alpha 1 \rightarrow 6$ fucosyltransferase by divalent metal ions may not necessarily be a property of the enzyme itself, but an artifact caused by the interaction of ions with TX-100. Similar effects have been reported by Shah and Ragupathy (111) for rat liver sialyltransferase, while Van Dijk et al. (113) report that calf kidney cortex sialyltransferase was inhibited by divalent metal ions in the presence of this detergent. The finding by Munro and Schachter (62) that the human serum enzyme was unaffected by divalent metal ions would also be relevant in this regard.

The search for a chaotropic agent that could solubilize sialyltransferase and still maintain activity was less successful, however. While 0.8% deoxycholate completely solubilized this enzyme, it also inhibited enzyme activity. The extent of this inhibition can be seen upon comparison with activity found in the presence of Cutscum or octylglucoside. Human serum sialyltransferase (110) and human lymphocyte sialyltransferase (165) were also inhibited by deoxycholate. On the other hand, Cutscum (0.5%) and octylglucoside were both unable to solubilize sialyl-

transferase to any extent; however, they did not interfere with enzyme activity. In fact, octylglucoside was the only detergent that provided enzyme activation. Unfortunately, octylglucoside was not available commercially until this project was complete and thus it was not used for any characterization studies.

Cutscum was used by Van den Lunden et al. (117) for the study of rat liver sialyltransferase and Van Dijk et al. (113) have reported that activity of calf kidney cortex sialyltransferase with Cutscum was essentially the same as with Triton X-100.

One question that arises from this study is why deoxycholate was required for maximum solubilization of both fucosyl- and sialyltransferases. Investigators who have studied glycosyltransferases from tissues such as placenta, liver, and salivary glands report that TX-100 alone is sufficient to solubilize these enzymes. However, Kirkpatrick et al. (169) report that deoxycholate was a much more efficient detergent than TX-100 in terms of protein solubilization. Thus it seems likely that sialyltransferase and fucosyltransferase from lymphocytes are more strongly bound than these enzymes are in other tissues, that is, the lipid composition of lymphoid cell membranes may be such that stronger detergents are required for their disruption. A study of the lipid constituents of lymphoid cell golgi membranes compared with other tissues would be informative in this regard.

Kuhns et al. (115) studied the orientation of galactosyltransferase in mammary gland golgi apparatus and determined that the active site of the enzyme faced into the golgi cisternae. Klips et al. (170) found that deoxycholate-treated brush border membrane vesicles showed discontinuities and release of vesicle contents. Assuming that deoxycholate functioned

in the golgi membrane system in the same way, the active site of the enzyme would be exposed to substrate more efficiently. The formation of membrane sheets would also allow TX-100 to attack the bilayer from both sides, making its action more efficient as well.

The data accumulated in this study allow some insight into the relationship of these enzymes to each other in the golgi membrane. As previously discussed, fucosyl- and sialyltransferases have been shown by many workers to be located in the golgi apparatus, probably as part of a multiglycosyltransferase complex that is something in the nature of an oligosaccharide-synthesizing assembly line. Bretz et al. (116) separated hepatic golgi apparatus into three morphologically and functionally distinct fractions, but they were unable to separate sialyl-, galactosyl-, and N-acetylglucosaminyltransferase activity, i.e., enrich one golgi fraction preferentially in terms of these enzyme activities. The authors concluded that these enzymes were located in close spatial relationship to each other, rather than being randomly distributed along the synthetic pathway. The requirement for very similar detergent treatment to solubilize all three enzymes under study in this project would also indicate that they were located in very similar membrane environments.

When these detergent studies were first initiated, the purification of these enzymes was a major consideration. The irreversible sensitivity of the fucosyltransferases to high salt concentrations, organic solvents, and ammonium sulfate made the purification of the cellular enzymes by conventional techniques extremely difficult. Affinity chromatography techniques were also precluded since high salt concentrations are often required.

III. Sulfhydryl Reagents

Liu et al. (54) also reported inhibition of placental sialyltransferase by 0.1 M iodoacetate and by 1 mM PCMB. These authors speculated that there was a methionine residue in the active site.

