ADVANCES IN CANCER RESEARCH

VOLUME 17

ADVANCES IN CANCER RESEARCH

Edited by

GEORGE KLEIN
Department of Tumor Biology
Karolinska Institutet
Stockholm, Sweden

SIDNEY WEINHOUSE
Fels Research Institute
Temple University Medical School
Philadelphia, Pennsylvania

Consulting Editor

ALEXANDER HADDOW
Chester Beatty Research Institute
Institute of Cancer Research
Royal Cancer Hospital
London, England

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CONTRIBUTORS TO VOLUME 17

Numbers in parentheses refer to the pages on which the authors' contributions begin.

ANTONIO CANTERO, *Laboratoire de Recherches, Institut du Cancer de Montréal, Hôpital Notre-Dame et Université de Montréal, Montréal, Canada* (1)

JOHN S. HARINGTON, *Cancer Research Unit, National Cancer Association of South Africa, Johannesburg, South Africa* (81)

ALFRED G. KNUDSON, JR., *Medical Genetics Center, Graduate School of Biomedical Sciences, and M. D. Anderson Hospital and Tumor Institute, University of Texas at Houston, Houston, Texas* (317)

FRANK LILLY, *Department of Genetics, Albert Einstein College of Medicine, Bronx, New York* (231)

CHARLES M. MCGRATH, *Department of Zoology and Its Cancer Research Laboratory, University of California, Berkeley, California* (353)

S. NANDI, *Department of Zoology and Its Cancer Research Laboratory, University of California, Berkeley, California* (353)

K. NAZERIAN, *United States Department of Agriculture, Agricultural Research Station, Regional Poultry Research Laboratory, East Lansing, Michigan* (279)

VIJAI N. NIGAM,[1] *Laboratoire de Recherches, Institut du Cancer de Montréal, Hôpital Notre-Dame et Université de Montréal, Montréal, Canada* (1)

THEODORE PINCUS,[2] *Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland* (231)

GERALD P. WARWICK, *Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, England* (81)

[1] Present address: Département de Biologie Cellulaire, Faculté de Médicine, Centre Hospitalier, Sherbrooke, Québec, Canada.

[2] Present address: Sloan-Kettering Institute for Cancer Research, New York, New York.

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POLYSACCHARIDES IN CANCER: GLYCOPROTEINS AND GLYCOLIPIDS[1]

Vijai N. Nigam[2] and Antonio Cantero

Laboratoire de Recherches, Institut du Cancer de Montréal,
Hôpital Notre-Dame et Université de Montréal, Montréal, Canada

I. Hetero-oligo- and Heteropolysaccharides—Glycoproteins

A. General

Although the existence of glycoproteins in tissues and serum has been known for a long time, studies of their chemistry, biochemistry, and function are relatively meager. Variations in the concentrations of serum glycoproteins have been implicated in a variety of diseased states, and reviews on serum glycoproteins with respect to cancer have appeared earlier in the *Advances in Cancer Research* (Winzler, 1953; Abelev, 1971). We will therefore omit major discussion of serum glycoproteins

[1] The first part of this review was published in Volume 16 of this serial publication. It included consideration of homopolysaccharide (glycogen) and mucopolysaccharides (glycosaminoglycans). This part concerns the hetero-oligo and heteropolysaccharide (glycoprotein and glycolipid) components of normal and cancer cells.

[2] Present address: Département de Biologie Cellulaire, Faculté de Médicine, Centre Hospitalier, Sherbrooke, Québec, Canada.

TABLE I

CARBOHYDRATES COMPOSITION OF REPRESENTATIVE GLYCOPROTEINS OF DIFFERENT CLASSES[a]

