SUGAR RESIDUES ON PROTEINS

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I. INTRODUCTION

In recent years it has become known that a large number of proteins are associated with covalently linked sugars. Such proteins, commonly designated as glycoproteins, are a complex group of macromolecules which appear to be widely distributed in nature. They have been found in virtually all forms of life — i.e., animals, plants, and microorganisms — and have been identified in the extra- and intracellular fluids, connective tissue, and cellular membranes. Interest in glycoproteins is mainly due to the diverse biological functions which they perform. These include, among others, immunological protection, enzymatic catalysis, hormonal control, hormone storage, blood clotting, ion transport, lubrication, surface protection, structural support, cell adhesion, and molecular recognition. Some well-known glycoproteins with well-defined functions include immunoglobulins,[1] gonadotropins,[2-9] thyroid-stimulating hormone,[10] ribonuclease,[11,12] antifreeze glycoprotein,[13] mucous glycoproteins,[14] avidin,[15,16] proteoglycans,[17] collagen,[18] and fibronectin[19] (Table 1). In recent years, significant advances have been made in regard to the biosynthesis, degradation, structure-function relationship of glycoproteins and their involvement in the manifestation of several disease processes. A number of review articles[1,20-24] and monographs[25-27] covering various aspects of glycoproteins have been published. It is the purpose of this article to review primarily the structural aspects of the glycoproteins, although brief reference will be made to proteoglycans and collagen and also to the biosynthesis and role of the carbohydrate in the biological processes.

The key step in the transformation of a protein into a glycoprotein is the glycosylation of the peptide chain during biosynthesis. Thus, glycoprotein by definition is a protein in which one or more saccharides are covalently attached to the peptide chain. Although this strict definition would technically include proteoglycans, they have been classified separately on the basis of repeating units in the saccharides and the number or size of the saccharide units. The carbohydrate content of glycoproteins ranges from less than 1% to over 80% of the molecule. Three types of sugars commonly found are (1) neutral sugars such as D-galactose, D-mannose, D-glucose, and L-fucose, (2) amino sugars as *N*-acetylglucosamine and *N*-acetylgalactosamine, and (3) acidic sugars as sialic acids. The latter are derivatives of neuraminic acid in which the amino group is always substituted by acetyl or glycolyl groups; therefore, these are referred to as *N*-acetyl- or *N*-glycolyl neuraminic acid. In addition to *N*-acetyl groups, sialic acids have been found which contain one to three *O*-acetyl groups. Other acidic sugars such as D-glucuronic and L-iduronic acids are essential components of proteoglycans. A recent report on the presence of D-glucuronic acid in a glycoprotein[28] would narrow the compositional differences between

Table 1
FUNCTIONS OF SOME WELL-CHARACTERIZED GLYCOPROTEINS

Glycoprotein	Source of isolation	Function
Immunoglobulins	Vertebrate plasma[1]	Immunoprotection
Chorionic gonadotropin	Urine[2-5]	Hormone
Follicle-stimulating hormone	Pituitary,[6] serum[7]	Hormone
Luteinizing hormone	Serum,[7] pituitary[8,9]	Hormone
Thyroid-stimulating hormone	Pituitary[10]	Hormone
Ribonuclease	Pancreas[11,12]	Enzyme
Antifreeze glycoprotein	Plasma (Antarctic fish)[13]	Freezing point, depressing
Mucous glycoproteins	Mucous secretions[14]	Lubrication, protective
Avidin	Hen egg[15,16]	Vitamin-binding
Proteoglycans	Cartilage[17]	Structural
Collagens	Skin[18]	Structural
Fibronectin	Cell surfaces[19]	Cell adhesion

glycoproteins and proteoglycans. Glycoproteins of plant and microbial origins contain D-xylose, L-arabinose, and L-rhamnose. D-xylose is also a linkage sugar in proteoglycans. Besides sulfated proteoglycans, several glycoproteins (such as those from mucous secretions and brain) contain sulfated sugar residues.

In view of the diversity in both the carbohydrate content and the types of monosaccharides present in glycoproteins, theoretically one can expect several possible permutations for the structural patterns of the carbohydrate units when more than one sugar is involved. However, an examination of the known structural patterns of the carbohydrate units in glycoproteins from various sources indicates that only a limited number of these patterns exist, thereby suggesting a limited number of biosynthetic pathways. However, within these fewer structural patterns a large variety of structures differing in size and the intersugar linkages are possible. The glycoproteins differ in the number of carbohydrate units present. For example, bovine ribonuclease B,[11] human chorionic gonadotropin,[4,5] and ovine submaxillary mucin contain 1, 8, and 800 carbohydrate units per molecule of the glycoprotein, respectively. Furthermore, there is no uniform pattern(s) of the distribution and location of the carbohydrate units on the polypeptide chain. From these observations, it follows that it is not feasible to classify glycoproteins from the standpoint of composition of carbohydrate and the number of carbohydrate units. However, a critical comparison of the structural features of the carbohydrate units, the nature of the linkages between the carbohydrate and the protein, and the sugars associated with these linkages allows to a certain degree the classification of glycoproteins.

II. COVALENT LINKAGE OF CARBOHYDRATE TO THE PEPTIDE CHAIN

The nature of covalent bond that links the carbohydrate to the peptide chain is a major aspect of the structural uniqueness of glycoproteins. The glycosylation of amino acids within a polypeptide chain, that is destined to be a glycoprotein, is apparently not a random event because it is known that glycosylation occurs at specific sites on the protein molecule. For instance, in human chorionic gonadotropin, of which the complete

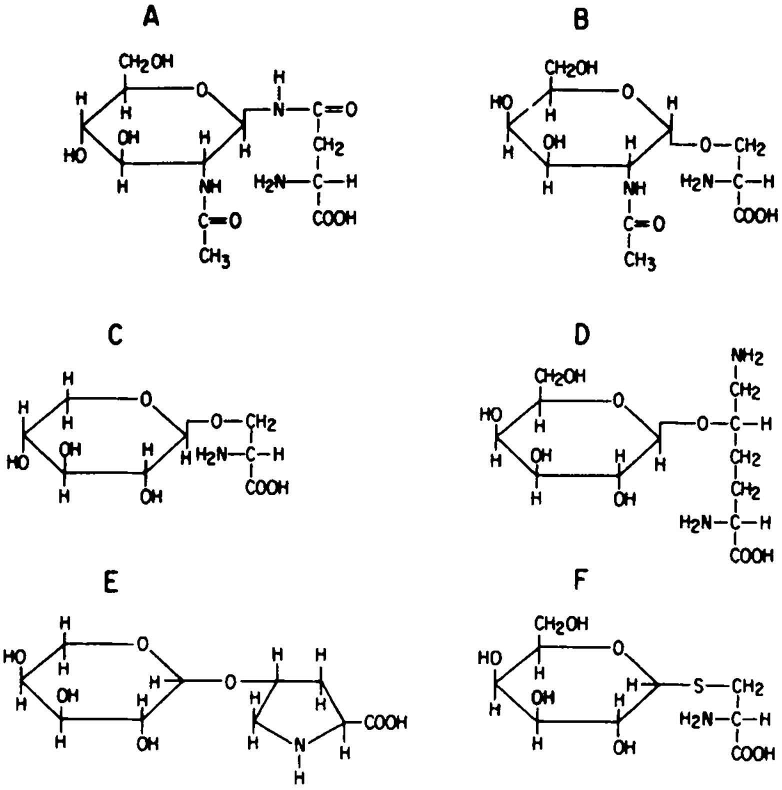

FIGURE 1. Structures of carbohydrate-peptide linkages. (A) *N*-Acetylglucosaminyl asparagine, (B) *N*-acetylgalactosaminyl serine, (C) xylosyl serine, (D) galactosyl hydroxylysine, (E) arabinosyl hydroxyproline, and (F) galactosyl cysteine.

primary structure is known,[2-5] the specific sites for carbohydrate attachment are at 4 of the 7 asparaginyl residues in the α and β subunits (i.e., 52 and 78 in the α-subunit and 13 and 30 in the β-subunit) and 4 of the 13 serine residues (i.e., 121, 127, 132, and 138) in the β-subunit.

Basically, there are three types of glycopeptide bonds which have been found to occur among the glycoproteins studied thus far. These can be differentiated strictly on the basis of the attachment of a given sugar to a specific functional group of an amino acid. First, when the attachment of the carbohydrate is via the amide group of asparagine, a glycosylamine or *N*-glycosidic bond* is formed. Second, whenever the carbohydrate is linked through the hydroxyl group of serine, threonine, hydroxylysine, or hydroxyproline, the glycosidic bond is of the *O*-glycosidic type. Third is the *S*-glycosidic bond which is formed when the carbohydrate is attached through the thiol group of cysteine. While the first two types of carbohydrate-peptide linkages are preponderant in nature, the *S*-glycosidic bond has been found in only a few cases.

A. Glycoproteins With *N*-Glycosidically Linked Carbohydrate

The *N*-glycosidic linkage or [2-acetamido-1-(β-L-aspartamido)-1,2-dideoxy-β-D-glucose] (Figure 1—A) is stable to mild alkaline conditions, a criterion which

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* *N*-glycosidic bond is a trivial term used here, and is synonymous with glycosylamine bond which is the more appropriate term for the linkage between the anomeric carbon of a sugar and the amide group of asparagine.

Table 2
CARBOHYDRATE-PEPTIDE LINKAGES IN GLYCOPROTEINS

I. *N*-Glycosidic linkage between *N*-acetylglucosamine and asparagine
  A. Virus, fungi, and plants
    1. Sindbis virus and glycoprotein[29]
    2. Vesicular stomatitis virus G protein[30]
    3. Taka-amylase *(Aspergillus oryzae)*[31]
    4. Mannan-protein *(Saccharomyces cerevisiae)*[32]
    5. Bromelain (pineapple stem)[33]
    6. Soybean agglutinin[34]
  B. Hen
    1. Ovalbumin[35]
    2. Avidin[15]
    3. Phosvitin[36]
    4. Ovotransferrin[37]
    5. Ovomucoid[38]
  C. Porcine
    1. Ribonuclease[39]
    2. Thyroglobulin[40]
    3. Lipases[41]
  D. Bovine
    1. Deoxyribonuclease A[42]
    2. Ribonuclease B[43]
    3. Fetuin[44]
    4. Thyroglobulin[45]
    5. Glomerular basement membrane[46]
    6. Immunoglobulin G[47]
    7. Visual pigment[48]
    8. Thyroid-stimulating hormone (β-subunit)[10]
    9. Corneal keratan sulfate[49]
  E. Human
    1. α_1-Acid glycoprotein[50]
    2. α_2-macroglobulin[51]
    3. Erythrocyte membrane asialoglycoprotein[52]
    4. Glycophorin[53]
    5. Immunoglobulin M, G, E, and A[1]
    6. Transferrin[54]
    7. Lactotransferrin[55]
    8. Ceruloplasmin[56]
    9. Thyroglobulin[57]
    10. Chorionic gonadotropin[4]
    11. Follicle-stimulating hormone[6]
    12. Thyroid-stimulating hormone (β-subunit)[58]

distinguishes this linkage from the serine/threonine-linked *O*-glycosidic linkage. The *N*-glycosidic bond is the most commonly occurring bond in glycoproteins (Table 2). Its presence initially was established in ovalbumin by the actual isolation of the *N*-acetylglucosaminyl asparagine complex. In order to establish this linkage, it is necessary to degrade the polypeptide chain by proteolysis, followed by the isolation of the glycopeptide(s) and its further cleavage from the amino and carboxy termini by amino- and carboxypeptidases and/or chemically by Edman degradation in such a way that only the linkage amino acid (i.e., aspartic acid) is left behind. Similarly, all the sugar residues (with the exception of *N*-acetylglucosamine) involved in the protein-carbohydrate linkage can be removed by appropriate exo- and endoglycosidases and/or by Smith degradation. Partial acid hydrolysis of the glycopeptide in some cases has yielded the

Table 2 (continued)
CARBOHYDRATE-PEPTIDE LINKAGES IN GLYCOPROTEINS

- II. Alkali-labile *O*-glycosidic linkages
  - A. *O*-Glycosidic linkage between *N*-acetylgalactosamine and serine or threonine
    - 1. Submaxillary glycoproteins
      - a. Porcine[59]
      - b. Canine[60]
      - c. Bovine[61]
      - d. Ovine[62]
    - 2. Human salivary glycoprotein[63]
    - 3. Others
      - a. Blood group substances[64]
      - b. Bovine cartilage keratan sulfate[65]
      - c. Bovine fetuin[66]
      - d. Epiglycanin (TA-3 cells)[67]
      - e. Human immunoglobulin A[1]
      - f. Glycophorin[53]
      - g. Erythrocyte membrane glycoprotein[68]
      - h. Chorionic gonadotropin (β-subunit)[5]
      - i. Antifreeze glycoprotein (Antartic fish)[13]
  - B. *O*-Glycosidic linkage between sugars (R) other than *N*-acetylgalactosamine and serine or threonine
    - 1. R = xylose
      - a. Chondroitin sulfate[69]
      - b. Dermatan sulfate[69]
      - c. Heparin[69]
    - 2. R = Mannose
      - a. Glucoamylase *(Aspergillus phoenices*[70]
      - b. Glucoamylase *(Aspergillus niger)*[71]
      - c. Mannan-protein *(Saccharomyces cerevisiae)*[72]
      - d. Envelope glycoprotein *(Cryptococcus laurentii)*[73]
      - e. Mycodextranase *(Penicillium melinii)*[74]
      - f. Baker's yeast cell wall glycopeptides[75]
      - g. Collagen (nereis)[28]
    - 3. R = Galactose
      - a. Cuticle collagen (nereis[28] lumbricus[76])
    - 4. R = L-fuocse
      - a. Human urinary glycopeptide[77]
      - b. Glycoproteins from normal and oncogenically transformed mammalian cell lines[78]

- III. *O*-Glycosidic (alkali-stable and *S*-glycosidic linkages)
  - A. *O*-Glycosidic linkage between galactose and δ-hydroxyl group of hydroxylysine
    - 1. Collagens
      - a. Vertebrate tissues[79]
      - b. Cuttle fish[80]
    - 2. Glomerular basement membrane
      - a. Bovine[81]
      - b. Human[82]
    - 3. Bovine lens capsule[83]
  - B. *O*-Glycosidic linkage between arabinose or galactose and hydroxyl group of hydroxyproline
    - 1. Plant cell wall[84]
    - 2. Chlamydomonas[85]
  - C. *S*-Glycosidic linkage between galactose or glucose and thiol group of cysteine
    - 1. Human urinary glycopeptide[86]
    - 2. Human erythrocyte glycopeptide[87]

N-acetylglucosaminyl-asparagine complex, although its recovery has been generally poor. The complex can be identified by its chemical composition and by an amino acid analyzer. The amino acid involved in the carbohydrate-protein linkage can also be identified during the amino acid sequencing of a protein. Glycoprotein hormones are well-known examples where the specific linkage asparagine residues have been identified by amino acid sequence studies.

The asparaginyl glycopeptides thus far known exhibit three different types of structural patterns:

1. High mannose type, commonly known as "simple" carbohydrate units, contain only two types of monosaccharides, mannose and *N*-acetylglucosamine (however, these are by no means simple as far as their structural analyses are concerned.).
2. Complex type are heterosaccharides containing more than two types of sugars.
3. Hybrid type are those carbohydrate units which share some of the features of the high mannose (simple) and complex carbohydrate structures.

The inner core — Manα1 → 3-(Manα1 → 6)Manβ1 → 4GlcNAcβ1 → 4GlcNac — has been found to be a common feature in most of the asparagine-linked carbohydrates. Thus, the designation of the type of carbohydrate structure is defined by the nature of the peripheral sugars attached to the inner core.

High mannose type asparaginyl glycopeptides contain only mannose residues in the peripheral region of the inner core. The number of such mannose residues varies from three in the carbohydrate units of human γM globulin, hen ovalbumin, α-amylase from *Aspergillus oryzae,* and bovine ribonuclease B (Figure 2—A), to nine in the linkage region of mannan from *Saccharomyces cerevisiae* (Figure 2—B). A comparison of these two types of structures indicates that the intersugar linkages of all six mannose residues in both types of structures are identical. The structure of the glycopeptide from soybean agglutinin (Figure 2 — C) appears to be unique since the mannose residue linked by α1,3 instead of α1,6 linkage to the β-linked mannose serves as the branch point. Furthermore, all the terminal mannose residues are α1,2-linked.

The complex type carbohydrate structures are characterized by the presence of saccharides other than mannose in their peripheral region. The sugars which are generally present in the outer region include galactose, mannose, L-fucose, *N*-acetylglucosamine, and sialic acid. A general structure for the complex type carbohydrate units based on the recently elucidated carbohydrate structures in various glycoproteins is illustrated in Figure 3. Structure for almost any complex type carbohydrate unit can be derived from this composite structure by appropriately substituting R_1 to R_7. The salient feature of this structure is the presence of either one or two oligosaccharide chains at each of the two outer mannose residues of the core structure. Thus, the number of such chains present in the carbohydrate structure may be two, three, or four. Each chain — i.e., A, B, C, or D — has a sequence SA$\xrightarrow{\alpha 2,3}$Gal$\xrightarrow{\beta 1,4}$GlcNAc $\xrightarrow[(3,4,6)]{\beta 1,2}$ in which R_4 to R_7 substituents are sialic acid residues. In some cases, these chains lack sialic acid and/or galactose due to microheterogeneity. The linkage of sialic acid to galactose is either α2-3 or α2-6. Fucose, whenever present, is attached by α1-6 linkage to *N*-acetylglucosamine involved in the carbohydrate-protein linkage although in α_1-acid glycoprotein, it has been found to be linked by α1-3 linkage to the *N*-acetylglucosamine in the outer chain. A comparison of the structures shown in Figure 3 reveals that the simplest carbohydrate structure is that of transferrin which is common to other glycoproteins, i.e., lactotransferrin, Cl_q complement, immunoglobulins, Sindbis virus and vesicular stomatitis virus G proteins, and human chorionic gonadotropin. The

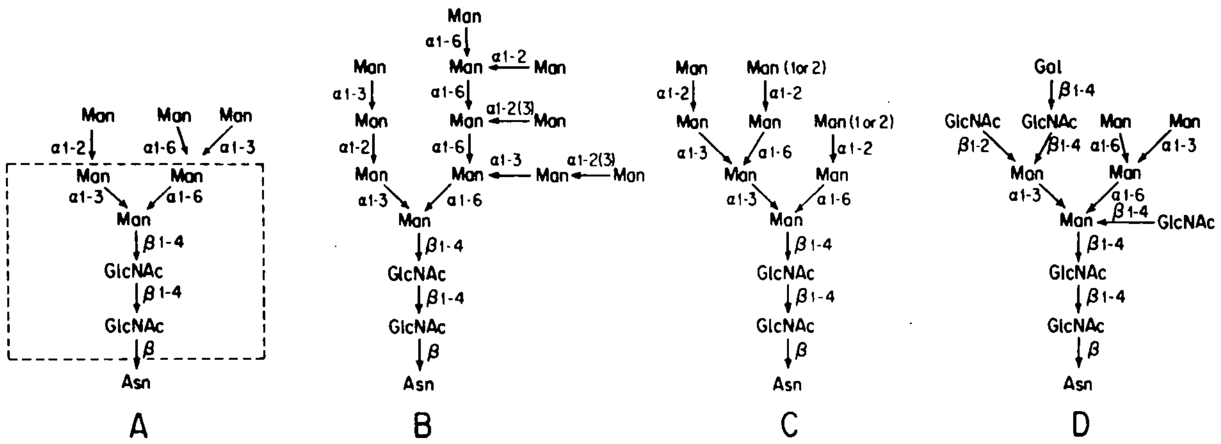


FIGURE 2. Structures of "high mannose" and "Hybrid" type glycopeptides. (A) Takaamylase A glycopeptide[31] (structure within dashed lines represents the inner core which is identical with the inner core of structures B, C, and D), (B) mannan-protein linkage region of *Saccharomyces cerevisiae*,[32] (C) glycopeptide from soybean agglutinin,[34] and (D) ovalbumin glycopeptide.[88]