Alhadeff et al. (108) reported that 1.5 mM DTT decreased liver asialofetuin sialyltransferase activity by 81% and 14 mM mercaptoethanol inhibited activity by 44%.

The inhibition due to PCMB treatment of all three enzymes from the different sources may well be the result of the formation of a bulky mercaptide residue in the vicinity of the active site, which blocked access of the enzyme to its substrates.

IV. Substrate Binding Sites

The presence of fucose in concentrations up to 0.1 M affected the activity of both fucosyltransferases only slightly. Thus it appears that the nucleotide sugar was recognized through its nucleotide, GDP, and not through the sugar. The only other nucleotide sugar with a GDP moiety is GDP-mannose may be compartmentalized there, eliminating the problem of using the wrong precursor.

On the other hand, sialyltransferase was completely inhibited by 25 mM sialic acid, indicating that the recognition was for the entire nucleotide sugar. Sialyltransferase activity was also increased by 25% in the presence of 5 mM galactose. This may have been due to a favorable change in the conformation of the enzyme caused by the binding of the

galactose to the acceptor site. This in turn would lead to increased CMP-sialic acid binding. Since affinity for the galactose was most likely weaker than for asialofetuin, the glycoprotein probably displaced it. This mechanism is based on the findings of Paulson et al. (106) and Rearick et al. (55) who have shown that β -D-galactoside $\alpha 2 \rightarrow 6$ and $\alpha 2 \rightarrow 3$ sialyltransferases had random order kinetics.

$\alpha 1 \rightarrow 2$ fucosyltransferase, on the other hand, was inhibited by galactose, and this may indicate that even though a galactose terminated oligosaccharide was required for acceptor activity, the recognition site may have had more nearly equal affinities for galactose and oligosaccharides.

V. Comparison of Fucosyl- and Sialyltransferases in Lymphoid Lines and Fibroblasts

The data presented in Tables 14 and 15 provide clear evidence that neither a deficiency nor altered levels of $\alpha 1 \rightarrow 6$ fucosyltransferase constitute the basic defect in cystic fibrosis. It is already known that a deficiency of the other fucosyltransferases, $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 4$ do not play a role in the disease process since Bombay and Lewis negative individuals exhibit no clinical abnormalities of any sort.

Although a deficiency in any particular sialyltransferase is not ruled out by these studies, since the acceptor used could detect four different enzyme activities, there is no evidence that levels in patients with cystic fibrosis are altered in any way.

The relative activities of these enzymes in cells derived from patients with cystic fibrosis and obligate heterozygotes do not appear to be significantly different from those of normal donors, and thus it appears unlikely that this plays any role in the disease process. The determination of the various sialyltransferase activities using specific acceptors would provide conclusive evidence on this point.

However, the comparison of enzyme activities within a given cell line, presented in Tables 13-16, must be approached with considerable caution since saturating concentrations of nucleotide sugar substrates were not used. The use of such high concentrations of these radioactive substrates would be prohibitively expensive, and presents a significant problem for all investigators who study these enzymes. Unlabeled substrates are commercially unavailable, and the methods reported in the literature are unreliable (H. Schachter, private communication) and are, at best, extremely time-consuming.

Although experiments using saturating levels of substrates should be conducted to determine accurately the total enzyme activity in these cells, such studies would not reflect the actual levels of fucose and sialic acid transfer under physiologic conditions. Firstly it should be remembered that full enzyme activity is rarely realized under normal conditions, as witnessed by the large number of heterozygotes for a number of lethal autosomal recessive diseases who exhibit only half maximal activity of an essential enzyme and yet enjoy good health. Secondly, the levels of fucose and sialic acid incorporation are determined in the main by the *in vivo* levels of their nucleotide sugar precursors. Studies of the levels of GDP-fucose and CMP-sialic acid would be informative in their own right and would allow studies to be conducted under physiologic conditions when relative rates of incorporation are desired.