Class	Members	Representative	Galactose (%)	Mannose (%)	GNAc[b] (%)	GalNAc[b] (%)	Sialic acid (%)	Fucose (%)	Total carbohydrates (%)
Plasma	Orosomucoid, fetuin, ceruloplasmin, haptoglobins, prothrombin, fibrinogen, transferrin, γ-globulins	Orosomucoid	6.5	4.8	15.2	0	10.8	1.0	38.3
Urinary	Glycoprotein of Tamm and Horsfall	Glycoprotein of Tamm and Horsfall	5.4	2.7	9.6	2.0	9.1	1.1	29.9
Hormones and related substances	Human chorionic gonadotropin, follicle-stimulating hormone, thyroglobulin, erythropoietin	Human chorionic gonadotropin	—	—	10.7	—	8.5	1.2	31.4
		Thyroglobulin	1.3	3.5	4.2	0	1.1	0.5	10.6
Blood-group active substances	Ovarian cysts, gastric mucosa, saliva, meconium, amniotic fluid, urine	Ovarian cyst	17.0	0	21.6	24.1	—	18.0	80.7
Mucous secretions	Submaxillary, sublingual, cervical, bronchial, gastric, and biliary secretions	Cervical (bovine estrus)	27.5	0	18.9	14.1	13.8	5.1	79.4
Connective tissue	Collagen, reticulin, basement membranes, lens capsule, soluble glycoproteins	Collagen (calf)	1.5 (hexose)		0.4 (hexosamine)		0	—	1.9
Enzymes	Cholinesterase, ribonuclease B, phosphatases	Ribonuclease B	6.3 (hexose)		5.0 (hexosamine)		0	—	11.3

[a] Data taken from papers by Spiro (1963) and Eylar (1965).

[b] GNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

and will concentrate on the chemistry, biochemistry, and histochemistry of tissue glycoproteins. Further, the reader may turn to a recently published book (Gottschalk, 1966) and recent review articles (Kent, 1967; Montgomery, 1970; Burger, 1971a; Marshall, 1972).

Glycoproteins are usually defined as protein–carbohydrate complexes in which oligo- or polysaccharides are joined by covalent linkage to specific amino acids of proteins. The carbohydrate portion mostly contains amino sugars (glucosamine, galactosamine, or sialic acid) and hexose (galactose, mannose, or fucose), and they can be distinguished from mucopolysaccharides by the absence of uronic acid and rarity of sulfate groups. Eylar (1965) and Spiro (1963) have each compiled a comprehensive list of well-defined glycoproteins of animal origin, and a partial list of these is collected in Table I. Forty-seven glycoproteins are known to be present in blood plasma. So far fifty glycoproteins are described as extracellular and twenty as intracellular. The presence of such a large number of extracellular glycoproteins in animals led Eylar (1965) to suggest that the addition of carbohydrate residues to proteins may provide them a "passport" to escape from the intracellular to the extracellular environment. However, the amount of carbohydrate in different glycoproteins is variable (0.1% in serum albumin and 82.0% in cervical mucin) and does not appear to be a critical factor in their extracellular or intracellular location. Among individual carbohydrates, hexose concentration varies from 0.3 to 28%, acetylhexosamine from 0 to 40%, and sialic acid from 0 to 30% in various glycoproteins.

B. Structure of Glycoproteins

In the structural determination of glycoproteins, various proteolytic enzymes have been employed to remove most of the amino acid. The glycopeptides are then subjected to gel filtration and are further purified by chromatography on ion-exchange resins or by electrophoresis. The glycopeptides are used to determine the number and composition of the carbohydrate units, and the structure of these units with respect to the sequence, linkage, and branching of the component monosaccharides. The chemical nature of the glycopeptide bond, the sugars and the amino acids involved in the covalent bonding, and the arrangement of carbohydrate units along the peptide chains are other structural features that are explored on other components of partially degraded glycoproteins, namely the glycopeptides. The stability of *N*-glycosidic linked glycopeptides to alkali is further used as a means to distinguish them from the *O*-glycosidic linked glycopeptides. In glycoproteins in which serine or threonine are *O*-glycosidically linked, alkali treatment produces 2-amino-

acrylic acid or 2-aminocrotonic acid through a characteristic reaction mechanism of β-elimination (Gottschalk, 1966).

The development of the useful techniques of sequential periodate oxidation and borohydride reduction (Smith degradation) has greatly helped in providing information about the nature of glycosidic bonds. For a description of the application of Smith degradation, the reader is referred to an article by Spiro (1966) and a review by Kent (1967).