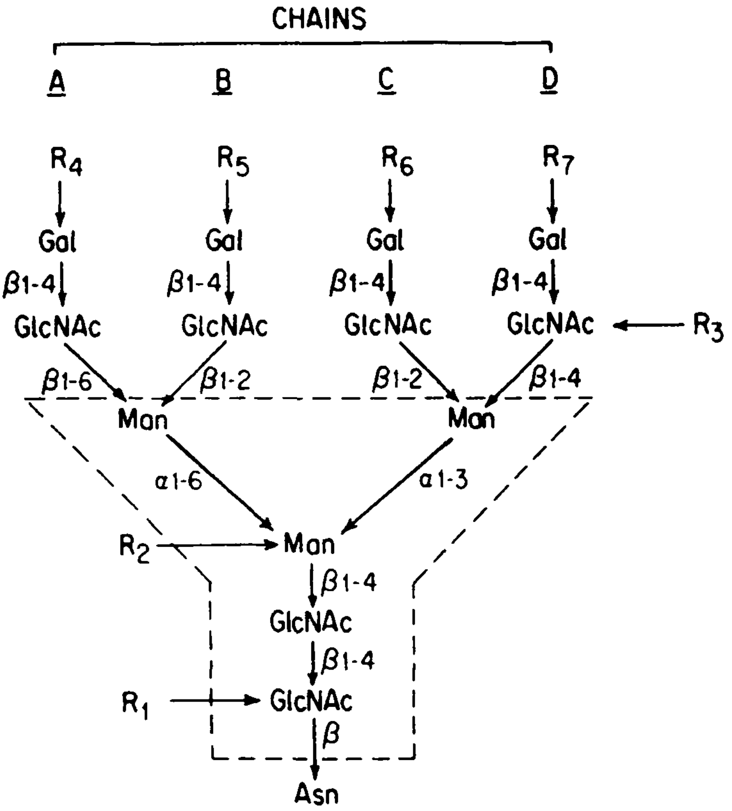


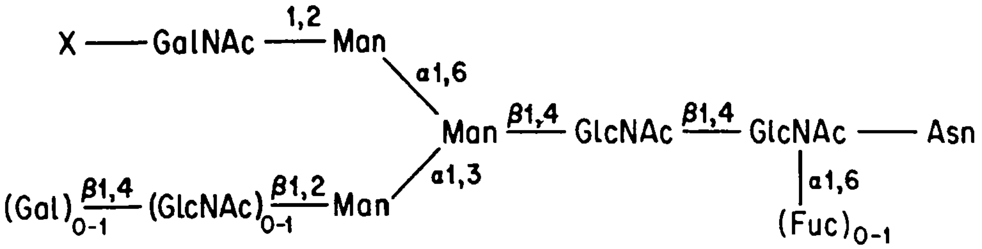
FIGURE 3. Structures of some "complex type" asparagine-linked glycopeptides. Sugars within the dashed lines indicate core region (unless otherwise indicated, R_1 through R_7 = H.) (1) α_1-Acid glycoprotein[89] (R_3 = Fuc α1,3, (2) human thyroglobulin B glycopeptide[90] (minus chain D; linkage of GlcNAc to mannose in chain B is β1,3; R_4 and R_5 = NANA), (3) human transferrin[54] (minus chains A and D; R_5 and R_6 = NANA α2,6), (4) human chorionic gonadotropin[4,5] (minus chains A and D; R_1 = Fuc α1,6; R_5 and R_6 = NANA α2,3), (5) human lactotransferrin[54] and Human Clq complement[91] (minus chains A and D; R_1 = Fuc α1,6; R_5 and R_6 = NANA α2,6), (6) human IgG, IgE, and IgA[1] (minus chains A and D; ± galactose; R_1 = Fuc α1,6; R_5 and R_6 = ± sialic acid α2,6), (7) human IgA_1-IIA_1 (minus chains A and D; R_2 = GlcNAc β1,4 or 6; R_5 = sialic acid), (8) human IgG-Hum B-3[1] (minus chains A and D; R_1 = Fuc α1,6; R_2 = ± GlcNAc β1,4; R_5 and R_6 = sialic acid α2,6), (9) bovine fetuin[92] (minus chain A; substitute chain D for chain A; R_5 = NANA α2,6; R_6 and R_7 = NANA α2,3), (10) sindbis virus glycopeptide S_1[29] (minus chains A and D; R_1 = Fuc α1,6; R_5 and R_6 = NANA α2,3), (11) vesicular stomatitis virus G protein[30] (minus chain A; substitute chain D for chain A; R_5, R_6, and R_7 = NANA α2,3).

linkage of sialic acid to galactose is α2-6 in most of the glycoproteins except viral glycoproteins in which sialic acid is linked to galactose by α2-3 linkage, the latter also being found in human chorionic gonadotropin. Calf serum fetuin has both α2-3- and α2-6-linked sialic acid residues. Fetuin contains the three branches linked to the core mannose residues by β1-2, β1-4, and β1-6 linkages. α_1-Acid glycoprotein also has these three branches but contains an additional 1,2-β-linked branch. The structures of the carbohydrate units of fetuin and thyroglobulin glycopeptide B are quite similar except the latter has one of the three outer branches linked to mannose by a β1→3 linkage.

The third or the hybrid type carbohydrate structure among the asparaginyl carbohydrate units contains features characteristic of both simple and complex carbohydrate units. For example, one of the carbohydrate units of ovalbumin has a high mannose content as well as complex type branches containing galactose and *N*-acetylglucosamine. Thus the structure (Figure 2—D) is a hybrid of the high mannose and complex carbohydrate type structures.

While most of the *N*-glycosidic carbohydrates have a common monosaccharide pattern, several exceptions have been noted. The glycoprotein hormones of the pituitary gland — i.e., luteinizing, follicle-stimulating, and thyroid-stimulating hormones — are the first examples in which *N*-acetylgalactosamine has been found as an integral component of *N*-glycosidic carbohydrate units.[93] The bovine and ovine luteinizing hormones contain three *N*-glycosidically linked carbohydrate units per molecule, each unit containing one residue of *N*-acetylgalactosamine. The position of the *N*-acetylgalactosamine has been established by detailed enzymatic and chemical studies.[94] It has been found that the *N*-acetylgalactosamine is located in one of the outer branches to 1,3- or 1,6-linked mannose residues. Furthermore, it is interesting to note that the *N*-acetylgalactosamine is substituted at the 0-4 position by an acid-labile group. This novel carbohydrate structure (see below) proposed for the bovine and ovine luteinizing hormones is not compatible with the one proposed for the carbohydrate of the α-subunits of human pituitary glycohormones. In the latter proposal, the *N*-acetylgalactosamine is shown to be present in the inner core located between the β-linked mannose and the linkage *N*-acetylglucosamine.[93]

A carbohydrate structure of bovine and ovine LH follows.



B. Glycoproteins with *O*-Glycosidically Linked Carbohydrates

1. Alkali-Labile O-*Glycosidic Linkage between* N-*Acetylgalactosamine and Serine or Threonine*

The glycopeptide bond *N*-acetylgalactosaminyl serine or threonine (Figure 1—B) is quite common and is present in ovine, bovine, porcine, and canine submaxillary glycoproteins, mucin from human submaxillary secretions, bovine cartilage, keratan sulfate, fetuin, antifreeze glycoprotein, epilglycanin (TA-3 cells), immunoglobulin A, glycophorin, and the β-subunit of human chorionic gonadotropin (Table 2). This bond is quite susceptible to cleavage under alkaline conditions by β-elimination. A generalized outline of the β-elimination reaction of a glycoprotein and the resulting products are given in Figure 4.

The β-elimination yields the intact carbohydrate unit containing the reducing sugar residue and the peptide chain in which the linkage amino acids, serine or threonine, are converted into unsaturated 2-aminopropenoic (α-amino acrylic) and 2-amino-2-butenoic (α-amino-crotonic) acids, respectively. The olefinic amino acids formed during the course of β-elimination have a molar extinction coefficient of about 6000 at 240 nm; therefore, their formation can be monitored spectrophotemetrically.[95]

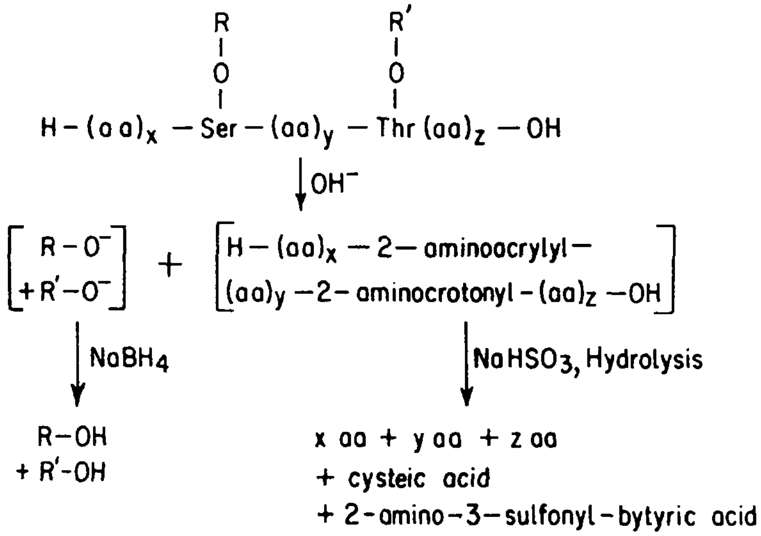


FIGURE 4. A generalized outline of the β-elimination reaction of a glycoprotein. R and R^1 represent carbohydrate units linked to serine and threonine, respectively; aa = peptide amino acids.

Furthermore, these olefinic amino acids are readily converted into α-keto acids upon hydrolysis of the peptide chain in 3 *N* HCl for 4 hr at 100°C. The pyruvic and α-ketobutyric acids from serine and threonine, respectively, can be determined quantitatively employing the lactic acid dehydrogenase assay system. This enzyme has differential Km for the two substrates, i.e., $5 \times 10^{-5} M$ for pyruvic acid and $8 \times 10^{-4} M$ for α-ketobutyric acid. Thus, whereas pyruvic acid is reduced by a low level of the enzyme, about a 40-fold excess is needed for the reduction of α-ketobutyric acid. A quantitative relationship between the loss of β-hydroxyamino acids and the formation of α-keto acids have been found when this technique is applied to glycopeptides.[96,97]

If the β-elimination reaction is carried out in the presence of sodium borohydride, the linkage sugar can be identified as sugar alcohol, and the unsaturated amino acid residues are converted into their corresponding saturated derivatives: alanine and α-aminobutyric acid from serine and threonine, respectively. It has been established that inclusion of palladium chloride (a hydrogenation catalyst) during the β-elimination is essential in obtaining stoichiometric yields of alanine and α-amino butyric acid.[98] In the absence of the catalyst, the loss of serine is approximately equal to the gain in alanine, but a negligible amount of α-aminobutyric acid is formed from threonine. The inclusion of sodium bisulfite in the reaction mixture during β-elimination results in the formation of cysteic acid and 2-amino-3-sulfonylbutyric acid residues. Upon hydrolysis, these can be identified and quantitated by an amino acid analyzer[99] or by gas liquid chromatography as their trimethylsilyl derivatives.[100] It has been found by using model compounds that the substitution at amino- and carboxyl-groups of serine or threonine to which the carbohydrate is attached, enhances the rate of β-elimination.

Although β-elimination results in the release of intact carbohydrate units, a phenomenon termed the "peeling reaction"[101] occurs if the conditions of the reaction are not carefully controlled. Generally, alkali treatment of *N*-acetylhexosaminylhydroxyamino acid linkages in the absence of sodium borohydride results in the formation of reducing *N*-acetylhexosamine. The product is unstable in alkaline environment, and is susceptible to undergoing another β-elimination resulting in its conversion to a furanosyl

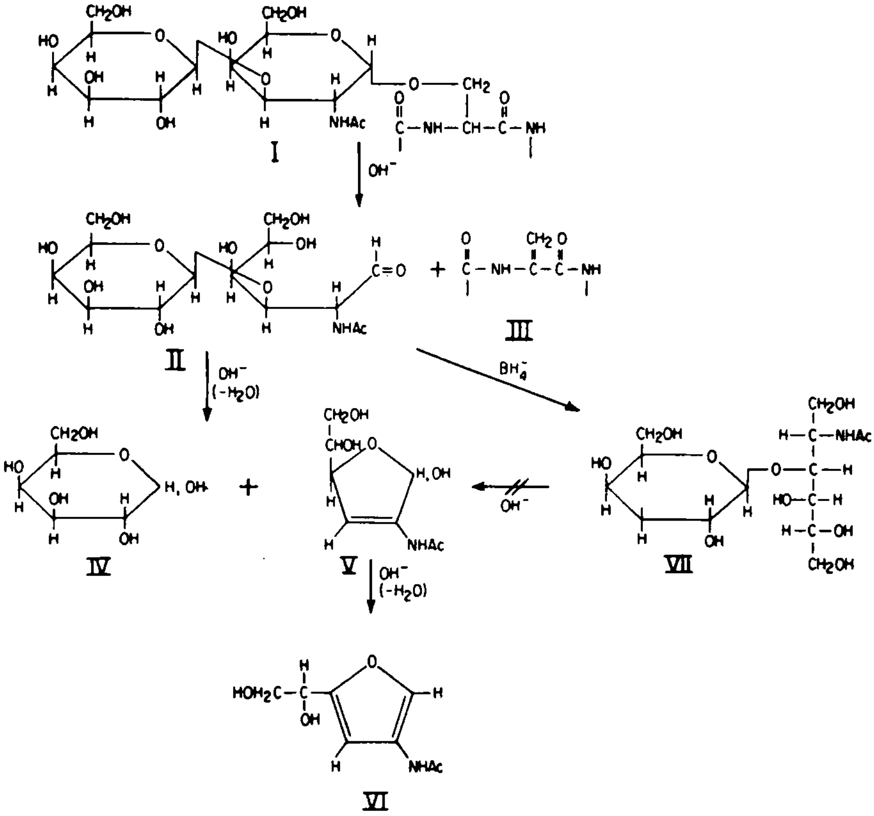


FIGURE 5. The peeling reaction during the β-elimination of carbohydrate units.

compound. Moreover, if carbon-3 of the linkage *N*-acetylhexosamine is substituted, the conversion to the furanosyl derivative is greatly enhanced even at low basicity and temperature. Therefore, structures involving *O*-β-D-(1 → 3)-linked *N*-acetylhexosaminyl units are very vulnerable to this reaction. The peeling reaction during the β-elimination of carbohydrate units containing carbon-3 substituted *N*-acetylgalactosamine linked to serine or threonine is illustrated in Figure 5. The structure Gal$\xrightarrow{\beta1\text{-}3}$GalNAc$\xrightarrow{\alpha}$Ser(Thr) (I) has been found in antifreeze glycoprotein, human IgA_1, cartilage keratan sulfate, and epiglycanin (TA-3 cells). As a result of β-elimination, galactosyl-*N*-acetylgalactosamine (II) and 2-aminopropenoyl peptide derivative (III) (or 2-amino-2-butenoyl derivative from threonine) are formed. The carbohydrate unit (II) is unstable in alkaline environment and may undergo another β-elimination by the release of a proton at carbon-2 of *N*-acetylgalactosamine. Consequently, galactose (IV) is cleaved off and *N*-acetylgalactosamine is rapidly converted into Morgan-Elson chromogens (V and VI). This peeling reaction (stepwise degradation) proceeds rapidly from the nonreducing end of the oligosaccharide if the sugar residues are 1 → 3 linked.

Generally, 1 → 2 linked sugars are alkali-stable, and 1 → 4 and 1 → 6 linkages are split less readily than 1 → 3 linkages. Accordingly, stepwise degradation of the oligosacchride will continue until the product is alkali-stable. The peeling reaction can be largely abated by lowering the concentration of alkali and by the inclusion of sodium borohydride in the reaction mixture. The conversion of galactosyl-*N*-acetylgalactosamine (II) into galactosyl-*N*-acetyl-galactosaminitol (VII) by sodium borohydride reduction imparts

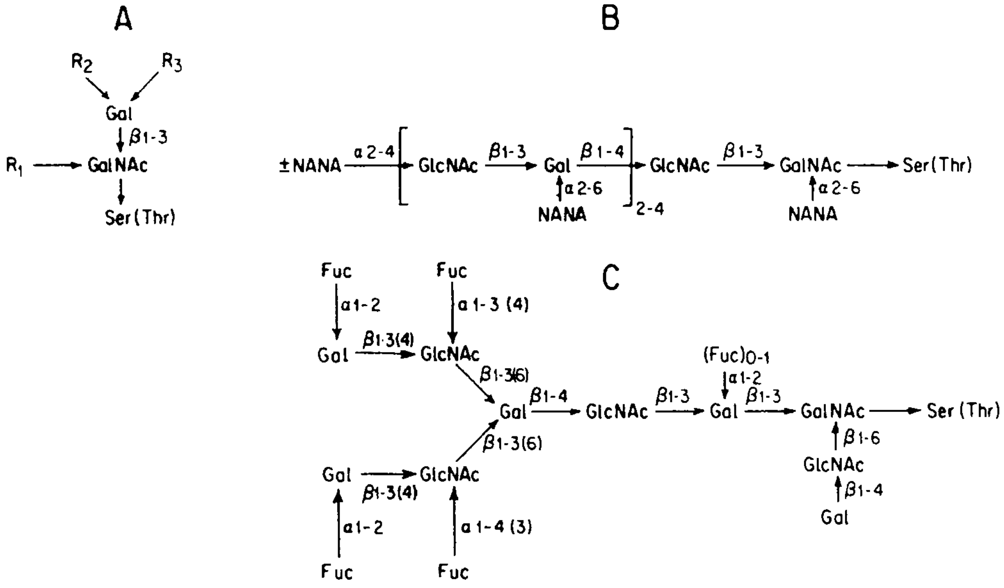


FIGURE 6. Structures of glycopeptides with linkages between *N*-acetylgalactosamine and serine (threonine). (A) Unless otherwise indicated, R_1, R_2, and R_3 = H: 1. Antifreeze glycoprotein,[13] human IgA_1,[1] cartilage keratan sulfate[65] and epiglycanin (TA-3 cells)[67] (R_1 = R_2 = R_3 = H); 2. human chorionic gonadotropin (β-subunit),[5] bovine fetuin[66] and human erythocyte membrane[68] (R_1 = NANA α2,6; R_2 = NANA α2,3); 3. canine submaxillary glycoprotein[60] (R_1 = NANA α2,6); 4. canine[60] and porcine[102] A^-, and A^+ submaxillary glycoproteins (R_1 = NANA α2,6; R_2 = Fuc α1,2); 5. porcine submaxillary glycoprotein A^+[102] (R_1 = NANA α2,6; R_2 = Fuc α1,2; R_3 = GalNAc α1,3); 6. canine submaxillary glycoprotein[60] (R_2 = ± Fuc α1,2; R_3 = Fuc α1,2 Gal β GlcNAc, O_4 β1,6); 7. human gastric mucin[101] (R_1 = Gal 1,4 GlcNAc 1,6; R_2 = Gal 1,3 GlcNAc 1,3). (B) Rat sublingual glycoprotein (acidic oligosaccharide).[103] (C) Human salivary glycoprotein (neutral oligosaccharide).[63]

alkali stability to the latter due to the loss of electron-attracting carbonyl function at carbon-1 of *N*-acetylgalactosamine.

The structures of the *O*-glycosidic carbohydrates involving *N*-acetylgalactosaminyl serine or threonine linkage (Figure 1—B) fall into three structural patterns (Figure 6):

1. The carbohydrate units of antifreeze glycoprotein, epiglycamine (TA-3 cells), and immunoglobulin IgA are made up of Gal$\xrightarrow{\beta1,3}$GalNAc (Figure 6—A). When R_1, R_2, and R_3, consisting of fucose, galactose, sialic acid, and *N*-acetylglucosamine are appropriately substituted, as shown in Figure 6—A, the disaccharide serves as the inner core of the carbohydrate structures of hCG-β, fetuin, glycophorin, submaxillary mucins, and human gastric mucin.
2. The acidic oligosaccharide of rat sublingual glycoprotein obtained by β-elimination contains an inner core of trisaccharide, GlcNAc$\xrightarrow{\beta1,3}$(NANA$\xrightarrow{\alpha2,6}$) ⟶ GalNAc at the reducing end (Figure 6—B). A repeating trisaccharide structure, GlcNAc $\xrightarrow{1,3}$ (NANA$\xrightarrow{\alpha2,6}$) Gal$\xrightarrow{\beta1,4}$ with 2 to 4 such units, is attached to the *N*-acetylglucosamine of the inner core, resulting in oligosaccharides with chain lengths of 9, 12, and 15 sugar residues, respectively. In addition, oligosaccharides containing 10 and 13 sugars have also been found because of the presence of sialic acid residues at the nonreducing terminal *N*-acetylglucosamine.
3. The structures of the mucin from human submaxillary secretions (Figure 6—C) and blood group substances constitute the third type containing GalNAc-Ser(Thr)

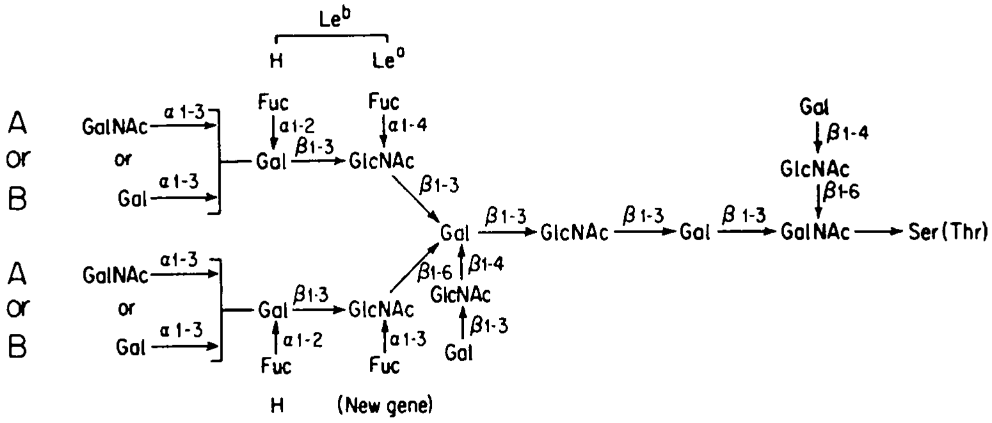


FIGURE 7. Composite heteropolysaccharide structure for blood group substances.

linkage. Although the structures of the human secretory mucin and blood group substances (Figure 7) share several characteristics in common, some distinct differences have been found. For example, the human salivary mucin has only a disubstituted galactose residue (rather than trisubstituted), as found in the blood group substances.[63] The differences in the intersugar linkages may be the result of individual variation and therefore may vary with the preparation and the blood group type of the mucin and the blood group substances.