It is also difficult to compare the various enzyme activities within the cell lines because different detergent treatments were used for the preparation of the cell extracts used to determine the activities of the fucosyl- and sialyltransferases. It was indeed unfortunate that no method could be found that allowed the determination of all three acti-

vities in the same preparation. In order to minimize this difficulty, enzyme activities were determined in homogenates prepared from cells grown in the same culture flasks, and P_1 fractions were used to assay total fucosyltransferase activities. The protein concentrations of both Cutscum and deoxycholate plus TX-100 P_1 fractions were almost identical; thus the data could be expressed as sugar transferred per mg of protein.

In any case, the data can be used to compare relative activities from different cell lines since assay conditions were identical from line to line.

In this study, the fucosyltransferases were studied at the same GDP-fucose concentrations and this allowed comparison of their relative activities. In all cases, the $\alpha 1 \rightarrow 6$ activity was higher, from 14 to 100-fold, than the $\alpha 1 \rightarrow 2$ enzyme. While this was not a predictable result, it is not surprising.

Immunoglobulins are a major secretory product of lymphoid lines and are synthesized by over 90% of the cell population (171). The cells synthesize gamma, mu, and alpha heavy chains as well as both lambda and kappa light chains. Fucose in $\alpha 1 \rightarrow 6$ linkage to the chitobiose core is a common feature of the carbohydrate moieties of the heavy chain classes cited above (8). Wilson et al. (104) reported that an aspariginyl-linked oligosaccharide prepared from a myeloma protein acted as an acceptor for the $\alpha 1 \rightarrow 6$ enzyme.

The $\alpha 1 \rightarrow 2$ enzyme, so far as is presently known, is involved mainly in the synthesis of H antigen on cell surface glycoproteins and glycolipids. Thus the enzyme would be involved only in synthesizing oligosaccharides during membrane synthesis, i.e., during cell division and for ongoing membrane repair. Thus the relatively low levels of this enzyme are not particularly surprising.

The data presented in Table 13 indicate that alterations in fucosyl- and sialyltransferase activities were not associated with any of the metabolic diseases surveyed. Additionally, the finding that fucosyltransferase activities seen in cell lines derived from a fucosidosis patient and an obligate heterozygote for this disease, NB 41 and NB 49, respectively, were well within normal limits indicates that fucosidase activity did not significantly interfere with detection of fucosyltransferase activity. Thus the amount of fucose actually transferred was probably close to that reported in the table.

Table 16 shows the effect of time in culture on fibroblast enzyme activities. The results were quite interesting, showing a great deal of variation in enzyme activities and their ratios over time. The patterns were similar to the patterns of sialic acid incorporation seen in L cells (mouse fibroblasts)(172). This author also showed that an important factor in determining the relative levels of sialic acid incorporation was cell density rather than time in culture.

The two items of note in the tissue data are the levels of $\alpha 1 \rightarrow 6$ activity found in fetal intestine and in liver. It would be of great interest to obtain more samples from fetal tissue to determine if this enzyme is increased in other organs as well.

The low liver activity was quite surprising since this enzyme was very active in pork liver (89). It is possible that the long periods at minus 70° adversely affect enzyme activity. Further studies to determine the stability of this enzyme toward long periods of exposure to low temperatures are indicated.