The *N*-glycosidic linkage involves *N*-acetylhexosamine in a secondary amide bond with L-asparagine. The linkage compound, 2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose has been obtained from egg

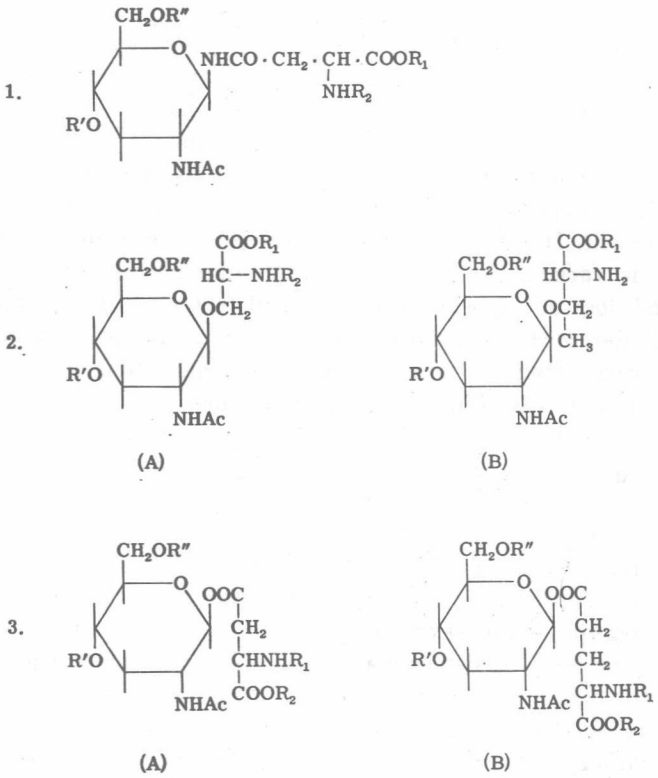

FIG. 1. Sugar–amino acid linkages in glycoproteins. (1) Acylglycosylamine linkage involving C-1 of *N*-acetylglucosamine and amide N of asparagine. (2) *O*-Glycosidic linkage involving C-1 of *N*-acetylgalactosamine and hydroxyl of serine (A) and threonine (B). (3) Glycosidic ester bond between *N*-acetylhexosamine and β-carboxyl of aspartic acid (A) or γ-carboxyl of glutamic acid (B). These linkages are not frequent. Unequivocal proof of their existence in glycoproteins is also lacking. Sugar-hydroxy-L-proline and sugar-hydroxy-L-lysine linkages found in extensin and collagen are not shown. R′, R″, R_1, and R_2 represent substitutions.

albumin and other glycoproteins. Since the *N*-acetylhexosamine–aspartamide link is somewhat more stable than the peptide bond toward acid hydrolysis, it has been possible to obtain glycopeptides containing this linkage. This type of linkage occurs in a large number of glycoproteins.

O-Glycosidic bonds between *N*-acetylgalactosamine and threonine have been demonstrated in a human colloid breast carcinoma glycoprotein (Adams, 1965). Such a linkage is also representative of red cell membranes where the glycosidic linkage involves serine or threonine residues of the peptide chain (Gottschalk, 1966). Recent investigations on ovine submaxillary gland glycoprotein by Carubelli, Bhavanandan, and Gottschalk (1965) tends to support *O*-glycosidic bonds involving serine or threonine and the disaccharide (α-*N*-acetylneuraminidyl-(2 → 6)-*N*-acetylgalactosamine) side branches instead of the Kennedy-proposed glycosidic ester bond between the disaccharide and the β- or γ-carboxyl groups of aspartic acid. In extensin and collagen, glycosidic linkages involving hydroxy-L-proline and hydroxy-L-lysine have been shown (Lamport, 1967; Butler and Cunningham, 1966; Spiro, 1967).

O-D-galactopyranosyl-(1 → 3)-S-D-galactopyranosyl linked to L-cysteine and triglucosyl-pyranosyl linked to L-cysteine have been detected in urine (Lote and Weiss, 1971) and in red cell membranes (Bevan *et al.*, 1972), respectively. However, it is not known if these products are catabolites of glycoproteins or they are biosynthesized.

Some of the common carbohydrate-peptide linkages are shown in Fig. 1.

Although no general structure can be described for all glycoproteins, there are some features common to many. Thus, among mucins, the viscous glycoproteins, a large number of carbohydrate side branches terminated either by sialic acid or fucose are attached to a single protein core (Fig. 2a); whereas in the globular glycoproteins, for example fetuin, carbohydrate residues form large structures, a few (3 or 4) of which are attached to the polypeptide chain (Fig. 2). Most studies still center on the structural determination of glycopeptide or oligosaccharide fractions

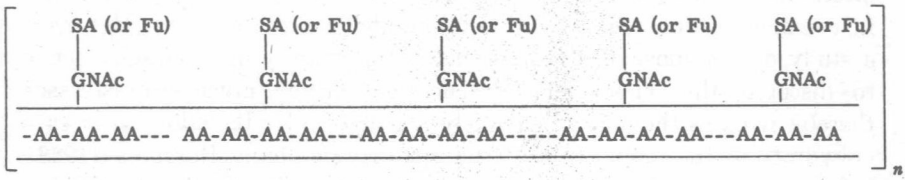

FIG. 2a. Hypothetical structure of a viscous glycoprotein (ovine and bovine submaxillary mucin). AA, amino acid; GNAc, *N*-acetylgalactosamine; SA, sialic acid; Fu, fucose.