2. *Alkali-Labile* O-*Glycosidic Linkage between Sugars Other Than* N-*Acetylgalactosamine and Serine or Threonine*

A large number of glycoproteins contain carbohydrate units which are linked to serine or threonine through xylose, mannose, galactose, and fucose (Table 2). The structures of such carbohydrate units are shown in Figure 8. In proteoglycans such as chondroitin sulfate, dermatan sulfate, and heparin, the carbohydrate-peptide linkage is Xyl$\xrightarrow{\beta}$Ser (Figure 1—C).

The oligosaccharide in the linkage region has the following structure: GlcUA$\xrightarrow{\beta 1,3}$Gal$\xrightarrow{\beta 1,3}$Gal$\xrightarrow{\beta 1,4}$Xyl→, and the linkage amino acid is always serine (Figure 8—a). The main carbohydrate chains are found to be linear polymers made up of repeating units of alternating hexosamine and hexuronic acid residues. In each the sugar residue attached to the nonreducing terminal galactose of the linkage region is glucuronic acid. The glycoproteins of *S. cerevisiae* and envelope glycoproteins of *Cryptococcus laurentii* are, in fact, mannan proteins containing linear chains of mannose attached to serine or threonine (Figures 8—b and 8—c). However, in the latter, xylose has also been found to be attached to the linkage mannose. Of the total carbohydrate chains releasable from mycodextranase of *Penicillium melinii* by β-elimination, about one half have mannosylserine (threonine) linkages while the other half are linked through mannosyl residues as Man$\xrightarrow{\alpha 1,2}$Glc$\xrightarrow{\alpha 1,2}$Man → Ser(Thr) (Figure 8—d). The di- and trisaccharides of galactose have been found linked to serine or threonine in cuticle collagen from *Lumbricus terrestris* and from *Nereis* (Figure 8—e). The presence of glucuronic acid in the collagen from the latter has been reported by the isolation of glucuronosyl-mannitol by alkali-borohydride treatment (Figure 8—f). It has been demonstrated that a glycopeptide from human urine contains carbohydrate linked by α-L-fucose residues to threonine (Figure 8—g). Recently, Fuc$\xrightarrow{\alpha}$Thr(Ser) glycopeptide

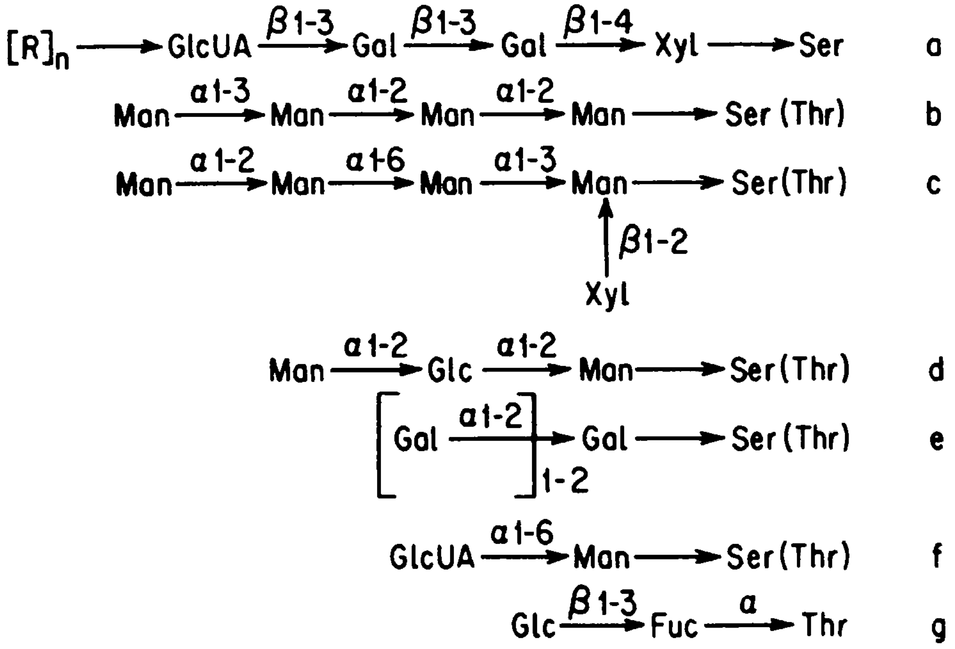


FIGURE 8. Structures of glycopeptides with linkages between sugars other than *N*-acetylgalactosamine and serine (threonine). (a) Chondroitin sulfate, dermatan sulfate, and heparin;[69] (b) mannan-protein *(Saccharomyces cerevisiae)*;[72] (c) envelope glycoprotein of *Cryptococcus laurenti*;[73] (d) myco-dextranase *(Penicillium mellinii)*;[74] (e) cuticle collagen (Nereis[28] and Lumbricus[76]); (f) cuticle collagen (Nereis);[28] (g) human urinary glycopeptide[77] and glycoproteins of mammalian cell lines.[78]

linkages have been identified in cellular glycoproteins from a variety of normal and oncogenically transformed mammalian cell lines. The evidence suggests that the level of Glc-Fuc-Thr containing glycoproteins varies as a function of the expression of the transformed morphological phenotypes.[78]

3. O- and S-Glycosidic Linkages Involving Hydroxylysine and Hydroxyproline and Cysteine

The carbohydrate-peptide linkage between galactose and hydroxylysine (Figure 1—D) has been found in a variety of collagens, basement membranes, and the lens capsule. The carbohydrate units of these structural glycoproteins contain both $\mathrm{Glc}\xrightarrow{\alpha 1,2}\mathrm{Gal}\rightarrow$ and galactose linked to hydroxylysine. The distribution of the carbohydrate between disaccharide and monosaccharide units is variable in these glycoproteins. Thus, of the total hydroxylysine linked carbohydrate units, the disaccharide units range from 94 to 97% in glomerular basement membrane and lens capsule collagens, 86 to 90% in invertebrate collagens, and 54 to 83% in vertebrate collagens.[18] The β-galactosyl hydroxylysine bond was first discovered by Butler and Cunningham in guinea pig skin tropocollagen.[79] Subsequently, its presence was determined in bovine glomerular basement and bovine lens capsule.[18] Unlike the serine (threonine)-linked glycopeptide, the β-galactosyl hydroxylysine bond is stable to mild as well as strong alkaline conditions. As a matter of fact, Glu-Gal-Hyl has been isolated from intact glomerular basement membranes and its pronase treated glycopeptides by treatment with 2 *N* NaOH at 105°C for 24 hr. The sugar-amino acid complexes have been separated by an amino acid analyzer.[18]

The plant cell wall contains structural glycoproteins in which the carbohydrate-protein linkage is between L-arabinose and hydroxyproline[84] (Figure 1—E). The linkage of galactose to hydroxyproline has been reported in glycoproteins from green algae *Chlamydomonas*.[85] The glycopeptide bond involving hydroxyproline is stable to alkaline conditions like the Gal-Hyl bond.

The presence of thioglycosidic glycopeptide bond between galactose and cysteine has been reported in a glycopeptide obtained from human urine[86] (Figure 1—F). In this glycopeptide a disaccharide of galactose is linked to cysteine. Furthermore, a triglucosylcysteine glycopeptide has been reported to occur in human erythrocyte suggesting a linkage between glucose and cysteine.[87]

III. AMINO ACID SEQUENCES AROUND CARBOHYDRATE UNITS

One of the intriguing questions in the field of glycoproteins which has attracted a great deal of attention concerns the factors which determine the specificity of incorporation of the carbohydrate in the protein. It has been of interest to know whether it is the overall conformation of the glycoprotein or the amino acid sequences around the linkage asparaginyl residues, or both, that direct the carbohydrate incorporation. Consequently, investigations have been carried out to elucidate the linear amino acid sequences around the *N*-glycosylated asparaginyl residue. These sequences have been deduced from the complete amino acid sequence studies in a number of glycoproteins which include ribonucleases, deoxyribonucleases, human chorionic gonadotropin, luteinizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone, IgE (Eu) myeloma immunoglobulin, hen egg avidin, and human plasma α_1-acid glycoprotein. Such amino acid sequences have been also determined in glycopeptides after their isolation from various glycoproteins. An examination of the known sequences around asparagine-linked carbohydrates in a variety of glycoproteins (Table 3) reveals that the sequence Asn-X-Thr(Ser), with X being the variable amino acid, is found invariant in each case. No similar specific common amino acid sequence on the *N*-terminal side of the asparagine residues has been found.

It was earlier proposed by Jackson and Hirs[11] that the nature of the amino acid X in Asn-X-Thr(Ser), whether polar or nonpolar, is the determinant for the type of carbohydrate unit that is attached to the polypeptide chain. It is now clear that this hypothesis is not tenable because there is no consistent relationship between X and the nature of carbohydrate units (Table 3). The presence of the tripeptide sequence above does not necessarily involve specific glycosylation of the asparagine residues because several proteins containing this sequence exist in both glycosylated and unglycosylated forms or found to be glycosylated at one site and unglycosylated at another such site. The data on a total of 111 tripeptide sequences from 266 proteins[125] have been statistically analyzed.[126] It was revealed that whereas for the prokaryotic proteins the frequency of occurrence of the tripeptide sequence was consistent with the statistical expectation, for the eukaryotic proteins the frequency of occurrence was approximately one third of the expected value. Moreover, of all such tripeptide sequences examined, the glycosylated forms were present only in the extracellular and membrane-bound proteins of the eukaryotes. It was suggested that the prokaryotes do not contain glycosylating enzymes necessary for the glycosylation of asparagine residues, implying that the asparagine-linked glycoproteins may have evolved in eukaryotes after the divergence from prokaryotes.

It is conceivable that the tripeptide sequence Asn-X-Thr(Ser) may endow certain special properties, such as in exerting a change in the conformation of the protein thereby facilitating glycosylation at specific sites. In studies employing exogenous acceptors,

Table 3
AMINO ACID SEQUENCES AROUND SEVERAL ASPARAGINE-LINKED CARBOHYDRATE UNITS

Glycoprotein (source)	Sequence[a]
Enzymes	
Bromelain (pineapple stem)[33]	Pro-Arg-Asn-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Glu-Ser-Ser
Ceruloplasmin (human)[56]	Ala-Ile-Tyr-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Asp-Thr-Thr Gln-Glu-Gln-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Val-Ser-Asn Thr-Lys-Glu-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Leu-Thr-Ala
Deoxyribonucleases A,B,C,D (bovine)[104-106]	Lys-Met-Ser-$\overset{\text{CS}}{\overset{\vert}{\text{Asn}}}$-Ala-Thr-Leu
Ribonucleases B,C,D (Bovine)[107,108]	Lys-Ser-Arg-$\overset{\text{CS}}{\overset{\vert}{\text{Asn}}}$-Leu-Thr-Lys
Ribonuclease (porcine)[11]	Ser-Ser-Ser-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Ser-Ser-Asn Tyr-Gln-Ser-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Ser-Thr-Met Ser-Arg-Arg-$\overset{\text{S}}{\overset{\vert}{\text{Asn}}}$-Met-Thr-Gly
Taka-amylase (*Aspergillus oryzae*)[109]	Leu-Val-Ser-$\overset{\text{S}}{\overset{\vert}{\text{Asn}}}$-Tyr-Ser-Ile
Lipases L_A and L_B (porcine)[41]	Thr-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Gly-Thr-Ile
Phospholipase A_2 (bee venom)[110]	Gly-His-Gly-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Lys-Ser-Ser
Hormones[b]	
CG-α,[2] LH-α,[111] TSH-α,[112] FSH-α[111] (human)	Leu-Val-Gln-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Val-Thr-Ser Lys-Val-Glx-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-His-Thr-Ala
LH-α (ovine),[8] TSH-α (bovine)[10]	Val-Pro-Lys-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Ile-Thr-Ser Arg-Val-Glx-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-His-Thr-Glx
CG-β (human)[3]	Arg-Pro-Ile-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Ala-Thr-Leu Ile-Thr-Val-$\overset{\text{C}}{\overset{\vert}{\text{Asn}}}$-Thr-Thr-Ile

Table 3 (continued)
AMINO ACID SEQUENCES AROUND SEVERAL ASPARAGINE-LINKED CARBOHYDRATE UNITS

Glycoprotein (source)	Sequence[a]
LH-β (human)[113]	C Glx-Pro-Ile-Asn-Ala-Thr-Val
LH-β (ovine)[9,114]	C Gln-Pro-Ile-Asn-Ala-Thr-Leu
FSH-β (human)[6,115]	C Gln-Leu-Thr-Asn-Ile-Thr-Ile
	C Ile-Ser-Ile-Asn-Thr-Thr
TSH-β (human)[58]	C Leu-Thr-Ile-Asn-Thr-Thr-Ile
TSH-β (bovine)[10]	C Leu-Thr-Ile-Asn-Thr-Thr-Val
Immunoglobulins	
IgG, H chain (human myeloma Eu)[116]	C Gln-Gln-Tyr-Asn-Ser-Thr-Tyr
IgG, H chain (rabbit)[117]	C Gln-Gln-Phe-Asn-Ser-Thr-Ile
IgG, L chain (mouse plasma cell tumor)[118]	C Ala-Ser-Gln-Asn-Ile-Ser-Asn
IgM, H chain (human macroglobulin Ou)[119]	C Phe-Gln-Glx-Asn-Ala-Ser-Ser S Leu-Tyr-Asn-Val-Ser-Leu
Other	
α_1-acid glycoprotein (human)[120]	C Pro-Ile-Thr-Asn-Ala-Thr-Leu
	C Glu-Glu-Tyr-Asn-Lys-Ser-Val
	C Phe-Thr-Pro-Asn-Lys-Thr-Glu
	C Cys-Ile-Tyr-Asn-Thr-Thr-Tyr
	C Gln-Arg-Glu-Asn-Gly-Thr-Ile
Avidin (hen)[16]	S Leu-Gly-Ser-Asn-Met-Thr-Ile
Bence-Jones protein[121] (human, K chain HBJ-4)	C Ala-Ser-Glx-Asn-Val-Ser-Asx

Table 3 (continued)
AMINO ACID SEQUENCES AROUND SEVERAL ASPARAGINE-LINKED CARBOHYDRATE UNITS

Glycoprotein (source)	Sequence[a]
Bence-Jones protein[121] (human, λ chain Ful)	Cys-Ser-Gly-$\overset{C}{\text{Asn}}$-Ser-Ser
Fibrinogen (human, γ chain)[122]	Gln-Val-Glu-$\overset{C}{\text{Asn}}$-Lys-Thr-Ser
Ovalbumin (hen)[123]	Glu-Lys-Tyr-$\overset{C}{\text{Asn}}$-Leu-Thr-Ser
Ovotransferrin (hen)[37]	Leu-Ile-His-$\overset{S}{\text{Asn}}$-Arg-Thr-Gly
Thyroglobulin (human)[124]	Ala-Leu-Gln-$\overset{S}{\text{Asn}}$-Ala-Thr-Arg
Visual pigment (bovine)[48]	Met-$\overset{S}{\text{Asn}}$-Gly-Thr-Gln

[a] C and S at the asparagine residues indicate the type of carbohydrate unit: C = complex and S = simple. In bovine deoxyribonucleases the heterosaccharide unit is of the simple type for B and D. In bovine ribonucleases the heterosaccharide unit is of the simple type for B, and the complex type for C and D.

[b] Abbreviations: CG = chorionic gonadotropin; LH = luteinizing hormone; FSH = follicle-stimulating hormone; TSH = thyroid-stimulating hormone.

Pless and Lennarz[127] have demonstrated that the unfolding of a protein facilitates glycosylation. Thus, ovalbumin, α-lactalbumin, and ribonuclease A containing the required tripeptide sequence were effective acceptor proteins. Other proteins devoid of this sequence were ineffective as acceptors for glycosylation. On the other hand, denaturation of four proteins such as deoxyribonuclease, elastase, carboxypeptidase A, and alcohol dehydrogenase which contain the required tripeptide sequence did not promote glycosylation of the unfolded peptides. Thus, the mere presence of Asn-X-Thr (Ser) tripeptide does not determine that a protein will serve as an acceptor. Since the acceptor activity of the exogenous protein is detected only after their unfolding by sulfitolysis or by reduction and alkylation, it implies the importance of the amino acid sequence around the asparagine. The findings of Kiely et al.[128] — that glycosylation of ovalbumin can occur while it is still attached to tRNA — also implicates the importance of the linear amino acid sequence in determining the specificity of glycosylation.

While the amino acid sequence around the *N*-glycosidically linked asparaginyl residues is found to be invariant, as discussed above, an invariant sequence of amino acids in the vicinity of *O*-glycosidically linked serine and threonine residues is not discernible (Table 4). The sequence information is rather limited and is restricted to glycoproteins in which the linkage sugar is *N*-acetylgalactosamine. However, from the limited number of known amino acids sequences of glycoproteins containing serine (threonine)-linked carbohydrate units, it appears that there is no well-defined specific sequence common to all. In IgG immunoglobulin and hCG-β the only characteristic

Table 4
AMINO ACID SEQUENCES AROUND SEVERAL SERINE- AND THREONINE-LINKED CARBOHYDRATE UNITS

Glycoprotein	Sequence[a]
Chorionic gonadotropin β-subunit[b] (human)[5]	Ser-$\overset{*}{\text{Ser}}$-Lys-Ala-Pro-Pro-Pro-$\overset{*}{\text{Ser}}$-Leu-Pro-Ser- Pro-$\overset{*}{\text{Ser}}$-Arg-Leu-Pro-Gly-Pro-$\overset{*}{\text{Ser}}$-Asp-Thr-Pro- Ile-Leu-Pro-Glu
Immunoglobulin G; Hinge region (rabbit)[129]	Ser-Lys-Pro-$\overset{*}{\text{Thr}}$-Lys-Pro-Pro-Pro-Glu
Submaxillary mucin (ovine)[130]	
T_4F peptide	Phe-$\overset{*}{\text{Ser}}$-$\overset{*}{\text{Ser}}$-Ala-Gly-Ile-Pro-Ala-$\overset{*}{\text{Thr}}$-Pro- Gly-$\overset{*}{\text{Ser}}$-$\overset{*}{\text{Thr}}$-$\overset{*}{\text{Thr}}$-Gly-Ala
T_4C peptide	$\overset{*}{\text{Ser}}$-Ala-Gly-Ala-Gly-$\overset{*}{\text{Thr}}$-Ala-Gly-Val-Asp- $\overset{*}{\text{Ser}}$-Glu-Glu-Thr-Ala-Arg
Glycophorin[c] (human erythrocyte)[53]	$\underset{(\text{Ser})}{(\text{Leu})}$-$\overset{*}{\text{Ser}}$-$\overset{*}{\text{Thr}}$-$\overset{*}{\text{Thr}}$-$\underset{(\text{Gly})}{(\text{Glu})}$-Val-Ala-Met-His-$\overset{*}{\text{Thr}}$- $\overset{*}{\text{Thr}}$-$\overset{*}{\text{Thr}}$-$\overset{*}{\text{Ser}}$-$\overset{*}{\text{Ser}}$-$\overset{*}{\text{Ser}}$-Val-Ser-Lys-Ser-Tyr
Antifreeze glycoprotein (Antarctic fish)[13]	Thr-Ala-Ala-Thr-Ala-Ala-$\overset{*}{\text{Thr}}$

[a] Asterisks indicate glycosylated amino acid residues. In each case *N*-acetylgalactosamine is the linkage sugar to the amino acid via *O*-glycosidic bond.
[b] This is the carboxy-terminal peptide region of the β-subunit (residues 120—145).
[c] The sequence is for the amino-terminal peptide region of glycophorin (residues 1—20).

feature which is shared by both is the presence of a high concentration of proline residues in the vicinity of serine or threonine. On the other hand, this is not true in the case of red cell membrane glycophorin and antifreeze glycoprotein since these do not contain proline residues. Recently, the sequence of 106 of the 750 residues in ovine submaxillary mucin in which there are as many as 800 carbohydrate chains (made up of sialic acid and *N*-acetylgalactosamine) has been determined. There again no constancy of sequence in the four amino acid residues on either side of serine or threonine has been noted. Therefore, it appears that the *N*-acetylgalactosaminyl transferases in different systems have different substrate specificities. Also, the recognition site for glycosylation may depend on the overall conformation rather than any particular linear sequence. It is interesting to note, however, that while the carboxy-terminal of hCG-β (residues 109-145) has 8 serine and 3 threonine residues, only 4 serine residues are glycosylated (Table 4). Obviously, glycosylation is a specific and not a random process.