References

1. Gottschalk, A (ed). Glycoproteins: Their Composition, Structure and Function. Elsevier, New York, 1972.
2. Johansen, PF, Marshall, RD and Neuberger, A. Biochem. J. 78:518, 1961.
3. Fletcher, AP, Marks, GS, Marshall, RD and Neuberger, A. Biochem. J. 87:265, 1963.
4. Marks, GS, Marshall, RD and Neuberger, A. Biochem. J. 87:274, 1963.
5. Baenziger, J and Kornfeld, S. J. Biol. Chem. 249:7270, 1974.
6. Spiro, RG and Bhoyroo, VD. J. Biol. Chem. 249:5704, 1974.
7. Arima, T, Spiro, MI and Spiro, RG. J. Biol. Chem. 247:1825, 1972.
8. Kornfeld, R and Kornfeld, S. Ann. Rev. Biochem. 45:217, 1976.
9. Kornfeld, R, Keller, J, Baenziger, J and Kornfeld, S. J. Biol. Chem. 246:3259, 1971.
10. Baenziger, J, Kornfeld, S and Kochwa, S. J. Biol. Chem. 249:1897, 1974.
11. Miller, F. Immunochemistry 7:423, 1970.
12. Yasuda, Y, Takahashi, N and Murachi, T. Biochem. 9:25, 1970.
- 13.(a) Carlson, D. J. Biol. Chem. 243:616, 1968.
- 13.(b) Aminoff, D, Baig, MM and Gathmann, WD. J. Biol. Chem. 254:1788, 1979.
- 13.(c) Lombart, CG and Winzler, RJ. Europ. J. Biochem. 49:77, 1974.
14. Lingappa, VR, Lingappa, JR, Prasad, R, Ebner, KE and Blobel, G. Proc. Natl. Acad. Sci., U.S. 75:2338, 1978.
15. Lodish, HF and Rothman, JE. Sci. Am. 240:48, 1979.
16. Rothman, JE and Lodish, HR. Nature 269:775, 1977.
17. Katz, FN, Rothman, JE, Knipe, DM and Lodish, HF. J. Supramol. Struc. 7:151, 1977.
18. Blobel, G and Dinnerstein, B. J. Cell Biol. 67:835, 852, 1975.
19. Kreibich, G, Czako-Graham, M, Grebenau, R, Mok, W, Rodriguez-Boulan, E and Sabatini, DD. J. Supramol. Struc. 8:279, 1978.

20. Waechter, CJ and Lennarz, WJ. *Ann. Rev. Biochem.* 45:95, 1976.
21. Roseman, S. *Chem. Phys. Lipids* 5:270, 1970.
22. Hagopian, A and Eylar, EH. *Arch. Biochem. Biophys.* 128:422, 1968.
23. Aubert, JP, Biserte, G and Loucheux-Lefebvre, MH. *Arch. Biochem. Biophys.* 143:410, 1976.
24. Nagarajan, M and Rao, VSR. *Current Sci.* 46(12):397, 1977.
25. Hanover, JA, Lennarz, WJ and Young, JD. *J. Biol. Chem.* 255: 6713, 1980.
26. Schachter, H, Jabbal, I, Hudgin, RL, Pinteric, L, McGuire, EJ and Roseman, S. *J. Biol. Chem.* 245:1090, 1970.
27. Letts, PJ, Pinteric, L and Schachter, H. *Biochim. Biophys. Acta* 372:304, 1974.
28. Letts, PJ, Meistrich, ML, Bruce, WR and Schachter, H. *Biochim. Biophys. Acta* 343:192, 1974.
29. Fleischer, B and Fleischer, S. *Biochim. Biophys. Acta* 219:301, 1970.
30. Ehrenreich, JH, Bergeron, JJ, Siekevitz, P and Palade, GE. *J. Cell Biol.* 59:45, 1973.
31. Sturgess, JM, Katona, E and Moscarello, MA. *J. Membrane Biol.* 12:367, 1973.
32. Hickman, S, Kukczcki, A Jr, Lynch, RG and Kornfeld, S. *J. Biol. Chem.* 252:4402, 1977.
33. Olden, K, Pratt, RM and Yamada, K. *Cell* 13:461, 1978.
34. Leavitt, R, Schlesing, S and Kornfeld, S. *J. Biol. Chem.* 252: 9018, 1977.
35. Ciccimara, F, Rosen, FS, Schneeberger, E and Merler, E. *J. Clin. Invest.* 57:1386, 1976.
36. Waring, G. *Biochem. Gen.* 16:69, 1978.
37. Hercz, A, Katona, E, Cutz, E, Wilson, JR and Barton, M. *Science* 201:1229, 1978.
38. Hart, GW, Brew, K, Grant, GA, Bradshaw, RA and Lennarz, WJ. *J. Biol. Chem.* 254:9747, 1979.
39. Sinohara, H and Marugama, T. *J. Mol. Evol.* 2:117, 1973.