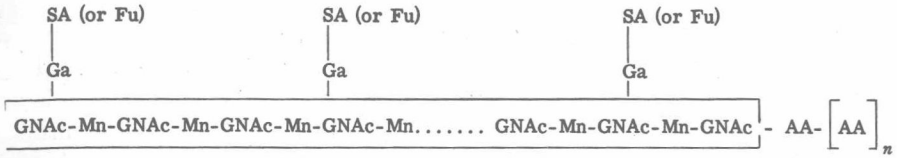

FIG. 2b. Hypothetical structure of a globular glyprotein (fetuin). AA, amino acid; GNAc, *N*-acetylglucosamine; Mn, mannose; Ga, galactose; SA, sialic acid; Fu, fucose.

isolated by enzymatic digestion and acid treatment. Although these studies help to define structural features of regions of glycoproteins, the complete structure of no glycoprotein, with the possible exception of ribonuclease, can be described with any accuracy.

C. BIOSYNTHESIS OF GLYCOPROTEINS

According to the recent work carried out on the biosynthesis of glycoproteins, it is believed that the polypeptide portion of protein undergoing synthesis is the receptor of *N*-acetylhexosamine at the ribosomal level. The completed polypeptide chain, once it is released, is able to add on other sugars to the hexosamine residues by specific transferases and the reaction occurs either in the soluble portion of the cell or on the smooth membranes of the endoplasmic reticulum. Other possibilities are that either the oligosaccharide chains are synthesized first in a stepwise manner and later transferred to polypeptide through the aid of a carrier, or the entire oligosaccharide chain is synthesized with the nucleotide attached and the oligosaccharide is then transferred to polypeptide by a nucleotide-linked glycosyltransferase. To give specificity to hexosamine (or hexose) linking to asparagine, serine, or threonine, it has been suggested that either (1) a specific sequence may be present around the amino acid to which carbohydrate is to be linked in the polypeptide chain to render specificity to the amino acid-carbohydrate bonding or (2) specificity may be determined by glycosyltransferase itself. If the first alternative is true, one would expect that many glycoproteins will share in common certain regions with similar amino acid sequence. However, a study of a number of glycoproteins in the vicinity of asparagine tends to discount this view. The presence of specific glycosyltransferases thereby remains the only other likely alternative for the addition of carbohydrate to the amino acid of the polypeptide chain. Roseman (1968) believes that glycosyltransferases comprise homologous families which differ in their specificity for acceptor, but transfer only one type of sugar. Thus, through the concerted action of specific transferases a glyco-

protein is built, the acceptor molecules themselves playing a major role in the formation of the completed product.

Since amino acid residues accepting carbohydrate may all be of one type or of different types in the same glycoprotein molecule and some may be glycosylated and others may not be, it is yet unknown what character of the amino acid sequence determines glycosylation of an amino acid residue. Although, in case of glycosylation of asparagine, it was postulated that the sequence asparagine-any amino acid-serine (or threonine) was essential, it has been noted that asparagine is not glycosylated in all such sequence present in glycoproteins (Marshall, 1972). Further, egg yolk phosvitin appears to be an exception to the above postulate as it contains the sequence

serine-asparagine-serine-glycine
|
sugar

The primary structure of the apoprotein that determines whether glycosylation of serine and threonine residues will occur also remains unknown and precise structural arrangements of amino acids in the vicinity of serine and threonine residues give little clue of any specific requirement. It is possible that stereochemical factors and conformation of the apoprotein may have a decision-making role when the necessary enzymes, substrates, and favorable kinetic factors are available.