The exact locations of hydroxylysine-linked carbohydrate units in collagens have been derived from their amino acid sequences. Collagens are triple helical structures composed of three of the four peptide chains designated α_1 (I), α_1 (II), α_1 (III), and α_2. Three types of collagens, i.e., type I, II, and III, have been defined on the basis of their peptide chain composition. Thus, the molecular formulas for type I, II, and III are $[\alpha_1 (I)]_2\, \alpha_2$, $[\alpha_1 (II)]_3$, and $[\alpha_1 (III)]_3$, respectively. The distribution of these collagens in the extracellular matrix of connective tissues has been reported.[131] Prior to the glycosylation of collagens, as a general rule lysine residues must be hydroxylated by lysyl hydroxylase. Since not all of the hydroxylysyl residues thus formed undergo glycosylation, considerable variations in the content and occurrence of hydroxylysines and glycosylated hydroxylysines have been observed. In spite of such variations, one site of glycosylation common to the 4 chains of the 3 interstitial collagens is hydroxylysine residue 87 within the sequence Gly-X-Hyl-Gly-Y-Arg. It has been suggested that the occurrence of the disaccharide at this site may have a functional role in the cross-linking of the collagens.[132] The collagen α_1 (II) has maximal glycosylation as is evident from the comparison of the amino acid sequences near glycosylated regions with those of the α_1 (I) and α_2 chains. It has been found that in procollagen α_1 (II) chains almost every lysine residue when present in the Y position of the Gly-X-Y- repeating triplet is hydroxylated and glycosylated unlike that in the α_1 (I) chain, where only residue 87 and none of the other lysyl residues are hydroxylated and, therefore, not glycosylated. It is postulated that the relatively high degree of glycosylation, as observed in type II collagen, is not due to the differences in the amino acid sequences which favor lysyl hydroxylase and glycosyltransferase reactions, but may be due to a slower rate of triple-helix formation.[132]

IV. ISOLATION OF GLYCOPEPTIDES

Detailed structural analysis of a glycoprotein apparently involves the structural determination of the protein and the carbohydrate parts. More specifically, it includes the determination of: (1) the number of saccharide units, (2) the location of carbohydrate attachment sites on the polypeptide chain, and (3) the nature of the glycopeptide bond. In addition, complete characterization of the carbohydrate units, whether linear or nonlinear, requires elucidation of the sequence of monosaccharides, the intersugar linkages, and finally the anomeric configuration of the glycosidic bonds.

It has been well recognized that the valid approach to the structural analysis of the carbohydrate units which are covalently bound to specific amino acid residues on a polypeptide chain is to isolate individual glycopeptides from a given glycoprotein and to characterize each glycopeptide. It is useful to obtain an accurate analysis of the monosaccharide constituents and the amino acids in the native glycoprotein preceding the isolation of glycopeptides.

The approach that one follows for the isolation of the glycopeptides depends upon whether the glycoprotein contains one or more carbohydrate chains along the polypeptide chain. When a single carbohydrate unit is present, one can use proteolytic enzymes with broad specificity, such as pronase, papain, subtilisin, and proteinase K for the degradation of glycoprotein and subsequently separate the only glycopeptide by techniques, such as gel filtration, ion exchange chromatography, and high voltage electrophoresis. On the other hand, when a glycoprotein contains more than one carbohydrate unit, the use of the above-mentioned proteases of indiscriminate specificity would yield a complex mixture of glycopeptides, posing difficulties in the isolation of individual glycopeptides representing different locations on the polypeptides backbone. In such circumstances it is advisable to use a rather specific chemical or enzymatic method for the degradation of the polypeptide chain in order to take advantage of the differences among the resultant glycopeptides in the nature and the number of their amino acids to facilitate separation. The determination of the number and/or the sequence of amino acids in each glycopeptide would then permit the designation of specific amino acids on the polypeptide chain as "anchors" to which oligosaccharide chains of known structure can be assigned. Following separation, the individual glycopeptides can be further degraded with nonspecific proteases mentioned above to remove the amino acids that may cause interference during carbohydrate structural analysis. The specific cleavage of the polypeptide chain can be brought about by cyanogen bromide, trypsin, protease *(Staphylococcus aureus)*, chymotrypsin, or thermolysin following which the polypeptides can be fractionated and purified. It should be noted that the cyanogen bromide method may not be desirable when the carbohydrate units contain acid-labile sialic acid or fucose residues since the cleavage reaction is carried out in formic acid. It is important to recognize the need for a specific reagent for the cleavage of glycoprotein for the preparation of glycopeptides.

The microheterogeneity in the carbohydrate units of glycoproteins is a well-recognized phenomenon. As a consequence of microheterogeneity, degradation of glycoprotein results in a heterogeneous mixture of glycopeptides. In several instances, it is advantageous to prepare glycopeptides from the asialo derivative of the glycoprotein in order to alleviate the problem of microheterogeneity. Subsequent to specific degradation of asialo derivative, the resulting mixture of peptides and glycopeptides can be fractionated. In addition, the use of a nonspecific cleavage reagent in the degradation of a glycoprotein results in a heterogeneous mixture of glycopeptides because of random hydrolysis of the polypeptide chain around the carbohydrate-attachment site. This problem may be circumvented in some instances by use of exhaustive pronase digestion followed by treatment of the glycopeptide by carboxypeptidase and aminopeptidase. Futhermore, the exact sequence of amino acids around the carbohydrate chains can be determined only on a well-defined glycopeptide, and the knowledge of these sequences are essential for the precise localization of the carbohydrate units in the molecule.

A number of procedures have been employed for the purification of glycopeptides. The commonly used methods include gel filtration, ion-exchange chromatography using Dowex-1 and Dowex-50, paper chromatography, and high-voltage paper electrophoresis. For high molecular weight glycopeptides, it is preferable to use Sephadex-, cellulose-, and polyacrylamide-based ion-exchangers. Some of the other techniques such as solvent partition on cellulose, counter-current distribution, zone electrophoresis, and charcoal-celite chromatography have been employed in special cases. Several of these techniques are used in combination since they are based on the differences in the various physicochemical parameters, such as molecular size, charge, and solubility.

In the structural elucidation of the carbohydrate moeities of α_1-acid glycoprotein, the glycopeptides have been obtained by initial digestion of the glycoprotein with trypsin. The tryptic peptides were then digested with pronase and leucine aminopeptidase following which the glycopeptides were purified by SE-Sephadex chromatography.[133] Traditionally, pronase has been used directly on several glycoproteins for the purpose of isolation of glycopeptides such as those from fetuin[92] and thyroxine-binding globulin.[134] In the former case, the glycoprotein contains both *N*- and *O*-glycosidically linked carbohydrate units. The separation of these two types of units has been attempted by repeated gel filtration followed by the purification of *N*-glycosidic glycopeptides on DE-52 cellulose. It was reported that two of the three glycopeptide fractions obtained after chromatography have been considered identical, although they differed in their peptide moieties. Procedures such as these when applied to the purification of glycopeptides may be useful in the elucidation of the structure of carbohydrate units. However, they present problems in the interpretation of the data if the glycopeptide mixture is inadequately resolved. The use of methods which allow selective degradation of the peptide chain with specific cleavage reagents as opposed to those used for random hydrolysis, yields precise information concerning the number and location(s) of the carbohydrate units. In fact, employing cyanogen bromide, trypsin, and chymotrypsin as specific cleavage reagents, the complete amino acid sequence and the locations of *N*- and *O*-glycosidically linked carbohydrate units have been elegantly documented in glycophorin.[53]

In our laboratory, the elucidation of the complete structure of human chorionic gonadotropin (hCG) was accomplished by sequence analyses of peptides and glycopeptides obtained from specific cleavage of the peptide chains from this hormone.[2,3] The intact hCG molecule (38,000 mol wt) is composed of two subunits designated hCG-α (mw 15,000) and hCG-β (23,000 mol wt), respectively. These subunits were dissociated in 8 *M* urea and separated by chromatography on DEAE-Sephadex and Sephadex G-100. Each subunit was reduced with 2-mercaptoethanol and aklylated with iodoacetamide for the conversion of cysteinyl residues into their carbamidomethyl derivatives. The subunits were desialyzed by mild acid hydrolysis and digested with trypsin. The various peptides and glycopeptides from the α-subunit were isolated by gel filtration on Sephadex G-25 and G-50 and by paper chromatography and/or paper electrophoresis. The tryptic peptides and glycopeptides from the β-subunit were digested further with chymotrypsin and thermolysin, and the secondary fragments were separated by ion-exchange chromatography on Dowex-1. The complete sequence of amino acids and the locations of carbohydrate units in the peptide subunits were established by Edman degradation of the isolated peptides and glycopeptides. It was determined from these studies that the α-subunit contained two asparagine-linked carbohydrate units. These were located at asparagine residues 52 and 78 in tryptic glycopeptides αT-8 (residues 51 to 63) and αT-11a (residues 76 to 91), respectively. On the other hand, the β-subunit contained six carbohydrate units, two which were linked to asparagine residues 13 and 30 in the βT-2 (residues 11 to 20) and βT-3 (residues 21 to 43) glycopeptides, respectively. The remaining four carbohydrate units were linked to serine residues 121, 127, 132, and 138 in the C-terminal glycopeptide βC-19 (residues 109 to 145). The amino acid and carbohydrate composition of intact hCG and of its subunits and various glycopeptides is given in Table 5. Each of the asparagine-linked carbohydrate units contained two residues of galactose, three of mannose, and four of *N*-acetylglucosamine as the neutral sugar components. Although the glycopeptides were isolated from asialo-hCG, studies on the intact molecule indicated that the 16 sialic acid residues in the native hormone were distributed evenly among the total number of carbohydrate units (2 sialic acid residues per carbohydrate unit). The sialic acid was found to be only in the form of *N*-acetylneuraminic acid by paper chromatography. Each of the serine-linked carbohydrate units contained two residues of sialic acid and one residue each of galactose and *N*-acetylgalactosamine.

The occurrence of four serine-linked carbohydrate units in βC-19 was confirmed by mild alkaline sodium borohydride treatment of the glycopeptide. The oligosaccharide released due to β-elimination contained four residues of *N*-acetylgalactosaminitol. Futhermore, the four linkage serine residues were converted into alanine or cysteic acid, depending on whether the β-elimination reaction was carried out in the presence of sodium borohydride or sodium bisulfite.

V. SUGAR RESIDUES AND THEIR DETERMINATION

As indicated earlier, glycoproteins mainly contain galactose, mannose, glucose, L-focuse, *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acids; occasionally other sugars such as arabinose, xylose, and rhamnose have been found to occur. The content and proportion of these sugars vary considerably. Collagens contain less than 1% of carbohydrate prosthetic groups made up of glucose and galactose. On the other hand, rat sublingual glycoproteins contain as much as 81% carbohydrate comprised of galactose, mannose, fucose, *N*-acetylhexosamines, and sialic acid.[135] Accordingly, any distinction between different classes of glycoproteins on the basis of how much carbohydrate they contain is strictly arbitrary.

One of the problems associated with carbohydrate analysis of glycopeptides or oligosaccharides lies in the expression of the units of composition. Although it is satisfactory to report the values in terms of residues per unit dry weight, caution must be exercised in protecting the sample from absorption of moisture during the storage and its subsequent analysis. A convenient way to avoid this problem is to dry the sample and to prepare a solution of known concentration. An aliquot of the solution is used for the determination of amino acids and sugars. Suitable aliquots from the same solution are then used for other chemical studies. This approach minimizes the errors which may be otherwise introduced due to reweighing the sample for each determination. The composition of the sugars can be expressed as moles per mole of amino acid or hexosamine residues per mole of glycopeptide. The precise concentration of the glycopeptide in the solution can be determined by amino acid analysis.

The second problem relates to the purity of the standard sugar compounds used for the analysis. It is imperative that each standard must be thoroughly tested for its purity. This can be conveniently done employing paper or thin-layer chromatography or gas liquid chromatography (GLC).

The third problem encountered in carbohydrate analysis is the destruction of sugars resulting from the hydrolytic conditions used for their release from the oligosaccharide. The selection of conditions of hydrolysis has to be such that the release of various sugars should be quantitative. Therefore, it is generally essential to follow the course of hydrolysis for a certain duration and choose the time at which maximal release and the least destruction of sugars occur. The quantitation of sugars by GLC procedures requires the inclusion of a suitable internal standard in the hydrolysate. L-Arabinose, 2-deoxy-D-glucose and inositol have been generally used as internal standards because these sugars are not found in the glycoproteins studied and they separate well from other sugars. In the alternative method, the given radioactive sugar is added to the glycoprotein before hydrolysis, and the recovery of this radioactivity after hydrolysis is a measure of destruction of the sugar. Such radioisotope dilution techniques have been employed for the estimation of mannose from ovalbumin[136] and galactose from γG immunoglobulin.[137]

A number of hydrolytic, chromatographic, and colorimetric methods for the release, identification, and quantitation of sugars in glycoproteins have been described.[25,99] There are several examples in the literature indicating considerable differences in the carbohydrate composition of known glycoproteins from the same source. This may be due to: (1) variations in the degree of purity of the glycoprotein obtained by different procedures, (2) inherent microheterogeneity, and (3) the techniques used for the determination of sugar components. As an example, for α_1-acid glycoprotein from human plasma, the values reported for percent sugar composition from 4 different laboratories are galactose, 6.5 to 11.2; mannose, 4.7 to 6.5; fucose, 0.7 to 1.5; *N*-acetylglucosamine, 12.2 to 15.2; and sialic acid, 10.8 to 14.7. It follows, therefore, that for meaningful comparison of the data, it is important to use similar analytical methods for the determination of sugars.

Several of the earlier procedures for the quantitation of sugars have been virtually replaced by the successful application of GLC techniques to carbohydrate analyses. Various aspects of GLC have been reviewed.[138,139] GLC allows separation, identification, and quantitation of all monosaccharides commonly found in glycoproteins in a single procedure. Due to improved sensitivity, resolution, and accuracy, sugars can be quantitated to about 1-pmol range. Among several other GLC techniques, the one that has been widely used is the alditol acetate method[140] which was later adapted to the analyses of neutral sugars.[141,142] The principal features of this technique are (1) hydrolysis of the glycoprotein with Dowex-50 (H+) resin or acid and (2) separation of the total neutral sugars from the hydrolysate followed by reduction and acetylation of the sugars to their corresponding alditol acetates. The volatile alditol acetates are separated and

Table 5
AMINO ACID AND CARBOHYDRATE COMPOSITION OF HUMAN CHORIONIC GONADOTROPIN AND ITS GLYCOPEPTIDES

								βC-19		
									β-elimination	
	Intact hCG	hCG-α	hCG-β	αT-8	αT-11a	βT-2	βT-3	Native	A	B
Amino acids[b]					Residues					
Lysine	10	6	4	1		1		1	N.D.	N.D.
Histidine	4	3	1		2					
Arginine	15	3	12			1	1	2	N.D.	N.D.
Aspartic acid	17	6	11	1	1	1	1	4	4	4
Threonine	18	8	10	2	2	1	5	2	2	2
Serine	21	8	13	2	1			8	4	4
Glutamic acid	18	9	9	1	1	1	1	2	2	2
Proline	29	7	22			1	2	10	10	10
Glycine	12	4	8				2	1	1	1
Alanine	13	5	8	1	1	2	1	1	5	2
Half-cysteine	22	10	12	2	3	1	4	1	1	1
Valine	18	7	11	2	1	1	2			
Methionine	4	3	1				1			
Isoleucine	6	1	5			1	2	1	1	1
Leucine	17	4	13			1		3	3	3
Tyrosine	7	4	3		2		1	1	1	1
Phenylalanine	6	4	2					1	1	1
Cysteic acid										4

Carbohydrate[c]										
Fucose	1	0	1				1			
Galactose	12	4	8	2	2	2	2	4	4	N.D.
Mannose	12	6	6	3	3	3	3			
N-Acetylglucosamine	16	8	8	4	4	4	4			
N-Acetylgalactosamine	4	0	4					4		
N-Acetylneuraminic acid	16	4	12	(2)	(2)	(2)	(2)	(8)		
N-Acetyl galactosaminitol									4	
Sulfonyl-*N*-acetylgalactosamine										4

[a] Values in column A were obtained after analyses of the peptide and carbohydrate components following β-elimination and sodium borohydride reduction. Values in column B were obtained after β-elimination and sodium bisulfite treatment. N.D. = not determined.

[b] The values for amino acid residues were obtained from sequence analyses.[2-5]

[c] Neutral sugars were quantitated by gas-liquid chromatography of their alditol acetates. Hexosamine values were determined using an amino acid analyzer. *N*-acetylneuraminic acid was measured by the thiobarbituric acid assay procedure. Values for sialic acid residues in the asialoglycopeptides which are given in parentheses were determined from the sialic acid composition of the native subunits.[2-5]

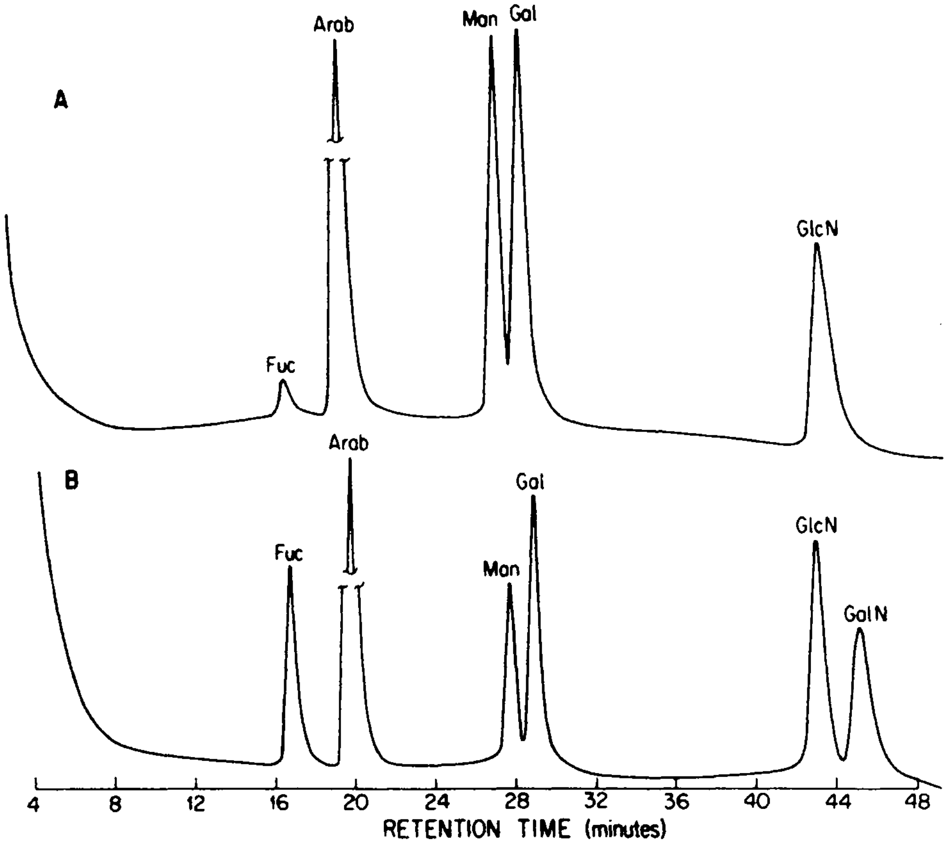


FIGURE 9. Gas chromatographic analysis of neutral and aminosugars as alditol acetates. (A) Sugar analysis of α_1-acid glycoprotein, (B) analysis of a known mixture of sugars.

quantitated using a suitable column support such as ECNSS-M or OV-17. This procedure has the advantage over that of trimethylsilylation in that the reduction at the anomeric carbon prevents ring isomerization; therefore a single peak is obtained for each sugar. Good separation of all neutral sugars and hexosamines was first reported by Neidermeier[143] employing 1% ECNSS-M on Gas Chrom Q and a linear temperature program between 140 and 230° C. In this study acid hydrolysis instead of hydrolysis with Dowex-50 resin was performed. We have found that resin hydrolysis can be used to quantitate neutral sugars and hexosamines in a single run if the original procedure of resin hydrolysis is modified. The neutral sugar fraction containing arabinose as the internal standard is separated essentially according to Lehnhardt and Winzler,[142] except that the Dowex-50 and Dow-1 resins are placed in separate glass columns joined together. Following elution with deionized water, the columns are separated and an aliquot of mannosamine solution (internal standard for hexosamines) is added to the Dow-50 column. This column is eluted with 2 *N* HCl. The neutral sugar fraction and the Dow-50 effluent are combined and processed for the preparation of alditol acetates. The separation of the sugars using this technique is shown in Figure 9.

While the GLC methods are quite suitable for the analysis of neutral sugars in glycoproteins, the quantitative determination of these components can be also carried out satisfactorily by automated borate-complex anion exchange chromatography.[99,144] For the determination of neutral sugars, the sample is hydrolyzed in 1-2 *N* hydrochloric or sulfuric acid for 6 to 8 hr at 100° C, and following neutralization of the hydrolysate, the sample is applied directly to the column packed with specially prepared anion exchange resin (Chromobead S®, low pressure, Technicon). The separation of neutral sugars is effected by gradient elution with borate buffers. The neutral sugars in the effluent are quantitated colorimetrically by the orcinol-sulfuric acid reaction. A good separation of all sugars except fucose and arabinose has been achieved by this method.[99]

The determination of amino sugars is generally carried out by using an amino acid analyzer. Hydrolysis of the sample is performed in 4 *N* HCL at 100°C for 4 to 6 hr. Glucosamine and galactosamine can be determined by employing the buffer systems used for the separation of amino acids. The determination of hexosamines and their alcohols required in certain cases can be carried out by chromatography on a long column of sulfonic acid resin eluted with citrate-borate buffers.[145]

The most commonly used approach to the composition of carbohydrates is determination of neutral sugars by GLC as alditol acetates, hexosamines by an amino acid analyzer, and sialic acid colorimetrically. It should be realized, however, that other techniques have been employed in special situations.