40. Huber, R, Deisenhofer, J, Coleman, PM, Matsushima, M and Palm, W. *Nature* 264:416, 1976.
41. Gibson, R, Schlesinger, S and Kornfeld, S. *J. Biol. Chem.* 254: 3600, 1979.
42. Morell, AG, Gregoriades, G, Scheinberg, IH, Hickman J and Ashwell, G. *J. Biol. Chem.* 246:1461, 1971.
43. Jancik, J, Schauer, R and Streicher, HJ. *Hoppe-Seyler's Z. Physiol. Chem.* 356:1329, 1975.
44. Hudgin, J, Pricer, WE, Ashwell, G, Stockert, RJ and Morell, AG. *J. Biol. Chem.* 249:5536, 1974.
45. Lunney, J, Ph.D. dissertation, The John's Hopkins University, 1976.
46. Stockert, R, Morell, A and Steinberg, I. *Biochem. Biophys. Res. Commun.* 68:88, 1976.
47. Stahl, P, Schlesinger, PH, Rodman, FS and Diebber, T. *Nature* 264:5581, 1976.
48. Lunney, J and Ashwell, G. *Proc. Natl. Acad. Sci., U.S.* 73:341, 1976.
49. Neufeld, EF and Cantz, MI. *Ann. N.Y. Acad. Sci.* 179:580, 1971.
50. Kaplan, A, Achord, DT and Sly, WS. *Proc. Natl. Acad. Sci., U.S.* 74:2026, 1977.
51. Martin, SS and Bosmann, HB. *Biochim. Biophys. Acta* 230:411, 1971.
52. Bosmann, HB and Hemsworth, BA. *J. Biol. Chem.* 245:363, 1970.
53. Roth, S and Shur, BD. *Biochim. Biophys. Acta* 415:473, 1978; Deppert, W and Walter, G. *J. Supramol. Struct.* 8:455, 1978.
54. Liu, CK, Schmeid, R, Greenspan, EM and Waxman, S. *Biochim. Biophys. Acta* 522:375, 1978.
55. Rearick, JL, Sandler, JE, Paulson, JC and Hill, RL. *J. Biol. Chem.* 254:4444, 1979.
56. Feizi, T, Kabat, EA, Vicari, G, Anderson, B and Marsh, WL. *J. Immunol.* 106:1578, 1971.
57. Watkins, WM. *Proc. Roy. Soc. (London)B* 202:31, 1978.
58. Shen, L, Grollman, EF and Ginsburg, V. *Proc. Natl. Acad. Sci., U.S.* 59:224, 1968.

59. Chester, MA, Yates, AD and Watkins, WM. *Europ. J. Biochem.* 69:583, 1976.
60. Hearn, VM, Smith, LG and Watkins, WM. *Biochem. J.* 109:315, 1966.
61. Kobata, A, Grollman, EF and Ginsburg, V. *Arch. Biochem.* 124: 609, 1968.
62. Munro, JR and Schachter, H. *Arch. Biochem. Biophys.* 156:534, 1973.
63. Schenkel-Brunner, H, Chester, MA and Watkins, WM. *Europ. J. Biochem.* 30:269, 1972.
64. Beyer, TA, Rearick, JI, Paulson, JC, Prieels, JP, Sadler, JE and Hill, RL. *J. Biol. Chem.* 254:12531, 1979.
65. Jato-Rodriguez, JJ and Mookerjee, S. *Arch. Biochem. Biophys.* 162:281, 1974.
66. Carlson, DM, David, J and Rutter, WJ. *Arch. Biochem. Biophys.* 162:100, 1973.
67. Watanabe, K and Hakamori, SI. *J. Exptl. Med.* 144:644, 1976.
68. Jentoft, N, Cheng, PW and Carlson, DM. The Enzymes of Biological Membranes, Vol. 2, A. Martonosi, ed., Plenum, New York, 1976.
69. Nelson, JD, Jato-Rodriguez, JJ and Mookerjee, S. *Arch. Biochem. Biophys.* 169:181, 1975.
70. Reddy, PRK, Tadolini, B, Wilson, J and Williams-Ashman, HG. *Mol. Cell. Endocrinol.* 5:23, 1976.
71. Davidsohn, I, Kovarick, S and Lee, CH. *Arch. Pathol.* 81:381, 1966.
72. Kim, YS, Issacs, R and Perdomo, JM. *Proc. Natl. Acad. Sci., U.S.* 71:4869, 1974.
73. Yunis, EJ, Yunis, JJ and Brand, K. *Blood* 25:96, 1965.
74. Chessin, LH, Bramson, S, Kuhns, WJ and Hirschhorn, K. *Blood* 25: 944, 1965.
75. Stellner, K, Hakamori, SI and Warner, GA. *Biochem. Biophys. Res. Commun.* 55:439, 1973.
76. Bosmann, HB, Hagopian, A and Eylar, EH. *J. Cell Physiol.* 72:81, 1968.
77. Buck, CA, Fuhrer, JP, Soslau, G and Warren, L. *J. Biol. Chem.* 249:1541, 1974.