More recently, interest has been centered on the possibility that isoprenoid-linked sugar pyrophosphates may be the immediate donors of sugars to glycoproteins (Lennarz and Scher, 1972). Such lipid-linked sugar carriers are known to participate in the synthesis of bacterial mucopeptides and lipopolysaccharides (Osborn, 1969). One of the reasons for finding a lipid carrier for sugars is based on the fact that polar nucleoside diphosphate sugars fail to penetrate the hydrophobic environment of the plasma and Golgi membranes, although these are the glycoprotein-rich membranes of the cell. Transfer of a sugar to a lipid from nucleoside diphosphate sugar will easily convert the sugar carrier to a hydrophobic molecule that can penetrate and glycosylate proteins associated with the lipid bilayer of the membranes.

Initial studies seem to indicate that polyprenol phosphates can serve as acceptor lipids for sugars in a glycosyltransferase reaction. However, it is far from clear which of the polyprenols (undecaprenol, dolichol, ficaprenol, retinol, vitamin K_1, etc.) are the specific carriers of different sugars. Many investigators (Caccam *et al.*, 1969; Zatz and Barondes, 1969; Tetas *et al.*, 1970; Molnar *et al.*, 1971; Behrens and Leloir, 1970; Baynes and Heath, 1972; DeLuca *et al.*, 1970; H. V. Johnson *et al.*, 1971)

using different mammalian systems have shown the transfer of certain sugars from their nucleoside diphosphate derivatives to endogenous and exogenous lipid acceptors. The linkage between sugar and lipid has been found to be susceptible to mild acid hydrolysis but resistant to alkaline hydrolysis (Alam and Hemming, 1971). Incorporation of sugar from nucleoside diphosphate sugar donor into the lipid has been shown to occur prior to its transfer to protein during glycoprotein biosynthesis. Behrens and Leloir (1970) have also shown that dolichol monophosphate hexose transfers hexose to a product seemingly a glycoprotein. However, dolichol monophosphate does not participate in the synthesis of ceramide-glucose or collagen (Behrens *et al.*, 1971).

Finally, the way in which the size of the carbohydrate chains is limited in glycoproteins remains obscure, and no information is available on the terminating signal required to indicate chain completion. Although it has been assumed that the introduction of α-L-fucosyl or α-sialyl moieties in 1C pyranose conformation may indicate chain completion, this has not been found universal for all glycoproteins (Marshall, 1972).

1. *Normal Tissues*

A number of investigators observed the incorporation *in vivo* of labeled glucose or glucosamine into liver glycoproteins. Boström *et al.*, (1958) showed that injection of labeled glucose into guinea pigs resulted in the labeling of neutral sugars and to a lesser extent of glucosamine and sialic acid of α_1-acid glycoprotein. An approximate half-life of 1 to 2 days was calculated for this glycoprotein. Spiro (1959) studied the incorporation of glucose-U-^{14}C into protein-bound glucosamine in tissues and plasma. The specific activity as well as total radioactivity of liver protein-bound glucosamine reached its maximum one and a half hours after injection. At three and three-quarter hours, serum glucosamine also reached the same activity. From kinetic studies of labeling, a precursor–product relationship for liver and serum glycoproteins was established. Shetlar *et al.* (1961) found that glucosamine-1-^{14}C injected intraperitoneally into rats or rabbits was rapidly incorporated into liver protein-bound glucosamine and into various serum fractions. In this case neutral hexoses had very little radioactivity (Shetlar, 1961). Kohn, Winzler, and Hoffman (1962) also investigated the metabolism of glucosamine-1-^{14}C and *N*-acetylglucosamine-1-^{14}C in liver of rats. The former was found to be a better precursor of glucosamine and sialic acid in liver glycoproteins. In contrast, glucose-1-^{14}C had very poor activity.

Draper and Kent (1963) reported the *in vitro* incorporation of ^{14}C from glucose-1-^{14}C into neutral sugars, hexosamine, and sialic acid of