Sialic acids occur in glycoproteins mainly as *N*-acetylneuraminic acid (NANA) and *N*-glycolyl neuraminic acid, although some other forms such as *N*-acetyl-*O*-acetyl neuraminic acids are known to be present. Various aspects of sialic acids have been reviewed.[25] The most widely used method for the determination of sialic acid is the Warren's thiobarbituric acid procedure[146] or some of its modifications.[147] This method, however, is nonspecific since different sialic acids do not give identical color yield. Furthermore, the presence of 2-deoxy sugars produces interfering chromogen with thiobarbituric acid. Other analytical methods for the quantitation of NANA include periodate-resorcinol procedure,[147a] spectrofluorometry,[148] and GLC.[149] The GLC properties of several *N*- and *O*-acetylated sialic acids[150] and their electron ionization mass spectra[151,152] have been published. Recently, a highly sensitive GLC-MS technique employing chemical ionization has been developed for the determination of NANA.[153] Chemical ionization of fully sialylated derivative of NANA yields a highly abundant $(M + 1)^+$ ion. The method developed is based upon the monitoring of the protonated molecular ions of fully sialylated NANA ($m/e = 814$) and β-methyl glycoside of NANA (internal standard, $m/e = 714$). The detection limit of NANA is of the order of 0.6 pmol. The technique is reported to be inherently sensitive as well as specific, and it is suggested that this method may be applicable to simultaneous identification and quantitation of other sialic acids.

VI. METHODS FOR STRUCTURAL STUDIES

The elucidation of complete structure of a glycopeptide as described above involves the determination of the sequence of sugars in the oligosaccharide, the anomeric configuration (α or β) of each sugar, and the intersugar linkages. These can be accomplished by a variety of chemical and enzymatic procedures. Thus, methods such as sequential periodate alone or combined with methylation or enzymatic hydrolysis, specific cleavage of oligosaccharides employing partial acid hydrolysis or acetolysis, and those which involve sequential degradation by the exo- and endoglycosidases, have been used. One of the significant developments, which has taken place in recent years, is the considerable refinement of the methylation procedure by the introduction of the Hakomori procedure of methylation and the introduction of GLC-MS for the identification of methylated sugars. Another important development has been in the availability of exo- and endoglycosidases. In fact, some of the presently proposed structures may have to be reexamined with the newer techniques since they yield much more accurate information.

A. Periodate Oxidation Methods

One of the approaches to study the structure of the carbohydrate component of glycoproteins is the sequential oxidation of the polysaccharide with periodic acid or its salt. This method,[154] commonly known as the "Smith degradation", offers in most instances valuable structural information concerning the intersugar linkages within a polysaccharide. In the application of this technique to structural studies, one does not require precious reference compounds for the identification of the products.

In this procedure the polysaccharide is oxidized with periodate, and the resulting polyaldehydic structure is reduced to polyalcohol with sodium borohydride. Whereas the noncyclic acetal linkages derived from the reduced periodate oxidized polysaccharide are susceptible to hydrolysis by acid under mild conditions, the glycosidic linkage of a residue that is not vulnerable to oxidation by periodate is comparatively stable to acid. This difference in the stability of the linkages yields specific glycosides of oligosaccharides characteristic of the parent polysaccharide.

The principal features of this technique can be summarized as follows: hexopyranosyl residues so substituted that the hydroxyl groups at C-2 and C-3 are free, yield after periodate oxidation, reduction, and hydrolysis, free or glycosylated erythritol and threitol (from carbons 3 to 6) and glycolic aldehyde (from carbons 1 and 2); such a situation exists for hexosides in which either *O*-4 or both *O*-4 and *O*-6 are substituted. If the hydroxyl groups on C-3 and C-4 are free—i.e., linkages involve *O*-2 and/ or *O*-6—free or glycosylated glycerol (from carbons 4 to 6) is obtained. Fucose, which is mostly located at the nonreducing terminus, yields propane-1,2-diol and glycolic aldehyde. *N*-Acetylhexosamines, either at the non-reducing termini or linked at C-6, give rise to 2-acetamido-2-deoxyglyceraldehyde and free or glycosylated glycerol.

It has been shown that periodate oxidation in certain situations is incomplete due to underoxidation resulting from inter-residue hemiacetals.[155,156] However, underoxidation can be conveniently prevented by treatment of the polyalcohol to another oxidation-reduction step.

If periodate treatment involved only the oxidation cleavage of vicinal diols, the interpretation of the results would be simpler. It is known, however, that there are other secondary reactions with periodate which result in the change in the products of primary oxidation. Therefore, extreme caution must be exercised so as to minimize these so-called overoxidation reactions.

It is customary to follow the oxidation by monitoring the reaction for the estimation of the amount of periodate consumed. This can be achieved either spectrophotometrically[157,158] or colorimetrically.[159] There are several methods which describe the determination of formaldehyde and formic acid which may be formed as a result of oxidation.[25] These two products should be estimated if materials are available in large quantities. The oxidized product is treated with ethylene glycol to eliminate excess periodate and is reduced with sodium borohydride. The reaction is stopped by addition of glacial acetic acid. The polyalcohol is then separated from salt by gel-filtration and hydrolyzed with mild acid. The products of hydrolysis are reduced, acetylated, and quantitated by GLC.

The Smith degradation procedure has been used in the elucidation of the structure of the carbohydrate moiety in several glycoproteins and glycopeptides such as α_1-acid glycoprotein,[160] fetuin,[44] Taka-amylase glycopeptide,[161] and glycopeptides from stem bromelain.[33] In our laboratory, this procedure was used for the structural determination of the carbohydrate units of hCG. Each asparagine-linked glycopeptide (100 nmol) was oxidized with 100 μℓ of 0.05 *M* $NaIO_4$ (~ five fold excess) in the dark for 60 hr at 4°C following which the pH was adjusted to 8.0 with 5 m*M* NaOH. To this solution $NaBH_4$ was added to attain a concentration of 0.25 *M*. The reduction was allowed to proceed at 4°C for 24 hr, at which time the reaction mixture was brought down to pH 4.0 by addition of glacial acetic acid. The product was lyophilized, dissolved in 0.1% NH_4HCO_3 (2.0 mℓ), and applied to a G-25 (fine) column (0.9 × 90 cm) which was eluted with the same buffer, after which 1 mℓ fractions were collected. The course of elution of the oxidized and reduced (OR) glycopeptide was followed by measuring absorbance at 230 nm. Appropriate fractions were pooled and lyophilized. This product (OR-1) of the first cycle of Smith degradation was dissolved in 2.0 mℓ of water. After the removal of suitable aliquots for amino acids, hexosamine, and neutral sugar analyses, and equal volume of 1 *N* HCL was added to the remainder solution in order to hydrolyze the acetal linkages in the polyalcohol structure. After 24 hr at room temperature, the mixture was adjusted to pH 7 with 0.5 *N* NaOH and lyophilized. The residue was chromatographed on a G-25 Sephadex column as before and the oxidized, reduced, and hydrolyzed (ORH) glycopeptide was isolated. The oxidation-reduction hydrolysis cycle was repeated two more times to yield OR-2 and OR-3 products.

The results from the 3 c of Smith degradation were as follows (numbers refer to residues per mole of aspartic acid):

Degradation cycle	Galactose	Mannose	Fucose	*N*-Acetylglucosamine
None	1.9	2.6	0—1	3.6 (4)
OR-1	0	1.6	0	3.4 (4)
ORH-1	0	1.3	0	2.3 (2)
OR-2	0	0.2	0	2.1
OR-3	0	0	0	0.9

These data indicated that the treatment of asialo-asparagine-linked glycopeptides of hCG with a fivefold excess of $NaIO_4$ caused the destruction of all galactose and fucose residues. Therefore, these residues were terminally located. In this same cycle (OR-1), two residues of mannose but none of the *N*-acetylglucosamine residues were destroyed. However, upon mild acid hydrolysis of OR-1, two residues of *N*-acetylglucosamine were lost from the glycopeptide, leaving one residue of mannose and two residues of *N*-acetylglucosamine per mole of aspartic acid (ORH-1). This finding suggested that the two mannose residues that were destroyed, were located internally to the two *N*-acetylglucosamine residues lost during acid hydrolysis. This observation was later confirmed by glycosidase treatment studies. It was evident that the two mannose residues which were destroyed by periodate were not substituted at 0-3. The C-4 oxygen of these mannoses were ruled out as a possible site of substitution by *N*-acetylglucosamine by isolation of glucosaminylglycerol from the glycopeptide after the first Smith cycle. This was done by passing the ORH-1 materials through a G-25 Sephadex column. Fractions subsequent to the elution of the core-glycopeptide were analyzed for glucosamine, and those showing its presence were pooled and lyophilized. The residues were dissolved in water, deionized, and lyophilized. Hydrolysis of this product and subsequent paper chromatography of the hydrolysate showed the presence of glucosamine and glycerol indicating GlcNAc→Man linkage. The substitution at *O*-2 of the two mannoses destroyed by periodate during the first Smith cycle was later established by methylation-GLC/MS analyses.

The oxidation of the core glycopeptide with periodate destroyed the remaining mannose residue, leaving a glycopeptide with two residues of *N*-acetylglucosamine per mole of aspartic acid (OR-2). The third treatment with periodate destroyed one of the two remaining *N*-acetylglucosamine residues and left a glycopeptide which contained just the *N*-acetylglucosamine residue involved in the linkage with asparagine (OR-3). Thus, the sequence of monosaccharides in the core glycopeptide was established as Man → GlcNAc → GlcNAc → Asn.

B. Methylation Analysis

Methylation analysis serves as one of the most powerful tools in deducing the structure of complex carbohydrates. The application of methylation analysis for sequence studies has been extensively reviewed.[162,163] The main features of this technique are (1) methylation of all hydroxyls in the oligosaccharide, (2) hydrolysis of the methyl derivative and conversion of the partially methylated sugars as their alditol acetates, and (3) their identification and quantitation by GLC-MS. The free hydroxyl groups in the partially methylated sugars represent the positions which are substituted in the native saccharide. The method, therefore, provides information on the structural details of intersugar residues but does not allow deduction of the sequence of sugars nor their anomeric linkages.

Several of the earlier classical methods for permethylation of carbohydrate, such as the Haworth (sodium hydroxide-methyl sulfate) and Purdie (silver oxide-methyl iodide) methods,[164] have been replaced by the more recent and most widely used technique described by Hakomori,[165] in which methylsulfinyl carbanion and methyl iodide are used. The prerequisite for methylation of glycopeptides and oligosaccharides is that these substances are soluble in dimethylsulfoxide. The solubilization in most cases is effected by ultrasonic treatment and/or warming to 70°C. The solubilized carbohydrate is reacted with carbanion that is prepared by heating sodium hydride in dimethylsulfoxide. The resulting carbohydrate alkoxide is treated with methyl iodide. Methylation of the hydroxyl group along with *N*-methylation of *N*-acetylhexosamine residues occurs.[166] Generally, the methylated polysaccharides are not quite soluble in hot water. While they are readily soluble in chloroform, it should be borne in mind that glycopeptides containing a high proportion of amino acids following methylation are not soluble in this solvent to any great extent. Solubilization of methylated glycopeptides in chloroform is a necessary step prior to its purification. Consequently, for methylation it is desirable to use glycopeptides containing the least number of amino acid residues. After extraction of the methylated product with chloroform, it is purified on silica gel[91] or on Sephadex LH-20,[4] and acetolysed.[167] Following acetolysis, the product is hydrolyzed, reduced, and acetylated, and the partially methylated sugar derivatives are identified and quantitated by GLC and GLC-MS.

Methylation of intact carbohydrate and of the derivatives formed during Smith degradation (previously discussed) provides more information on the structural features of complex carbohydrates. When the polyalcohol intermediate obtained after oxidation and the reduction of the carbohydrate is subjected to mild acid hydrolysis, a glycoside of the sugar residue that is not vulnerable to periodate oxidation can be isolated. The aglycon in such a glycoside is generally glycerol, threitol, or erythritol and is derived from the adjacent sugar residue. If the polyalcohol is methylated and the product is hydrolyzed with dilute acid and realkylated with trideuteromethyl or ethyl iodide, the alkylated glycosides can be characterized by GLC-MS as such or after hydrolysis. This approach was used to determine the fine structure of a polysaccharide from *Rhizobium meliloti*. For more details on this technique the reader is referred to Lindberg and Lonngren.[163]

In the analyses of methylated sugars, GLC and GLC-MS have proven to be important and effective techniques in the elucidation of the structure of complex carbohydrates. The separation and quantitation of methylated sugars by GLC[168,169] and the use of mass spectrometry in the structural analysis of carbohydrates[170] have been extensively reviewed. In addition, an excellent practical guide to the methylation analysis of carbohydrates has been published providing information on the relative retention times of a number of partially methylated alditol acetates, as well as the mass spectra of several such compounds.[171] The advantage in the GLC procedure for partially methylated alditol acetates is that the quantitation does not require response factors[172] except for the *N*-acetamido sugars. The quantitative analysis of methylated *N*-acetamido sugars by GLC, however, may bring about serious errors because of different response factors between various *N*-acetamido sugar derivatives, as well as the difference between the response factors for these derivatives and those for neutral sugars.

The partially methylated alditol acetates can be generally identified on the basis of their relative retention times and can be quantitated to some extent. However, certain derivatives have almost identical retention times, and the identification becomes difficult. As an example, the relative retention times for 3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetyl derivatives of glucose and mannose on an OV-225 column are 1.83 and 1.82 min, respectively. Also, on the same column, 3-*O*-methyl- and 4-*O*-methyl-mannose pentaacetates have identical mobility (relative retention time = 6.8 min).[171] Thus, it is not always possible to identify all methylated sugars solely on the basis of retention times. Here, mass spectrometry combined with GLC is most useful in the unequivocal identification of the substitution pattern of methoxy and acetoxy groups in the partially methylated alditol acetates.

The combined GLC-MS of partially methylated alditol acetates has been used for the elucidation of carbohydrate structures of several glycoproteins, e.g., human thyroglobulin β-glycopeptide,[90] thyroxine-binding globulin,[173] Clq complement,[91] and fetuin.[92] The technique used in our laboratory for the hCG carbohydrate is described below.

The four *N*-glycosidic glycopeptides were digested separately with pronase and recovered by chromatography on Sephadex G-50. Pronase digestion resulted in the removal of nearly all of the amino acids except the linkage asparagine residue. This step was found to be necessary to facilitate the subsequent extraction of permethylated glycopeptide by chloroform and minimize interference during GLC-MS analyses. Each pronase-digested glycopeptide was dried over P_2O_5 overnight, dissolved in dimethylsulfoxide (0.5 mℓ), and 2 *N* sodium methyl sulfinyl carbanion (0.25 mℓ) was added to this solution with stirring. After 2 hr, methyl iodide (0.25 mℓ) was added dropwise with cooling in a 20° C water bath. The methylation was allowed to proceed for 1 hr, after which the mixture was partitioned between water (8 mℓ) and chloroform (5 mℓ). The complete methylation procedure was conducted under N_2 in a dry box. The aqueous phase was further extracted with 5 mℓ of chloroform for five times. The chloroform extracts were combined, washed with 5 mℓ of water, and evaporated to dryness. The residue was dried over P_2O_5 under high vacuum, dissolved in 0.5 mℓ of chloroform methanol (2:1 v/v), and chromatographed on a column (1.1 × 48 cm) of Sephadex LH-20 packed in the same solvent. Approximately 5 to 10 μℓ aliquots of effluent fractions (2 mℓ) were spotted on silica gel thin-layer plates, and the methylated glycopeptide was identified by charring with methanolic-H_2SO_4. The methylated glycopeptide eluted at the void volume of the column.

The partially methylated hexitol acetates and 2-deoxy-2-*N*-methylacetamidohexitol acetates were prepared from methylated glycopeptide by acetolysis/hydrolysis procedure of Stellner et al.[167] with the following modifications: (1) reduction of the free partially methylated hexoses and 2-*N*-methylacetamido hexoses was performed with 10 mg $NaBH_4$ in 1 mℓ of 1:1 (v/v) ethanol/water instead of in water, and (2) acetylation of the resulting partially methylated hexitols and 2-deoxy-2-*N*-methylacetamidohexitols was effected in a mixture of 0.5 mℓ each of pyridine and acetic anhydride for 1 hr at 100° C. The columns used for GLC were packed with either stationary phase A (3% OV-225 on Supelcoport, 80 to 100 mesh) or phase B (3% OV-17 on Chromosport WHP,

Table 6
LINKAGE ANALYSIS-RELATIVE RETENTION TIMES[a] OF METHYLATED SUGARS AS THEIR ALDITOL ACETATES OBTAINED FROM METHYLATED *N*-GLYCOPEPTIDES OF hCG-α AND hCG-β

Methylated sugar	αT-8	αT-11a	βT-2	βT-3	Literature value[b]	Values from reference compounds
2,3,4-Tri-*O*-methyl-fucose	—	—	—	0.60	0.58	0.61[c]
2,3,4,6-Tetra-*O*-methyl-galactose	1.19	1.19	1.17	1.19	1.19	1.14[d]
3,4,6-Tri-*O*-methyl-mannose	1.80	1.83	1.81	1.82	1.82	1.83[e]
2,4-Di-*O*-methyl-mannose	4.42	4.41	4.33	4.26	4.51	4.44[f]
2-Deoxy-3,6-di-*O*-methyl-2-*N*-methyl-acetamido-glucose	1.47	1.48	1.50	1.52	—	1.50[g,h]
2-Deoxy-3-*O*-methyl-2-*N*-methylacetamido glucose	—	—	—	2.58	—	—

[a] For neutral sugars with respect to 2,3,4,6-tetra-*O*-methyl glucose, and for amino sugars with respect to 2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-glucose.

[b] See Jansson et al.[171]

[c–h] Relative retention times obtained from the following references compounds: c, Fuc $\xrightarrow{1,2\alpha}$ Gal; d, salivary mucin oligosaccharide; e, Man $\xrightarrow{1,2\alpha}$ Man; f, α_1-acid glycopeptide; g, chitotetraose; h, Man $\xrightarrow{1,4\alpha}$ GlcNAc.

80 to 100 mesh). The partially methylated alditol and hexosaminitol acetates were separated on phase A and B, respectively, and identified by comparison of their retention times with the reported values relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, as well as by comparison with those of authentic standards. The linkage analyses of the sugars contained in each of the four asparaginyl glycopeptides of hCG were obtained after their identification as partially methylated alditol acetates (Table 6). From these studies it was apparent that in each glycopeptide galactose residues were unsubstituted; therefore, they were located at the nonreducing termini. Two of the three mannose residues were monosubstituted, i.e., 1 → 2 substitution each. The third mannose residue was substituted at *O*-3 and *O*-6 positions. All of the *N*-acetyl-glucosamine residues had substituents at *O*-4.

In βT-3 glycopeptide the fucose residue was unsubstituted; hence it was at a terminal position. Furthermore, in this glycopeptide, one of the four *N*-acetylglucosamines was substituted at *O*-4 and *O*-6. Methylation of the core glycopeptide — i.e., the product of hydrolysis offer the first Smith degradation cycle — indicated the presence of terminal mannose at the nonreducing end and of 1,4-di-*O*-substituted *N*-acetylglucosamine.

C. GLC-MS Analysis

GLC or partially methylated alditol acetates alone may not provide sufficient information on the identity of sugar derivatives. GLC-MS analysis allows the identification of those derivatives due to their property of specific fragmentation on electron impact. The mode of fragmentation of various partially methylated alditol acetates using deuterium-labeling techniques has been extensively studied by Bjorndal et al.[174] and Boren et al.[175] The mass spectra of stereoiosomeric partially methylated alditol

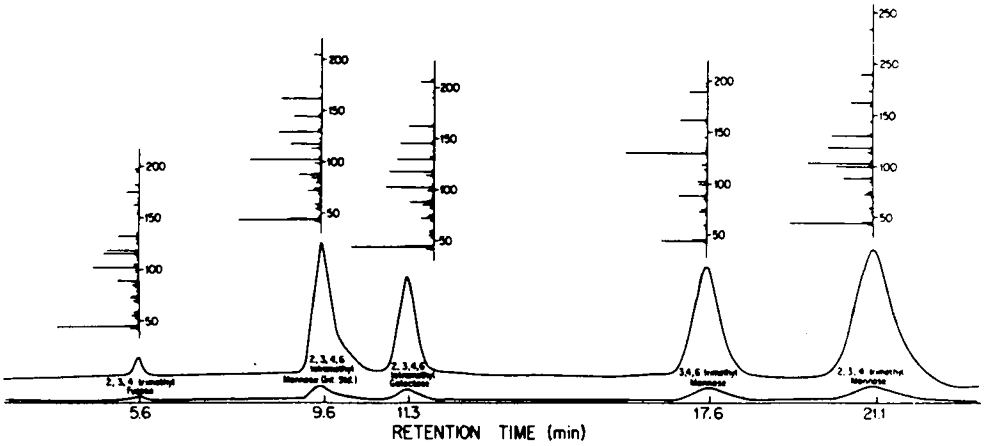


FIGURE 10. The separation and identification of methylated alditol acetates by GLC-MS.

acetates are almost identical; therefore, it is not possible to identify the parent sugar (e.g., glucose, galactose, and mannose) of these derivatives. Molecular ions are rarely observed in the mass spectra. The primary fragments are formed as a result of fission between carbon atoms in the alditol chain. As a general rule, fission between methoxylated and acetoxylated carbon atoms is preferential. The fission between two methoxylated carbons is preferred over the fission between one methoxylated and one acetoxylated carbons. The fission between two acetoxylated carbons is the least abundant. For more detailed information, on the exception to this rule and the mode of secondary fragmentation, the reader is referred to the review by Lonngren and Svensson.[170] In GLC-MS analysis, the partially methylated alditol acetates are separated in terms of their total ion concentration. The identity of each separated component can be established from its mass spectrum (Figure 10). A collection of reference mass spectra has been published[171] and is extremely useful in the identification of partially methylated alditol acetates. The relative retention time of sugar derivatives and their mass spectral analysis provide in most cases conclusive identification of each sugar component.