78. Pann, C and Kuhns, WJ. Nature, N.B. 240:22, 1972.
79. Mendicino, J and Rao, AK. Europ. J. Biochem. 51:547, 1975.
80. Kean, EL. J. Biol. Chem. 245:2301, 1970.
81. Schachter, H and Roden, L. Glycoprotein Biosynthesis. In: Metabolic Conjugation and Metabolic Hydrolysis, Vol. 3, WH Fishman, ed, Academic Press, New York, 1973, pp. 1-149.
82. Kornfeld, R. J. Biol. Chem. 242:3145, 1967.
83. Bauer, CH, Vischer, P, Grünholz, HJ and Reutter, W. Cancer Res. 37:1513, 1977.
84. Baenziger, J and Kornfeld, S. J. Biol. Chem. 249:7260, 1974.
85. Fournier, B, Strecker, G, Spik, G, Montreuil, J, Schmid, K, Dorland, L, Haverkamp, J, Schut, BL and Vliegenthart, J. Fourth International Symposium on Glycoconjugates, Woods Hole, Mass., 1977, RW Jeanloz, ed., Academic Press, New York, 1979.
86. Reading, CL, Penhoet, EE and Ballou, CE. J. Biol. Chem. 253: 5600, 1978.
87. Dawson, G and Tsay, CG. Nature of Storage Material in Fuco-
sidosi. In: Third International Symposium on Glycoconjugates,
Brighton, England, 1975, abstr.
88. Rovis, L, Anderson, B, Kabat, EA, Gruezo, F and Liao, J.
Biochem. 12:5340, 1973.
89. Jabbal, I and Schachter, H. J. Biol. Chem. 246, 5154, 1971.
90. Bosmann, HB, Hagopian, A and Eylar, EH. Arch. Biochem. Biophys.
128:470, 1968.
91. Whitehead, JS, Fearney, FJ and Kim, YS. Cancer Res. 39:1259,
1979.
92. Louisot, P, Levrat, C and Gilly, R. Clin. Chem. Acta 48:373,
1973.
93. Munro, JR, Narasimhan, S, Wetmore, S, Riordan, JR and Schachter,
H. Arch. Biochem. Biophys. 169:269, 1975.
94. Nakamura, A and Tsunemitsu, A. J. Dental Res. 54:417, 1975.
95. V8lk1, A, Seybold, J and Kern, HF. Cell Tiss. Res. 186:111,
1978.
96. Bella Jr, A and Kim, YS. Arch. Biochem. Biophys. 147:753, 1971.