glycoproteins of sheep colonic mucosa. More recently, using glucosamine-^{14}C as a glycoprotein precursor, Athineos *et al.* (1964), Molnar *et al.* (1964), Lawford and Schachter (1966), and Li *et al.* (1968) showed incorporation of the label into plasma glycoproteins or their precursors. Generally the label was first observed in the trichloroacetic acid-soluble fraction, then in macromolecules and finally in the plasma (Robinson *et al.*, 1964). Molnar *et al.* (1964) and Lawford and Schachter (1966) concluded that the hexosamine is incorporated initially into polypeptides undergoing synthesis on the ribosomes and further hexosamine residues are added after detachment from the ribosomes and during the movement of the polypeptide along the channels of rough and smooth surfaced endoplasmic reticulum. Carbohydrates occupying the terminal positions were assumed to be added within the smooth-surfaced endoplasmic reticulum. Hallinan, Murty, and Grant (1968) have confirmed that initiation of carbohydrate prosthetic group attachment occurs while the glycoprotein is still bound to sites of synthesis on the endoplasmic reticulum-bound ribosomes. Free ribosomes or polysomes were inactive in glycoprotein synthesis initiation. Li *et al.* (1968) isolated two radioactive peptides from the deoxycholate soluble fraction of rat liver microsomes after an injection of glucosamine-1-^{14}C. These glycopeptides were rich in mannose and *N*-acetylglucosamine, but poor in sialic acid and galactose. It was apparent that oligosaccharide chains in these glycopeptides lacked sialic acid–galactose-*N*-acetylglucosamine chains which are peripheral of sugar units in plasma glycoproteins. The isolated glycopeptides thereby appeared to be representative of the inner core of the mature plasma glycoprotein. The isolation of these glycopeptides spoke against the hypothesis that oligosaccharides are first synthesized at the nucleotide level and then transferred to the polymer.

The above investigations on glycoprotein synthesis *in vivo* have been accompanied closely by studies on glycoprotein synthesis in cell-free systems. Sarcione (1964) studied the simultaneous incorporation of leucine-^{3}H and galactose-^{14}C into glycoproteins in cell-free liver fractions. Incorporation of galactose was observed initially in the deoxycholate soluble proteins of microsomal fraction, and virtually none in the ribosomes themselves. Leucine, on the other hand, was incorporated initially in the ribosomal protein followed by its progressive appearance in the deoxycholate-soluble fraction. It was therefore concluded that galactose residues are added to glycoprotein of rat liver after the protein leaves the polysome complex. Sarcione and Carmody (1966) later provided evidence for the existence of an enzyme system in the deoxycholate soluble fraction that catalyzed incorporation of galactose from UDP-galactose into liver microsomal protein. Since the addition of increasing amounts of ribosomes to the deoxycholate soluble fraction stimulated incorpora-

tion of galactose, it was interpreted that ribosomes provide additional acceptor protein.

Johnston *et al.* (1966) and McGuire *et al.* (1965) also studied enzymes that catalyze transfer of galactose from UDP-galactose to glycoprotein I (prepared by pretreating orsomucoid with purified sialidase and β-galactosidase). The enzyme is particulate and is present in rat tissues. The partially purified enzyme from goat colostrum was active with UDP galactose and UDP-*N*-acetylgalactosamine as donors and glycoprotein I and *N*-acetylglucosamine as acceptors. Further purification of goat colostrum gave another enzyme that could transfer *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to certain glycoproteins denuded of their carbohydrate. Based on the structure of the glycoprotein formed, it was shown that *N*-acetylglucosamine is transferred to the terminal mannose units of the glycoprotein. It was thus apparent that the addition of each hexose requires an individual glucosyltransferase and an appropriate substrate, and requires a glycoprotein containing a particular terminal monosaccharide residue. The oligosaccharide is thus not built in a random fashion but according to an ordered sequence leading to the formation of the appropriate saccharide unit of the glycoprotein.

O'Brien *et al.* (1966) reported the attachment of sialic acid to glycoprotein stripped of its sialic acid by sialidase treatment. Rat liver microsomes were shown to carry out the incorporation of sialic acid from CMP-sialic acid to an endogenous acceptor. The reaction was found to stop when the acceptor sites were filled and 50 μmoles of sialic acid had been incorporated per gram of liver. Addition of puromycin to the reaction digest had no effect on the incorporation of sialic acid. Similar to the observations of Sarcione (1964), the product of endogenous transfer with CMP-sialic acid-^{14}C was within the microsomes and could be solubilized by sonication and detergents. Furthermore, sonic extracts gave rise to radioactive precipitin lines with antiserum against plasma proteins showing the validity of incorporation of sialic acid into plasma proteins.

Purification and properties of animal sialic acid transferases has been comprehensively described in a joint article by Roseman and others (Roseman *et al.*, 1966).

Recently a cell-free microsomes–cell sap system from guinea pig liver has been described by Simkin *et al.* (1968) that is capable of incorporating *N*-acetylglucosamine into microsomal fraction in the presence of UTP.

2. *Tumor Cells*

Since the microsomal fraction of Ehrlich ascites tumor cells contains significant amounts of sialic acid-bound glycoprotein (Wallach and