In GLC-MS analyses of partially methylated alditol acetates, the peaks are identified on the basis of relative retention times on standard GLC columns. The chromatograms are recorded with respect to total ions. The precise identification of peaks is facilitated by mass fragmentography which relates a single m/e species and normalizes its relative abundance over the entire fragmentogram. The relative abundance of mass fragments obtained from various partially methylated alditol acetates of asparagine- and serine-linked carbohydrate units of hCG (Table 7) resulted in unequivocal identification of each sugar derivative.

D. Sequence Determination Using Glycosidases

The periodate oxidation and methylation techniques provide information concerning the intersugar linkages and a limited information on the sequence of sugars by Smith degradation. The anomeric configuration and complete sequence of sugars cannot be deduced from these techniques. Although nuclear magnetic resonance and infrared spectroscopy can be used, a large amount of the sample is needed for such studies in addition to having access to the instruments.

Glycosidases have proven to be extremely useful both in the determination of the sequence of sugars and their anomeric linkages. Two major classes of glycosidases are

Table 7
RELATIVE ABUNDANCES OF MASS FRAGMENTS DERIVED FROM GLC/MS[a] ANALYSES OF PARTIALLY METHYLATED ALDITOL ACETATES OF ASPARAGINE- AND SERINE-LINKED CARBOHYDRATE UNITS OF hCG

Glycopeptide		Relative abundance (m/e)[b]											
		1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol											
N-glycosidic	m/e:	43	45	71	87	101	117	129	145	161	205		
αT-8		100	17	16	20	57	77	57	48	40	43		
αT-11a		100	17	38	24	62	49	32	29	28	14		
βT-2		100	35	13	21	51	49	36	34	31	17		
αT-3		100	29	19	38	92	111	74	62	59	32		
		1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methyl-mannitol											
	m/e:	43	45	71	87	101	117	129	161	189			
αT-8		100	32	27	66	43	23	179	91	59			
αT-11a		100	24	37	67	45	12	196	78	49			
βT-2		100	32	30	68	27	11	270	102	76			
βT-3		100	32	21	82	38	24	294	118	68			
		1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl-mannitol											
	m/e:	43	45	71	87	101	117	127	129	189	201	233	261
αT-8		100	13	20	28	26	69	16	72	40	12	13	11
αT-11a		100	14	20	53	27	169	24	125	49	8	17	0
βT-2		100	5	7	34	17	64	13	99	52	6	17	3
βT-3		100	7	11	50	29	119	17	114	49	7	14	1
		1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methyl-fucitol											
	m/e:	43	45	71	87	101	117	131	161	175			
βT-3		100	10	13	13	69	81	46	14	30			

1,4,5-Tri-*O*-acetyl-3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol

	m/e: 43	45	87	116	124	129	142	158	170	173	202	233
αT-8	100	48	14	156	4	6	4	52	1	0	1	38
αT-11a	100	16	13	86	6	7	11	38	0	0	1	16
βT-2	100	27	22	156	9	8	22	80	0	1	0	42
βT-3	100	38	15	127	8	5	18	65	1	1	0	34

1,4,5,6-Tetra-*O*-acetyl-3-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol

	m/e: 43	45	87	116	124	142	158	202	261
βT-3	100	7	29	357	21	68	252	4	36

1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol[c]

	m/e: 43	45	71	87	101	117	127	129	161	201	233
O-glycosidic[d]	100	32	25	21	47	104	19	78	44	2	18

3,6-Di-*O*-acetyl-1,4,5-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol

	m/e: 43	45	71	87	88	101	116	117	129	130	161	246
	100	16	32	20	29	30	3	9	6	19	7	8

[a] Gas liquid chromatography/mass spectrometry.

[b] Mass/charge.

[c] The relative retention time (T = 2.03) with respect to 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol was identical to the reported value.

[d] The *O*-glycosidic (serine-linked) carbohydrate units obtained from alkaline sodium borohydride treatment of hCG-β subunit were analyzed.

recognized: (1) exoglycosidases which hydrolyze sugars located at the nonreducing termini of oligosaccharides and (2) endoglycosidases which hydrolyze specific glycosidic linkages of sugars which are not present at the nonreducing termini of the oligosaccharides of various glycoproteins.

1. Studies with Exoglycosidases

A number of exoglycosidases have been purified to homogeneity and some of these have been obtained in crystalline forms. Their isolation, purification, and properties have been described.[176] Several of these enzymes which hydrolyze a wide variety of glycosyl linkages have been effectively used in the elucidation of the carbohydrate structure of various glycoproteins (Table 8).

In most instances, synthetic substrates — e.g., ortho- or para-nitrophenyl or 4-methylumbelliferyl glycosides — have been used during the purification of exoglycosidases. The enzymes are designated as α-D, β-D, α-L, or β-L according to their ability to cleave sugar residues with specific anomeric and D or L configurations. When these enzymes are used for the sequence analysis of the natural substrates such as oligosaccharides and glycopeptides, the configurations assigned to the sugar residues which are released correspond to those of the synthetic glycosides. Most of the exoglycosidases exhibit a remarkable degree of specificity with respect to the anomeric linkages and a broad specificity for the anomeric substituents and are, therefore, quite suitable for structural studies. However, it should be realized that certain exoglycosidases are highly specific for the anomeric linkages and the anomeric substituents. As an example, 1,2-α-L-fucosidase from *Aspergillus niger* is extremely specific for 1,2-α-L-fucosidic linkage to galactose.[188] This enzyme not only hydrolyzes the synthetic 2-*O*-α-L-fucopyranosyl-D-galactose but also releases fucosyl residues from milk oligosaccharides and porcine and canine submaxillary mucins in which fucose is found to be α1,2 linked to galactose. The enzyme does not hydrolyze *p*-nitrophenyl α-L and β-L fucopyranosides nor a variety of other fucosyl glycosides including 2-*O*-, 3-*O*-, or 4-*O*-α-L-fucopyranosyl-fucoses. An enzyme with similar specificity has been isolated from *Clostridium perfringens.*[189] An enzyme such as this is most useful when one is concerned with the selective cleavage of specific fucopyranosyl residues if it is established that the linkage is an α1,2 and none else. On the other hand, the failure of this enzyme to release other α-L-fucopyranosyl residues from a saccharide would not imply that fucose was not located at the nonreducing terminus nor can it be concluded that its anomeric configuration was different. Recently, Baenziger and Fiete,[92] in their report on the structure of fetuin oligosaccharides, have emphasized the importance of the exoglycosidase specificities in sequence analysis and have cautioned against erroneous interpretation of results. These authors encountered unexpected specificities for both diplococcal and jack bean β-galactosidases and diplococcal β-D-*N*-acetylglucosaminidase, which have been generally considered to be enzymes with rather broad specificities.

Whenever glycosidases are to be used in structural studies, the preparations must be tested for contamination with proteases and other glycosidases. Consequently, the purity of a given glycosidase preparation is examined for the contaminating glycosidases and proteases employing various synthetic glycosides and proteins, such as azocoll or azalbumin, respectively, as the substrates. It is advisable to check the purity using a known glycoprotein as the substrate and that, too, under the conditions of the assay. The carbohydrate compositional analysis of oligosaccharide usually allows the prediction of the exoglycosidases to be used. For example, when glycopeptides contain fucose and sialic acid, these sugars are mostly located at the nonreducing termini. Recently, however, fucose has been shown to be glycosidically linked to threonine (or serine) and substituted at *O*-4 by a glucopyranosyl residue.[77,78] Therefore, neuramini-

Table 8
EXOGLYCOSIDASES COMMONLY USEFUL FOR STRUCTURAL DETERMINATION OF CARBOHYDRATE UNITS OF GLYCOPROTEINS

Enzyme	Source and purification	Glycoproteins studied[a]
Neuraminidase	*Vibrio cholerae*,[177] *Clostridium perfringens*,[178] *Diplococcus pneumoniae*[179]	Sublingual glycoprotein,[103] submaxillary glycoprotein,[178,180] fetuin,[181] erythrocyte membrane glycoprotein,[178,182] α_1-acid glycoprotein,[178,179] thyroglobulin,[183] chorionic gonadotropin,[184] intimal glycoprotein,[185] human factor VIII,[186] glomerular basement membrane[187]
α-Fucosidase	*Aspergillus niger*,[188] *Clostridium perfringens*,[189] rat epididymis,[190] abalone liver,[191] almond emulsin[192]	Porcine[188,189] and canine[188] submaxillary glycoproteins, macroglobulin G,[190] luteinizing hormone,[190] milk oligosaccharides[192]
α-Galactosidase	*Aspergillus niger*[76,193]	Earthworm cuticle collagen[76]
β-Galactosidase	*Diplococcus pneumoniae*,[179] *Aspergillus niger*,[193] *Clostridium perfringens*,[194] *Escherichia coli*,[195] *Phaseolus vulgaris*,[196] jack bean[197,198]	Chorionic gonadotropin,[4] fetuin,[181] human factor VIII,[186] α-1 acid glycoprotein,[50,199] erythrocyte membrane glycoprotein,[200] immunoglobulins[201]
α-Mannosidase	*Phaseolus vulgaris*,[196] jack bean,[202-203] *Aspergillus niger*,[204] *Turbo cornatus*,[205] hog kidney[206]	Chorionic gonadotropin,[4] thyroglobulin,[57] immunoglobulins,[201] α_1-acid glycoprotein,[50,204] ovalbumin,[207] vesicular stomatis virus G protein[208]
β-Mannosidase	*Achatina fulica*,[209] Hen oviduct[43]	Vesicular stomatitis virus G protein,[208] ovalbumin,[209] ribonuclease B[43]
β-*N*-Acetylglucosaminidase	*Aspergillus niger*,[193] *Phaseolus vulgaris*,[196] *Diplococcus pneumoniae*,[199] pig epididymis,[210] jack bean[211]	Thyroglobulin,[57] chorionic gonadotropin,[184,193] erythrocyte membrane glycoprotein,[200] ovalbumin,[193,211] fetuin,[193,196,212] immunoglobulins,[201,213] transferrin[214]
α-*N*-Acetylgalactosaminidase	*Clostridium perfringens*,[215] *Aspergillus niger*,[216] *Lumbricus terrestris*,[217] porcine liver[218]	Submaxillary glycoproteins,[215-218] stomach blood group A glycoproteins[218]

[a] Either intact glycoproteins or glycopeptides derived therefrom were used as substrates. The source of enzyme used for a given glycoprotein is specified in the referenced articles.

dases and fucosidases are initially used in such cases. The sequence of other residues can be established by employing several other enzymes sequentially in appropriate combinations. In each case, the time-course of the release of each sugar should be followed.

The use of exoglycosidases in combination with periodate/Smith degradation procedures allows the elucidation of the complete structure of the carbohydrate unit.

This approach has been widely used. The choice of the exoglycosidase to be used is also made simpler if the intersugar linkages are known. Thus, from the information on the intersugar linkages obtained from methylation analysis and the use of exoglycosidases on the intact glycopeptides and the products of Smith degradation, complete structures of asparagine-linked carbohydrate in thyroglobulin (β-glycopeptide),[90] fetuin,[92] and thyroxine-binding globulin[173] have been determined.

The sequence and anomeric configurations of monosaccharides were determined by individual and sequential treatment of hCG subunits and their glycopeptides with specific glycosidases.[4,5] In these studies, neuraminidase, β-D-galactosidase, β-D-*N*-acetylglucosaminidase, and α-D and β-D-mannosidases were used. The sequence of peripheral carbohydrate in the asparaginyl glycopeptides was found to be NANA $\xrightarrow{\alpha}$ Gal $\xrightarrow{\beta}$ GlcNAc $\xrightarrow{\beta}$ Man $\xrightarrow{\alpha}$ from the release of each sugar with specific enzymes. The core region of the glycopeptides had the sequence Man $\xrightarrow{\beta}$ GlcNAc $\xrightarrow{\beta}$ GlcNAc → Asn, as determined from the release of one residue each of mannose and *N*-acetylglucosamine by β-D-mannosidase and β-D-*N*-acetylglucosaminidase, respectively. This mannose residue could not be released by α-D-mannosidase. The various techniques, including periodate oxidation, methylation and mass spectral analyses, and exoglycosidase treatment, were applied to the *O*-glycosidically linked carbohydrate of hCG-β subunit. The complete structure of the *N*-glycosidic (Figure 11) and *O*-glycosidic (Figure 12) carbohydrate of hCG were consistent with the experimental results.

2. *Studies with Endoglycosidases*

The endoglycosidases (Table 9) cleave specific sugar linkages located in the inner regions of the saccharides. More attention has been focused on endo-β-*N*-acetylglucosaminidases as compared to other endoglycosidases. As a rule, endoacetylglucosaminidases cleave *N*-*N*^{1}-diacetylchitobiose moieties of asparagine-linked oligosaccharides of various glycoproteins. They have been designated as endo-D, H, C-I, C-II, and L depending upon the source of their origin and their substrate specificities. It should be borne in mind that the indiscriminate use of these enzymes without regard to their specificity of action and their attendant contaminants may result in erroneous conclusions.

Although the substrate specificities for each of the endoglycosidases have been described in the pertinent literature cited in Table 9, some comments will be made in relation to the available information on this subject. The specificities of endo-H from *Streptomyces plicatus*[220] and endo-CII from *C. perfringens*[222] appear to be similar but not identical. Recently, employing a variety of oligosaccharides as substrates, Trimble et al.[226] have shown that the rates of hydrolysis of the various substrates by endo-H are in the following order of magnitude: $(Man)_4$ $(GlcNAc)_2$ Asn > $(Man)_3$ $(GlcNAc)_2$ Asn > $(Man)_5$ $(GlcNAc)_2$ Asn >>>> $(Man)_3$ $(GlcNAc)_2$ Asn $(aa)_x$ > $(Man)_2$ $(GlcNAc)_2$ Asn >> $(Man)_1$ $(GlcNAc)_2$ Asn. Thus, it can be readily recognized that endo-H hydrolyzes the acetyl chitobiosyl linkages in $(Man)_x$ $(GlcNAc)_2$ Asn, where X = 1 to 5, in varying effectiveness. Furthermore, it has been reported that endo-H can effectively hydrolyze oligosaccharide chains with as many as 50 or more mannosyl residues.[35]

The endo-D from *Diplococcus pneumoniae*[219] and endo-CI from *C. perfringens*[222] show similar substrate specificities. These enzymes effectively cleave the acetylchitobiosyl linkages in $\mathrm{Man} \xrightarrow{\alpha 1,3} (\mathrm{R}_1 \xrightarrow{\alpha 1,6}) \mathrm{Man} \xrightarrow{\beta 1,4} \mathrm{GlcNAc} \xrightarrow{\beta 1,4} (\mathrm{R}_2 \xrightarrow{\alpha 1,6} \mathrm{GlcNAc} \longrightarrow \mathrm{Asn}$, where R_1 = Man or oligosaccharide → Man, and R_2 = H or Fuc.[222,227] The endo-L enzyme from *S. plicatus* effectively hydrolyzes $(Man)_1$-$(GlcNAc)_2$ Asn, but is not very effective against substrates containing more than two mannose residues.[221]

The endo-β-galactosidase has been shown to cleave those galactosyl linkages in

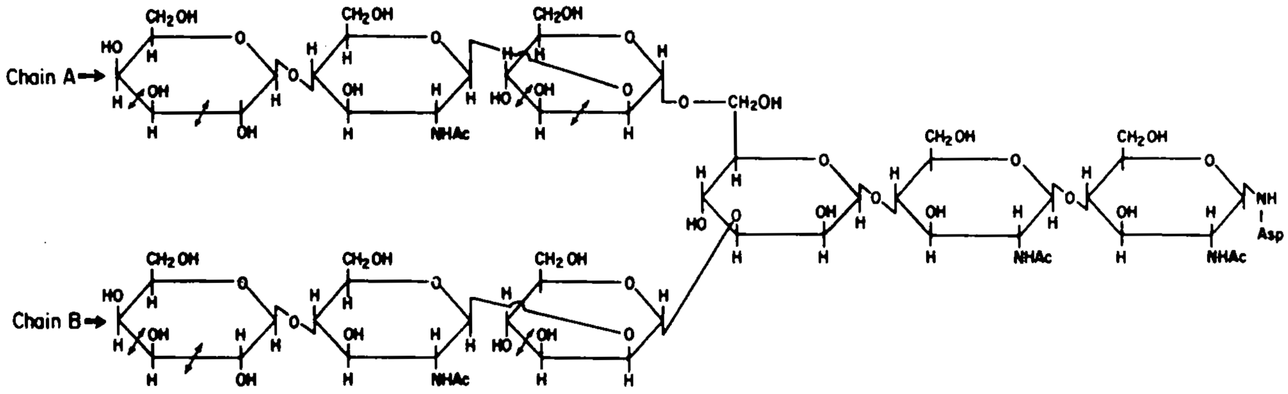


FIGURE 11. The structure of the *N*-glycosidically linked carbohydrate of human chorionic gonadotropin.

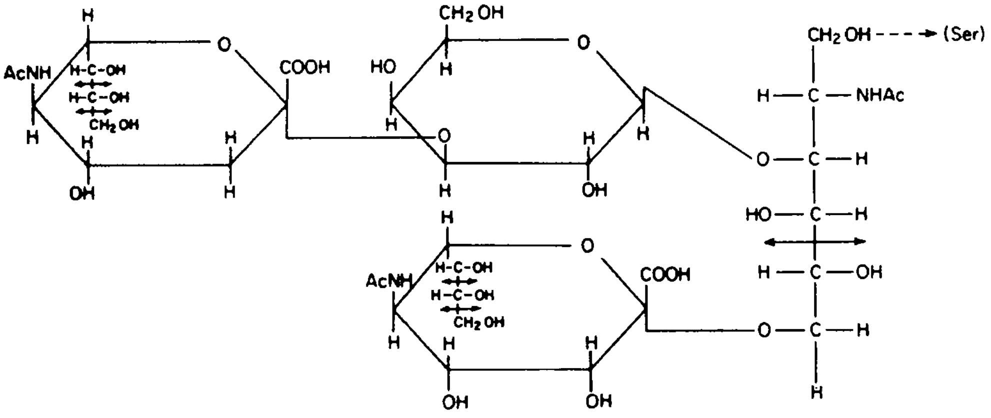


FIGURE 12. The structure of the *O*-glycosidically linked carbohydrate of human chorionic gonadotropin.

Table 9
ENDOGLYCOSIDASES USEFUL IN THE STUDY OF CARBOHYDRATE UNITS OF GLYCOPROTEINS

Enzyme and source	Glycoproteins studied[a]
Endo-β-*N*-acetylglucosaminidases	
Endo-D (*Diplococcus pneumoniae*)[219]	Mouse myeloma IgG, thyroglobulin[219]
Endo-H (*Streptomyces plicatus*)[220]	Ovalbumin, deoxyribonuclease, ribonuclease B, invertase, thyroglobulin, immunoglobulin M, carboxypeptidase Y, mungbean nuclease[221]
Endo-C$_I$ (*Clostridium perfringens*)[222]	Bovine IgG, ovalbumin, thyroglobulin (B unit)[222]
Endo-C$_{II}$ (*Clostridium perfringens*)[222]	Ovalbumin, thyroglobulin (A unit)[222]
Endo-L (*Streptomyces plicatus*)[220]	Ovalbumin[221]
Other endoglycosidases	
Endo-β-galactosidase (*Escherichia freundii*)[223]	Blood group substances, Keratan sulfate[223]
Endo-β-*N*-acetylgalactosaminidase (*Diplococcus pneumoniae*)[224]	Antifreeze glycoprotein, asialo-fetuin, asialo porcine submaxillary mucin, mouse myeloma glycoprotein, human bronchial glycoprotein[224]
Endo-amidase (almond emulsin)[225]	Stem bromelain[225]

[a] Either glycoproteins or their glycopeptide derivatives were used as substrates. Differences in the substrate specificities are described in cited references.

keratan sulfate and blood group substances which are linked by GlcNAc$\xrightarrow{\beta 1,3}$ on the nonreducing end.[223] An endo-α-galactosaminidase from *D. pneumoniae* has been isolated.[224] The unsubstituted galactose and an α-linkage between GalNAc and serine (threonine) are required for this endo-activity. It has been reported that an amidase from almond emulsin has the property of releasing oligosaccharide from stem bromelain glycopeptide by cleaving the glucosaminyl asparagine bond.[225] Although the enzyme has not been purified to any great extent, if well-characterized in the future, it should be extremely useful in the isolation of intact asparagine-linked oligosaccharides.