97. Chou, TH, Murphy, C and Kessel, D. Biochem. Biophys. Res. Commun. 74:1001, 1977.
98. Beyer, TA, Sadler, JE and Hill, RL. J. Biol. Chem. 255:5364, 1980.
99. Beyer, TA and Hill, RL. J. Biol. Chem. 255:5373, 1980.
100. Mitranic, MM, Sturgess, JM and Moscarello, MA. J. Membrane Biol. 19:397, 1974.
101. Prieels, JP, Beyers, T and Hill, RL. Biochem. Soc. Trans. 5: 838, 1977.
102. Louisot, P, Belon, P and Broquet, P. Compt. Rend. Acad. Sci., Ser. D. 283:401, 1976.
103. Bella, A and Kim, YS. Biochem. J. 125:1157, 1971.
104. Wilson, JR, Williams, D and Schachter, H. Biochem. Biophys. Res. Commun. 72:909, 1976.
105. Nathenson, SG and Muramatsu, T. Glycoproteins of Blood Cells and Plasma. Jamienson, JA and Greenwalt, TJ, eds., Lippincott, Philadelphia, 1971, pp. 245-262.
106. Paulson, JC, Rearick, JI and Hill, RL. J. Biol. Chem. 252: 2363, 1977.
107. Ng, SS and Dain, JA. J. Neurochem. 29:1075, 1085, 1977.
108. Alhadeff, JA, Cinino, G, Janowsky, A and O'Brien, JS. Biochim. Biophys. Acta 484:307, 1977.
109. Hudgin, RL and Schachter, H. Can. J. Biochem. 49:829, 1971.
110. Klohs, WD, Bernacki, RJ and Korytnyk, W. Cancer Res. 39:1231, 1979.
111. Shah, SN and Raghupathy, E. Proc. Soc. Exptl. Biol. Med. 155: 516, 1977.
112. Bernacki, RJ. Europ. J. Biochem. 58:477, 1975.
113. Van Dijk, W, Lasrhuia, AM and van den Eijnden, DH. Biochim. Biophys. Acta 584:129, 1979.
114. Sandler, JE, Rearick, JL, Paulson, JC and Hill, RL. J. Biol. Chem. 254:4434, 1979.
115. Paulson, J, Beranek, WE and Hill, RL. J. Biol. Chem. 252:2356, 1977.
116. Bretz, R, Zretz, H and Palade, GE. J. Cell Biol. 84:87, 1980.

117. Van Den Eijnden, DH, Stoffyn, P, Stoffyn, A and Schiphorst, WECM. Europ. J. Biochem. 81:1, 1977.
118. Schachter, H, McGuire, EJ and Roseman, S. J. Biol. Chem. 246: 5321, 1971.
119. Kean, E and Bighouse, KJ. J. Biol. Chem. 249:7813, 1974.
120. Dalziel, K. Acta Chem. Scand. 11:6, 1957.
121. Kuhlenschmidt, MS, Peters, SP, Pinkard, OD, Glew, RH and Sharp, H. Biochim. Biophys. Acta. 429:359, 1976.
122. Mookerjee, S, Marshall, JW, Collins, JM and Ratnam, S. Biochem. Biophys. Res. Commun. 78:309, 1977.
123. Jarkovsky, Z, Marcus, DM and Grollman, AP. Biochem. 9:1123, 1970.
124. Dische, Z. Ann. N.Y. Acad. Sci. 106:259, 1963.
125. Tonelli, Q and Meints, RH. J. Supramol. Struc. 8:67, 1978.
126. Gottschalk, A. Biochim. Biophys. Acta 23:645, 1957.
127. Boat, TF, Davis, J, Stern, RC, Chang, PW. Biochim. Biophys. Acta 540:127, 1978.
128. Fishman, PH, Moss, J and Vaughan, M. J. Biol. Chem. 251:4490, 1976.
129. Remold, HG. J. Exptl. Med. 138:1065, 1973.
130. Tatsumura, T, Sato, H, Mori, A, Komori, Y, Yamamoto, K, Fukatani, G and Kuno, S. Cancer Res. 37:4101, 1977.
131. Bernacki, RJ and Kim, U. Science 195:577, 1977.
132. Khilani, P, Chou, TH, Lomen, PL and Kessel, D. Cancer Res. 37:2557, 1977.
133. Kessel, D, Khilani, P and Chou, TH. Cancer Treatment Rept. 62: 147, 1978.
134. Bauer, C, Köttgen, E and Reutter, W. Biochem. Biophys. Res. Commun. 76:488, 1977.
135. Bauer, C, Reutter, WG, Erhart, KP, Köttgen, E and Gerok, W. Science 201:1232, 1978.
136. Kessel, D and Allen, J. Cancer Res. 35:670, 1975.
137. Potter, JL, Matthews, LW, Lemm, J and Spector, S. Ann. N.Y. Acad. Sci. 106:692, 1963.