The isolation, purification, and properties of endo-β-*N*-acetylglucosaminidases have been described.[228] These enzymes have been used for studying the structure of complex carbohydrates of several glycoproteins. Thus, oligosaccharides have been isolated from ovalbumin[88] and fetuin[92] by the use of endo-D and endo-H, respectively. The usefulness of endo-H in the determination of molecular weight of the protein moiety of glycoproteins and their inherent number of oligosaccharide chains has been described. Thus, after the treatment of carboxypeptidase Y with endo-H, the molecular mass of the carbohydrate-free protein was found to be 51,000 daltons. The native enzyme was shown to contain four oligosaccharide chains each averaging 14 mannose residues.[229] Furthermore, the endo-enzymes, due to their differential substrate specificities, have proven to be particularly suitable for determining the size of the oligosaccharide intermediates during the biosynthesis of asparagine-linked carbohydrates.[208,230]

E. Other Methods

1. Hydrazinolysis

The reaction of proteins with hydrazine was initially proposed as a method for the determination of carboxy-terminal amino acids. However, it is only rarely used for the analysis of carboxy-terminal groups. Its potential use in the isolation of carbohydrate units has been recognized. When α_1-acid glycoprotein was treated with anhydrous hydrazine containing hydrazine sulfate as a catalyst, it resulted in the cleavage of peptide, acetamido, and aspartamido linkages leaving the glycosyl bonds intact.[231] This general procedure has been modified by Bayard and Montreuil[232] so that hydrazinolysis of a glycoprotein containing asparagine-linked carbohydrate units gives rise to intact oligosaccharide units with reducing *N*-acetylglucosamine residues. The glycoproteins or glycopeptides containing asparagine-linked carbohydrate are heated with anhydrous hydrazine at 100° C for 30 hr in sealed tubes. The hydrazine is removed by repeated evaporation with toluene. Because the acetamido group of the *N*-acetylhexosamines are susceptible to hydrazinolysis, the primary amino groups are formed which are re-*N*-acetylated using acetic anhydride under alkaline conditions prior to the isolation and purification of the oligosaccharides by conventional techniques. Paper chromatography has been used for oligosaccharides from Cl_q subcomponent of the human complement.[91] The intact oligosaccharides may also be separated from the acetylated amino acid hydrazides by molecular seiving. The linkage sugar, *N*-acetylglucosamine, can be identified as *N*-acetylglucosaminitol by reduction of the oligosaccharide with sodium borohydride followed by hydrolysis.

2. Alkaline Hydrolysis and Deamination

Analogous to hydrazinolysis, isolation of intact *N*-deacetylated oligosaccharides from glycoproteins containing *N*-glycosidic linkage can be effected by alkaline hydrolysis. The inclusion of sodium borohydride during alkaline hydrolysis accelerates the rate of cleavage of GlcNAc → Asn Bond.[233] Lee and Scocca[234] used 1 *N* NaOH containing 1 *M* sodium borohydride at 100° C for 4 to 6 hr for the release of oligosaccharides from glycopeptides of ovalbumin, α-amylase, and bromelain. Under these conditions, isolation of intact oligosaccharides from bromelain glycopeptides was difficult. The authors suggested that the linkage *N*-acetylglucosamine possibly underwent peeling reaction due to a substituent fucosyl residue at C_3. Recently, these conditions have been modified to accelerate the rate of GlcNAc → Asn cleavage and to simultaneously protect the released oligosaccharides by reduction. Thus, treatment of α_1-acid glycoprotein and thyroxine-binding globulin glycopeptides with 1 *N* NaOH containing 4 *M* $NaBH_4$ at 80° C for 24 hr resulted in an increased yield of intact oligosaccharides.[134]

N-Deacetylation is a prerequisite for deamination. Usually, following alkaline hydrolysis of a glycoprotein or glycopeptide, the deamination of the oligosaccharide is carried out with 1% sodium nitrite in sodium acetate buffer for 4 hr at 24° C.[235] Isemura and Schmid[236] have employed this reaction in the structural analysis of the carbohydrate moiety of α_1-acid glycoprotein. When 2-amino-2-deoxy-hexopyranosyl residues are part of the chains, oligosaccharides terminating in 2,5-anhydrohexose residues are formed and can be isolated by chromatography. Thus, because of the specific cleavage reaction, carbohydrates containing 2-amino-2-deoxy-D-gluco- and D-galactopyranosidic linkages upon deamination yield oligosaccharides of the parent compound with 2,5-anhydro-D-mannose and 2,5-anhydro-D-talose, respectively, at the reducing termini. When the sample size is limited, it is preferable to reduce the deaminated product with sodium borotritide so that the anhydro derivative at the reducing end is converted into its corresponding radioactively labeled alcohol. By using this deamination technique, we could account for both anhydro-mannose and -talose derivatives in glycopeptide of ovine luteinizing hormone.[94]

3. *Deglycosylation of Glycoproteins*

Glycoproteins containing a large number of saccharides are generally resistant to proteolysis because in such glycoproteins, the proximity, charge, or size of the carbohydrate units cause hindrance to the action of proteases and prevent the cleavage of peptide chains. Therefore, deglycosylation of a protein should be useful in the determination of amino acid sequence. Recently, a method for deglycosylation of glycoproteins has been described.[237] In this procedure, it has been demonstrated that the cleavages of all the linkages of neutral and acidic sugars — but not of the peptide bonds and the glycopeptide linkages — occur when glycoproteins are exposed to hydrogen fluoride for 1 hr at 0° C. A more severe treatment with anhydrous hydrogen fluoride, i.e., 3 hr at 23° C, results in the cleavage of the *O*-glycosidic linkages of amino sugars, but peptide bonds and GlcNAc → Asn linkages remain intact. Furthermore, since little or no degradation of the sugars occurs during the cleavage of glycosidic bonds by anhydrous hydrogen fluoride, this technique allows the quantitation of sugars contained in the carbohydrate components. It is suggested that this method may be useful in the determination of amino acid sequence and the analysis of carbohydrate components in glycoproteins.

4. *Chromium Trioxide Oxidation*

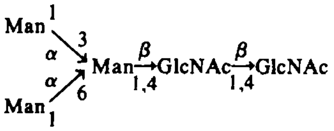
Although anomeric configurations of sugars in glycopeptides and oligosaccharides have been assigned by the use of specific glycosidases, it is possible to deduce the anomeric configurations chemically by oxidation of acetylated carbohydrates with chromium trioxide. The principle of this technique and its use and limitations in the determination of anomeric configurations has been discussed in detail.[163] When a fully acetylated aldopyranoside in which the aglycon is equatorially oriented in the most stable chair conformation (usually the β-anomer) is oxidized with chromium trioxide in acetic acid, it is converted into 5-aldulosonate. On the other hand, the anomer with an axial aglycon (usually the α-anomer) is comparatively less susceptible to oxidation. The oligosaccharides are usually reduced prior to acetylation. A portion of the acetylated material is oxidized with chromium trioxide by sonication for 1 hr at 50° C. The oxidized product and the remaining portion are then analyzed for sugar composition by GLC of the alditol acetates according to the procedure described earlier. Information as to which sugars are oxidized is obtained from a comparison of the two analyses.

VII. BIOSYNTHESIS OF GLYCOPROTEINS

It is clear from the foregoing discussion that asparagine-linked and serine/threonine-linked saccharides constitute the two major types of carbohydrate units of glycoproteins. Collagen and proteoglycans have carbohydrate linked to hydroxy-lysine and serine/threonine, respectively. Recently, considerable progress has been made in our understanding of the mechanisms of assembly of carbohydrates in glycoproteins. This is particularly true about asparagine-linked carbohydrate, in which several exciting advances have taken place. The most important discovery has been the recognition of the role of polyprenol phosphates in the glycosyl transfer reactions. The phosphorylated derivatives of C_{55} polyisoprenoid alcohol (undecaprenol) were initially found to be involved in the biosynthesis of cell wall peptidoglycans (lipopolysaccharides).[238] Although other polyprenols such as solanesyl phosphate (C_{45}) and farnesyl phosphate (C_{15}).and certain lipids like the retinol phosphate and vitamin K are known to be lipophilic glycosyl donors, the most prevalent ones are the phosphorylated derivatives of dolichols. The dolichols are a family of long-chain isoprenyl alcohols containing 16 to 20 isoprene units of which the first unit is saturated. These compounds have been found in animal tissues, and their function appears to be analogous to ficaprenol that is commonly found in bacteria. As a result of the contributions from various laboratories, it is now well established that dolichol monosaccharides are synthesized in mammalian tissues and serve as intermediates in the synthesis of polysaccharides and glycoproteins.[24] The various phosphorylated sugar-dolichols thus far found in eukaryotic cells include mannopyranosyl-β-D-dolichol phosphate, glucopyranosyl-β-D-dolichol phosphate, xylopyranosyl-α-D-dolichol phosphate, *N*-acetylglucosaminyl-β-D-dolichol pyrophosphate. These are formed by the action of GDP-α-D-mannose, UDP-α-D-glucose, UDP-β-D-xylose on dolichol phosphate, respectively, and of UDP-α-D-*N*-acetylglucosamine on dolichol phosphate. Apparently, these reactions are accompanied with the inversion of anomeric configuration. It may be pointed out that sugar nucleotides serve as glycosyl donors directly or via dolichol in the synthesis of glycoproteins. Two types of mechanisms of the assembly of carbohydrates have been commonly found in glycoproteins. One of these involves the preassembly of oligosaccharides on dolichol phosphate before it is transferred *en bloc* to the polypeptide chain. The other mechanism involves the addition of the linkage sugar directly to the polypeptide chain followed by the stepwise addition of the monosaccharide individually from the sugar nucleotides by appropriate glycosyl transferases. Whereas the former mechanism occurs in asparagine-linked carbohydrate, the latter is found in serine/threonine-linked carbohydrates and in the synthesis of collagen disaccharide.

A. Biosynthesis of Asparagine-Linked Carbohydrates

The core of the asparagine-linked carbohydrates generally consists of a pentasaccharide made up of a tri-mannose and an *N*-*N*′-diacetyl chitobiose units. However, a few exceptions have been noted. The immunoglobulin IgE (C-1) has been reported to contain only one *N*-acetylglucosamine residue in the core.[1] In pituitary glycoprotein hormones the distal *N*-acetylglucosamine is replaced by *N*-acetylgalactosamine.[93] The pentasaccharide core, having the structure



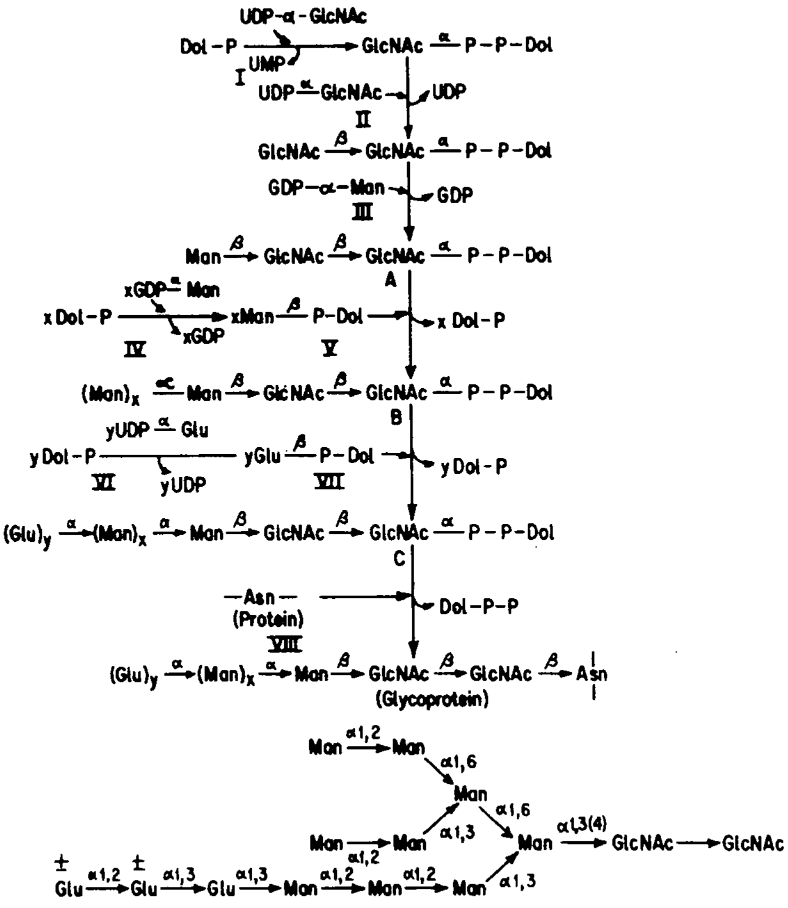


FIGURE 13. Biosynthesis of lipid-linked oligosaccharide for *N*-glycosylation of glycoproteins.

is synthesized as a larger polysaccharide on dolichol phosphate. The biosynthetic intermediate probably consists of 1 to 3 glucose, 9 mannose, and 2 *N*-acetylglucosamine residues, and is transferred as a unit to the nascent polypeptide chain. An outline of the postulated biosynthetic pathway of the assembly of dolichol-linked polysaccharide is shown in Figure 13. This scheme is derived from the results from various laboratories on different tissue sources. Several of the transferases in this lipid pathway have been solubilized and partially purified, such as the dolichol phosphate mannose transferases and the GlcNAc-1-P transferase.[238a] The synthesis of dolichol-linked oligosaccharides is initiated with the formation of *N*-acetylglucosaminyl pyrophosphoryl dolichol by the action of an *N*-acetylglucosaminyl transferase (I). Following the attachment of *N*-acetylglucosamine to dolichol phosphate, one residue each of *N*-acetylglucosamine (II) and mannose (III) is added stepwise. It is postulated that the direct transfer of these sugars from their respective nucleotide derivatives is effected by *N*-acetylglucosaminyl and mannosyl transferases, respectively. Following the formation of the core trisaccharide pyrophosphoryl dolichol, the addition of mannose and glucose residues occurs via their sugar phosphoryldolichol derivatives. The mannosyl phosphoryldolichols (V) formed by the transfer of mannose residues from GDP-mannose to dolichol phosphate (IV) serve as mannosyl donors.

The recent evidence suggests that the β-linked mannose and the next four α-linked mannoses come directly from GDP-mannose rather than from mannosyl phosphoryl dolichol.[238b-d] Similarly, the synthesis of the final product occurs by the addition of one to three glucose residues (VI and VII) by glucosyl transferases. The product is transferred *en bloc,* presumably by an oligosaccharide transferase, to specific asparagine residues of a protein (VIII) resulting in its glycosylation. The final structure shown in Figure 13 has been proposed for the oligosaccharide moiety of lipid-linked oligosaccharide prior to its *en bloc* transfer to vesicular stomatitis virus G protein[239] and placental glycoproteins.[304] Partial structures for the in vitro synthesized lipid-linked oligosaccharide by bovine thyroid slices[240] and porcine intimal layer[241] have been found to be compatible with this proposed structure.

It has been established that the dolichol-bound oligosaccharide serves as the donor of the oligosaccharide moiety to suitable asparagine residues on acceptor proteins. The transferase required for the incorporation of oligosaccharide into the protein has been solubilized and purified and the transfer to asparagine peptides demonstrated.[238e] Moreover, there is no direct evidence that an *N*-glycosidic bond is formed between an acetamido group of asparagine and *N*-acetylglucosamine on the transfer of the oligosaccharide, although such a linkage has been established in glycoproteins by the isolation of *N*-acetylglucosaminyl-asparagine. Earlier studies employing myeloma microsomes and hen oviduct membranes have indicated that the endogenous protein acceptors and their glycosylated products are tightly bound to the membrane.[24] Although these tissues synthesize immunoglobulin (Kappa type light chain) and ovalbumin, respectively, in studies utilizing endogenous acceptors, it has been found that these two secretary glycoproteins are not labeled to any great extent.[242,243] Consequently, it was postulated that the dolichol pathway might be involved predominantly in the synthesis of membrane glycoproteins. It is now known that at least certain secretory proteins undergo glycosylation via the dolichol pathway. One such evidence is provided by the studies on glycosylation of ovalbumin using an antibiotic inhibitor of glycosylation. Tunicamycin blocks the synthesis of *N*-acetylglucosaminylpyrophosphoryl dolichol which is the first crucial step in the synthesis of dolichol pyrophosphate oligosaccharide.[244] It was found that whereas the glycosylation of ovalbumin was inhibited in the presence of tunicamycin, the rate of synthesis of the unglycosylated ovalbumin was not affected by the presence of antibiotic, implying that dolichol pyrophosphate oligosaccharide is an essential intermediate in the glycosylation of ovalbumin. Additional evidence in support of the dolichol pathway for glycosylation of secretory glycoproteins comes from the experiments in which the denatured forms of ovalbumin, α-lactalbumin, and ribonuclease A were found to serve as effective acceptors of the oligosaccharide from dolicholpyrophosphoryl-oligosaccharide.[127] These three proteins contained the necessary tripeptide sequence Asn-X-Ser(Thr) for *N*-glycosylation. More recently it has been shown that a variety of chemically synthesized tripeptides of the sequence Asn-X-Ser and Asn-X-Thr serve as substrates for the oligosaccharide transferase if both the amino- and carboxy-termini are blocked.[245] The glycosylation of asparagine residues in certain proteins apparently is effective when the protein is in an unfolded state. However, several proteins containing the necessary tripeptide sequence upon denaturation have proven to be ineffective carbohydrate acceptors.[127] Furthermore, a protein containing more than one tripeptide sequence in the molecule may be glycosylated at one site, but unglycosylated at other site(s). Although these observations have provided useful information concerning some aspects of the structural requirements for glycosylation — e.g., the necessity of a tripeptide sequence in an unfolded state — the precise requirements for substrate specificity of the transferase are not yet understood.

It is noteworthy that most of the glycoproteins do not contain glucose and only a fewer mannose residues. Therefore, the polysaccharide thus transferred to the polypeptide apparently undergoes some processing before the rest of the chains are completed. Thus, studies with vesicular stomatitis virus[208] and Sindbis virus[230] have revealed that during the assembly of carbohydrate units which are essential components of the envelope glycoproteins of these viruses, glucose and several mannose residues are cleaved off by the processing enzymes. Besides the membrane glycoproteins, oligosaccharide processing also occurs in secretory glycoproteins as has been demonstrated in the biosynthesis of immunoglobulin G by mouse plasmacytoma cell line.[246]

The biosynthetic pathways involved in the processing of the carbohydrate bound to an asparagine residue are summarized in Figure 14. After the transfer of oligosaccharide to the asparagine residue (I), processing apparently occurs rapidly. As a first step the terminal α-1,2 and α-1,3-linked glucose residues are cleaved off by two glucosidases, resulting in a polymannose structure (II). The presence of α-1,2 and α 1,3 glucosidases in rough and smooth microsomal membranes of rat liver has been reported,[247] and it has been shown that these enzymes cleave glucosyl residues from the lipid-linked oligosaccharide synthesized in vitro.[248] The high mannose type oligosaccharide may undergo a limited or extensive processing. In case of limited processing, the polymannose structure may lose one or three mannose residues to yield high mannose type glycopeptides of soybean agglutinin and Taka-amylase A (Figures 2 — C and 2 — A), respectively. It should be noted, however, that the external branching mannose in soybean agglutinin glycopeptide is found to be linked 1,3 instead of 1,6 to the β-linked mannose. The control mechanisms for the limited, as against extensive, processing of the oligosaccharide are unknown. In the synthesis of the complex type oligosaccharide, the polymannose structure is converted into an oligosaccharide intermediate (V) via two different mechanisms. The pathway indicated by solid[249] and dashed[250] lines have been proposed by different investigators from in vitro and in vivo studies, respectively. The core region of most asparagine-linked oligosaccharides (III) is derived from polymannose (II) by the cleavage of six mannose residues. Two *N*-acetyl glucosamine residues are then transferred successively to the two outer mannoses by the action of *N*-acetylglucosamine transferases I and II, respectively. The former transferase has been shown to be specific for the terminal α-1,3-linked mannose to form structure (IV) which serves as a substrate for transferase II. The specificity of this transferase for mannose α-1,6 terminus has not been unequivocally established. However, it has been possible to demonstrate the preferential incorporation of *N*-acetylglucosamine residues into the appropriate glycopeptide derivatives of immunoglobulin G by *N*-acetylglucosaminyl transferase I and II from Chinese hamster ovary cells.[251] Whereas the wild type cells contain both the transferases, the lectin-resistant cells do not contain *N*-acetylglucosaminyl transferase I.