138. Dische, Z, Pallavicini, C, Cizek, LJ and Chun, S. Ann. N.Y. Acad. Sci. 93:526, 1962.
139. Knauff, RE and Adams, JA. Clin. Chim. Acta 19:245, 1968.
140. Boat, TF, Kleinerman, JI, Carlson, DM, Maloney, WH and Matthews, LW. Am. Rev. Resp. Dis. 110:428, 1974.
141. Boat, TF, Cheng, PW, Iyer, RN, Carlson, DM, Maloney, WH and Matthews, LW. Arch. Biochem. Biophys. 177:95, 1976.
142. Pearson, RD and Lubin, AH. Pediat. Res. 13:834, 1979.
143. Hösli, P and Vogt, E. Biochem. Biophys. Res. Commun. 79:741, 1977.
144. Alhadeff, JA, Tennant, L and O'Brien, JS. Clin. Genet. 10:63, 1976.
145. Alhadeff, JA, Watkins, P and Freeze, H. Clin. Genet. 13:317, 1978.
146. Alhadeff, JA and Cimino, G. Clin. Genet. 13:207, 1978.
147. Hunt, LA and Summers, DF. J. Supramol. Struc. 7:213, 1977.
148. Butterworth, J, Scott, F, McCrae, WM and Bain, AD. Clin. Chim. Acta 40:139, 1972.
149. Scanlin, TF, Matakic, SS, Pace, M, Santer, UV and Glick, MC. Biochem. Biophys. Res. Commun. 79:869, 1977.
150. Isenberg, JN, Sahlstrom, SJ and Sharp, HL. Am. Rev. Resp. Dis. 113:567, 1976.
151. Antonowicz, I, Seppell, WG and Shwachman, H. Pediat. Res. 6:803, 1972.
152. Guha, AK, Kutty, KM, Chandra, RK and Way, RC. Clin. Biochem. 10:153, 1977.
153. Mookerjee, S, Michaels, MA, Hudgin, RL, Moscarello, MA, Chow, A and Schachter, H. Can. J. Biochem. 50:738, 1972.
154. Glade, PR and Beratis, NG. Progr. Med. Genet. 1:1, 1976.
155. Kuhn, NJ. Biochem. Soc. Trans. 5:840, 1977.
156. Spiro, RG. J. Biol. Chem. 239:567, 1964.
157. Kim, YS, Perdomo, J and Nordberg, J. J. Biol. Chem. 246:5466, 1971.

158. de Salegui, M and Plonska, E. Arch. Biochem. Biophys. 129:49, 1969.
159. Lowry, OH, Rosebrough, NJ, Farr, AL and Randall, RJ. J. Biol. Chem. 193:265, 1965.
160. Udenfriend, S, Stein, S, Böhlen, P, Dairman, W, Leimgruber, W and Weigela, M. Science 178:871, 1972.
161. Glade, PR and Broder, SW. Preparation and Care of Established Human Lymphoid Cell Lines. In: In Vitro Methods in Cell-Mediated Immunity. B Bloom and PR Glade, eds., Academic Press, New York, 1971, p. 561.
162. Beratis, NG, Turner, BM, Weiss, R and Hirschhorn, K. Ped. Res. 8:475, 1975.
163. Baron, C and Thompson, TE. Biochim. Biophys. Acta 382:276, 1975.
164. Metzler, DA. Biochemistry, the Chemical Reactions of Living Cells, Academic Press, New York, 1977, p. 119.
165. Shapiro, BL, Lam, LFH and Fast, LH. Science 203:1251, 1979.
166. Pauszka, T and Koscielak, J. FEBS Letters 41:348, 1974.
167. Strous, GJAM and Kramer, MF. Biochim. Biophys. Acta 451:201, 1976.
168. Paulson, JC, Pricels, JP, Glasgow, LR and Hill, RL. J. Biol. Chem. 253:5617, 1978.
169. Kirkpatrick, FH, Gordesky, SE and Marinetti, GV. Biochim. Biophys. Acta 345:154, 1974.
170. Klip, A, Grinstein, S and Semenza, G. Biochim. Biophys. Acta 558:233, 1979.
171. Glade, PR and Hirschhorn, K. Am. J. Pathol. 60:483, 1970.
172. Glick, MC. Biology and Chemistry of Eucaryotic Cell Surfaces, Vol. 7, FY Lee and EE Smith, eds., Academic Press, New York, 1974.