From their studies on the biosynthesis of vesicular stomatitis virus G glycoprotein complex type oligosaccharide, Tabas and Kornfeld[250] have postulated that the four exposed α-1,2-linked mannoses are cleaved following the removal of terminal glucose residues, resulting in an intermediate product (III—A). It was shown that this product accumulated when the virus was grown in the lectin-resistant Chinese hamster ovary cells which are deficient in *N*-acetylglucosaminyl transferase I. However, *N*-acetylglucosamine was specifically transferred to the mannose residue linked α-1,3 to the β-linked mannose of the oligosaccharide (IV—A) when the virus was grown in the wild type Chinese hamster ovary cells which contain both *N*-acetylglucosamine transferases I and II. The authors have proposed that the transformation of IV—A into V is dictated by the transfer of the first *N*-acetylglucosamine residue which signals an α-mannosidase to cleave the two terminal mannose residues, following which *N*-acetylglucosaminyl transferase II adds the second *N*-acetylglucosamine to the α-1,6-linked mannose.[250] In

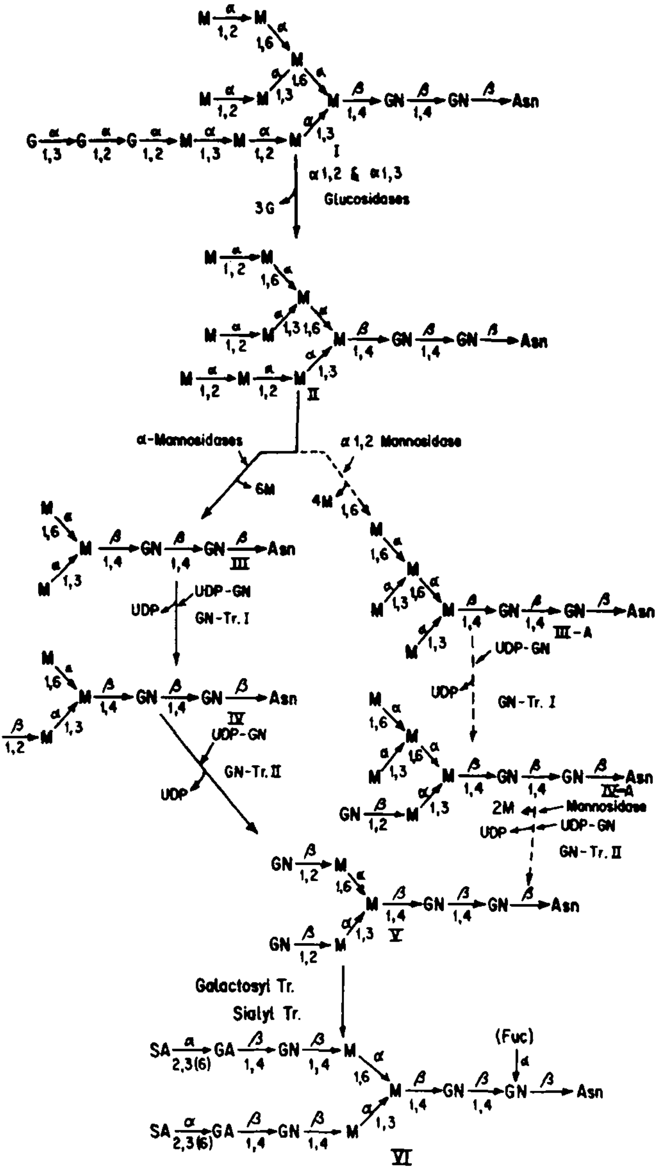


FIGURE 14. The processing of carbohydrate and the assembly of asparagine-linked carbohydrate units of glycoproteins.

vitro studies utilizing rat liver Golgi-rich membranes suggest that an *N*-acetylglucosaminyl transferase I-dependent α-D-mannosidase is involved in the processing of IV—A to V.[252]

It should be noted that while either of these pathways lead to the same end product (V), information on the absolute structural requirements for substrate specificity of *N*-acetylglucosaminyl transferase I has remained ambiguous. This enzyme adds *N*-acetylglucosamine specificially to the mannose residue linked α-1,3 to the β-linked mannose both in vitro (III → IV) and in vivo (III—A → IV—A), but not to the other α-1,3-linked mannose of III—A. *N*-Acetylglucosaminyl transferase I does not appear to discriminate between substrates III and III—A. The specificity of the *N*-acetylglucosaminyl transferase II obviously is more stringent since only structure IV seems to be its preferred substrate. In vivo, the two external mannose residues (IV—A) must be cleaved off preceding the incorporation of the second *N*-acetylglucosamine into the oligosaccharide. It has been shown that α-D-mannosidase acts on IV—A, resulting in the cleavage of only the two outer mannose residues after the addition of *N*-acetylglucosamine residue. However, the relationship between *N*-acetylglucosamine transferase I and the mannose processing enzymes is not understood.

The elongation of the carbohydrate chains proceeds by the addition of *N*-acetylglucosamine, galactose, fucose, and sialic acid by the action of specific transferases which are located in the Golgi apparatus.[253] The formation of intermediates IV by the addition of the first *N*-acetylglucosamine residue to the terminal mannose is critical to the entire elongation process. Fucose incorporation occurs only after the addition of first *N*-acetylglucosamine residue (IV → VI) or after the attachment of both of the residues (V → VI).[249] Further elongation of chains occurs by the addition of galactose followed by the incorporation of sialic acid by galactosyl and sialyl transferases, respectively.

In the complex type *N*-glycosidically linked carbohydrate, galactose seems to be invariably attached to *N*-acetylglucosamine in a β-1,4 linkage (Figure 3). The galactosyltransferase that catalyzes the transfer of galactose in β-1,4 linkage has been isolated from a variety of sources, and the properties of the enzyme have been thoroughly characterized.[249] There is no evidence for the specific addition of a galactose residue to the α-1,6 as against the α-1,3 mannose terminus. However, glycopeptides from bovine immunoglobulin G are found to contain galactose residue linked only to the α-1,6 terminus.[47]

The linkages between sialic acid and galactose have been found to be primarily α-2,3 and α-2,6 in a number of glycoproteins (Figure 3). However, the presenc of sialyl-α-2,4-galactose[254,255] and sialyl-α-2,2-galactose[236] linkages in α_1-acid glycoprotein have also been reported. The occurrence of enzymes catalyzing the synthesis of both sialyl-α-2,3 lactose and sialyl-α-2,6 lactose have been detected in colostrum[256] and mammalian liver.[257] Recently, the α-2,6 sialyl transferase from colostrum has been purified to homogeneity, and its kinetic properties have been described.[258,259] Apparently, the final step in the elongation process—i.e., the incorporation of sialic acid residues in α-2,3 and α-2,6 linkages — is catalyzed by these enzymes. The transferases which incorporate sialic acid into the oligosaccharide of asparagine carbohydrate in linkages other than α2,3 and α2,6 have not been reported.

It is generally recognized that the translation of peptide moieties occurs on membrane-bound ribosomes,[260] and it has been proposed that the nascent peptide carries a "signal sequence" at its amino terminal end which facilitates the binding of ribosomes to a receptor site on the endoplasmic raticulum and the creation of a channel for the passage of the nascent peptide into the intravesicular space of the endoplasmic reticulum.[261,262] Considerable interest has been centered upon localization of subcellular sites for the initiation of glycosyl transfer to asparagine and the processing of the bound oligosaccharide. The findings of Kiely et al.[128] indicate that glucosamine and mannose are present on the nascent ovalbumin peptide bound to the ribosome, suggesting that the *en bloc* transfer of the oligosaccharide (from dolichol intermediate) to asparagine occurs when the nascent peptide is still attached to the ribosomes. Whether the attachment of the oligosaccharide to asparagine occurs only at the nascent peptide stage or also after the release of peptide from ribosomes is not firmly established. Rothman and Lodish[263] have suggested that the transfer of core oligosaccharide is affected at the nascent peptide stage during the synthesis of vesicular stomatitis virus envelope glycoprotein, and the process of penetration of the nascent peptide into endoplasmic reticulum is coupled to glycosylation.

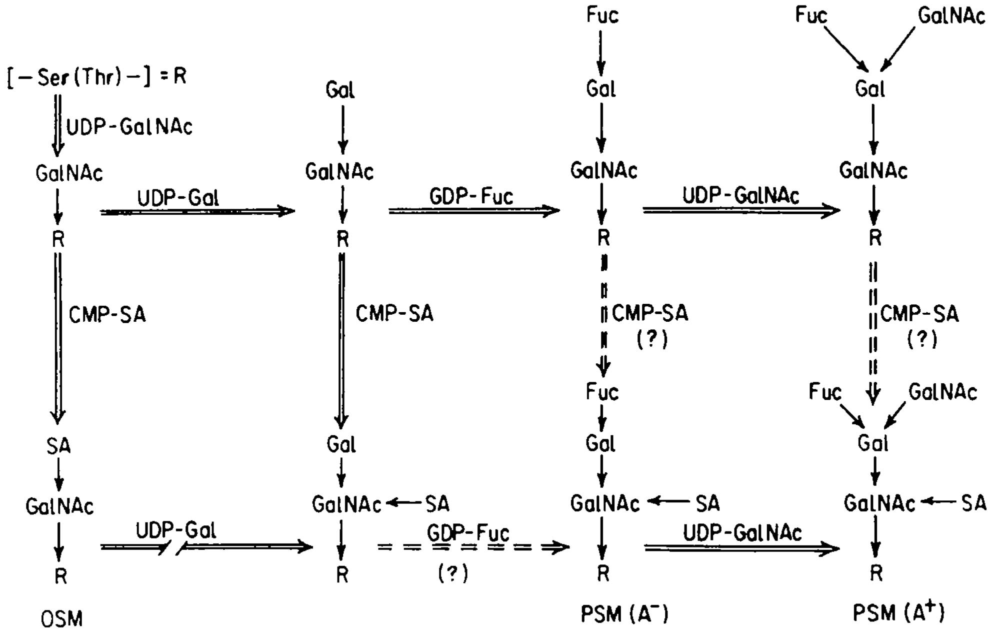


FIGURE 15. The biosynthesis of *O*-glycosidically linked carbohydrate units of submaxillary glycoproteins.

B. Biosynthesis of *O*-Glycosidically Linked Carbohydrate

A number of studies have provided information concerning the biosynthesis of *O*-glycosidically linked carbohydrate units of glycoproteins. Of the various types of such units (Figures 6 to 8), synthesis of four types — serine (threonine). *N*-acetylgalactosamine type, serine-xylose type, hydroxylysine-galactose type, and serine (threonine)-mannose type of oligosaccharides — has been most extensively studied. Except for the synthesis of the last type of oligosaccharide, there is no evidence for the involvement of a preassembled lipid intermediate for glycosylation. The initial incorporation of the linkage sugar as well as the elongation of the oligosaccharide chains of the other three types of carbohydrate units apparently result from stepwise addition of sugar residues directly from their nucleotide derivatives.

Our knowledge about the overall biosynthetic pathway of serine (threonine)-*N*-acetylgalactosamine type oligosaccharides is due to studies on secretory glycoproteins (mucins) of salivary glands and of mucous glands of respiratory, GI, and genitourinal tracts. Many mucous glycoproteins obtained from the mucous glands of different species (rat, dog, pig, and primates) show human blood group activities. Excellent reviews on the synthesis of blood group substances[264] and of mucins[253] have appeared. Since ovine and porcine submaxillary mucins have been well characterized[14] and the assembly of their major oligosaccharides by glycosyltransferases has been elucidated,[253] the biosynthetic pathway for the carbohydrate of these mucous glycoproteins (Figure 15) will be briefly discussed as a typical example of the assembly of carbohydrate chains in mucins.

Porcine submaxillary glands contain at least five glycosyl transferases[253] which are involved in the synthesis of oligosaccharide side chains[59,102] of mucin. The first step in glycosylation of the peptide backbone is the attachment of *N*-acetylgalactosamine to serine (threonine) residues by *N*-acetylgalactosaminyl transferase.[265] Following this initial glycosylation, the fate of the oligosaccharide is dictated by the attachment of either sialic acid or galactose to the *N*-acetylgalactosamine residue (Figure 15). If sialic acid is incorporated before galactose, the synthesis of the oligosaccharide stops. Consequently, the product is a disaccharide which is the major component of ovine submaxillary mucin.[62] On the other hand, if galactose is transferred before sialic acid, synthesis proceeds toward the formation of major oligosaccharides of porcine submaxillary mucin. Although sialyl- and galactosyltransferases for these reactions are present in both the ovine and porcine submaxillary glands, relatively little galactosyl transferase activity is present in the ovine glands. As such, the oligosaccharide terminating with sialic acid is preponderant in ovine submaxillary mucin.

After the incorporation of galactose other sugars can be added; thus, fucose and sialic acid can be attached to galactose and linkage *N*-acetylgalactosamine residues, respectively. The final step in the synthesis of porcine submaxillary mucin is the addition of the terminal *N*-acetylgalactosamine residue which confers blood group A^+ activity.

The specificities of the various transferases required in the synthesis of mucin have been reviewed.[253] The porcine A blood group-specific *N*-acetylgalactosaminyl transferase has been purified from porcine submaxillary glands, and its kinetic properties have been described in detail.[266,267] More recently two different sialyltransferases have been purified from the same tissue. One of these is the β-D-galactoside $\alpha \rightarrow 3$ sialyltransferase which transfers sialic acid from CMP-*N* acetylneuraminic acid and forms only α-2,3 linkages with asialoglycoproteins containing the sequence Gal $\xrightarrow[1,3]{\beta}$ GalNAc$\xrightarrow{\alpha}$Thr(Ser).[268] The other enzyme is the α-*N*-acetylgalactosaminide α-2,6 sialyltransferase which catalyzes the transfer of sialic acid from the donor nucleotide to acceptor substrates containing the structure R $\rightarrow$ 3 GalNAc$\xrightarrow{\alpha}$Thr(Ser), where R is H or a β-galactosyl residue.[269]

The carbohydrate chain or chordroitin sulfate, dermatan sulfate, and heparin are assembled by the stepwise addition of sugars.[270,271] It has been established that the sequence of sugars in the linkage region of proteoglycans is 3-*O*-β-D-glucuronosyl-3-*O*-β-D-galactosyl-4-*O*-β-D-galactosyl-D-xylosyl serine (Figure 8—a). The glycosylation of the proteins is initiated by the attachment of xylose from UDP-xylose to serine residues by a xylosyl transferase. Two galactose residues of β-1,4 and β-1,3 linkages are sequentially added by two different galactosyltransferases. The elongation of the chains proceeds by the addition of alternating hexosamine and hexuronic acid residues. However, the transfer of the glucuronic acid residue linked β-1,3 to galactose is catalyzed by a glucuronosyl transferase distinct from hexuronosyl transferases involved in the formation of the remainder of the polysaccharide chain. Thus, even in dermatan sulfate where L-iduronic acid is the major uronic acid of the repeating disaccharide, the uronic acid residue linked to galactose is glucuronic acid, indicating its special position in the molecule.

The assembly of carbohydrate prosthetic group of collagens and glomerular basement membrane is mediated by the direct transfer of galactose and glucose by two enzymes.[18,272] The first enzyme is a galactosyl transferase which attaches galactose to the unsubstituted hydroxylysine residues of either native collagen or glomerular basement membrane freed of oligosaccharide by periodate oxidation and mild acid hydrolysis. The second enzyme glucosyltransferase is specific for the transfer of glucose to galactosylhydroxylysyl derivatives.

In contrast to the biosynthesis of carbohydrate moieties of mucins, proteoglycans, and collagens, the synthesis of *O*-glycosidically linked carbohydrate of yeast cell wall mannan involves the participation of a lipid-linked intermediate in the initial glycosylation of the protein. Thus, mannosylphosphoryldolichol serves as the donor of the first mannose residue to be attached to serine or threonine.[273-275] Interestingly, it is only this first mannose residue which is transferred via the dolichol route. Subsequent elongation of the chain occurs by the addition of mannose from GDP-mannose. The cell wall mannan of the yeast *S accharomyces cerevisiae* contains both *N*- and O-glycosidically linked carbohydrate units. More recently it has been shown that the initial glycosylation of both asparagine and serine (threonine) residues involves the dolichol-linked intermediates.[276]

VIII. THE ROLE OF CARBOHYDRATE IN BIOLOGICAL FUNCTION

Although in recent years the complete structures of the carbohydrate units of a number of glycoproteins of well-defined functions have been elucidated in detail, much remains to be known concerning the role of carbohydrate in the biological function of glycoproteins.

It has been firmly established that the information contained in the heteropolysaccharide structure of blood-group substances (Figure 7) dictates the serological specificity of these antigens by virtue of specific sugars linked to a common core. Analogous to the heteropolysaccharide structure for the blood-group substances, it is conceivable that carbohydrate units linked to asparagine (Figures 2 and 3) and those linked to serine (threonine) (Figures 6 and 8) may contain specific information relative to a given function. In these structures the attachment of sugar residues (or oligosaccharide chains) to a common core is generally specific; therefore, they may be the determinants for specific function. As an example, human chorionic gonadotropin contains only asparagine-linked oligosaccharide in the α-subunit and both the asparagine- and serine-linked oligosaccharides in the β-subunit. The hormonal activity is expressed only when the two subunits are associated with each other. However, in vitro and in vivo studies indicate that the removal of the carbohydrate from hormone results in the impairment of biological (but not immunological) activity. This indicates that major immunological determinants are not associated with the carbohydrate, but the latter has a role in the biological function. On the other hand, it has been shown that the catalytic activity of certain enzymes is not associated with the carbohydrate. For instance, bovine[277] and ovine[278] ribonucleases A are devoid of carbohydrate, yet show the same specific activity as their glycosylated analogue, ribonuclease B.

A good deal of research interest is centered upon the role of certain sugars in the clearance and survival of circulating plasma glycoproteins. It is now well established that the nature of exposed or terminal sugar residues of the carbohydrate units determine the viability of many native and modified glycoproteins in the plasma. The concept that sugars serve as determinants for specific recognition and clearance in vivo was first advanced by Ashwell and Morell[279] on the basis that certain desialized glycoproteins with terminal galactose residues were rapidly removed from the circulation and taken up by the liver, where they were eventually degraded by the lysosomes. Furthermore, it was shown that the presence of intact sialic acid residues on the receptor sites of the hepatic plasma membrane was required for the initial binding of the asialoglycoproteins prior to their transport into the cell and subsequent lysosomal catabolism. These authors have characterized such a hepatocyte recognition system that mediates the clearance of glycoproteins with terminal galactose residues. It was subsequently shown[280] that the receptor described by Ashwell and Morell[279] also recognizes a glucose analogue covalently bound to bovine serum albumin. As such, this system has been termed the galactosyl/glucosyl recognition system confined to the parenchymal cells of the liver. A number of investigators have later shown that the concept of the recognition system can be extended to include the clearance of glycoproteins with terminal GlcNAc and mannose residues.[281-284] It has been demonstrated[285] that glycoproteins with terminal mannose and GlcNAc appear to compete for the same receptor site in vivo. Moreover, it was observed from the in vitro experiments employing alveolar macrophages that glycoproteins or glycoconjugates with glucose, mannose, and GlcNAc at the nonreducing termini competed in a reciprocal manner in their ability to bind to isolated cells.[286] This system — referred to as the mannosyl/glucosyl recognition system — is located in the nonparachymal cells of the liver and has been shown to be associated with the clearance of lysosomal enzymes in circulation.[287]

From the foregoing discussion it appears that the survival of glycoproteins containing the complex type of carbohydrate units (Figure 3) rests in the information contained in the saccharides. Whether the in vivo catabolism of such glycoproteins is regulated by the glycosidases present in the circulation in conjunction with specific recognition sites for the saccharides on the liver plasma membrane is not understood. The precise mechanism of the regulation of glycoprotein catabolism should be forthcoming when the different recognition systems are well characterized. It should be noted that removal of sialic acid from transferrin does not enhance the clearance of this glycoprotein from the circulation, indicating that some alternate pathways of catabolism of glycoproteins may be present.

It has been suggested that a novel receptor on fibroblasts that recognizes hexose phosphate is involved in the uptake of lysosomal glycoproteins β-glucurosidase, β-hexosaminidase, and β-galactosidase.[288,289] Mammalian glycoproteins rarely contain phosphorylated sugars. However, the presence of terminal D-glucose-6-phosphate residues in some forms of hemoglobulin has been reported.[290] In addition, phosphomannosyl residues have been found in rat brain glycoproteins.[291] Hieber et al.[292] and Distler et al.[293] have shown that mannose residues of testicular β-galactosidase are phosphorylated at *O*-6 position and regulate the uptake of this enzyme by fibroblasts.

In recent years considerable attention has been focused upon the relationship of the carbohydrate of biologically active glycoproteins such as glycoenzymes and glycohormones to the specific functions which they perform. Since hCG is a well-characterized glycohormone, the role of its carbohydrate in the immunological properties and mechanism of hormone action was investigated in this laboratory.[294-295] Both the subunits are essential for the full immunological activity of the hormone since the individual subunits show only a small percentage of the activity of hCG. The hormone binds to the plasma membranes with an association constant of 10^{10} M^{-1}.[296] It is capable of stimulating steroidogenesis and cyclic AMP formation in rat testicular tissues.[297,298]

In order to study the role of carbohydrate in immunological activity (binding to plasma membrane receptors, steroidogenesis, and cyclic AMP formation), hCG was sequentially degraded by specific exoglycosidases such as neuraminidase *(Vibrio cholerae)*, β-D-galactosidase *(Aspergillus niger)*, β-D-*N*-acetylglucosaminidase *(A. niger)*, and α-D-mannosidase *(A. niger)*. The amounts of the sugars released from hCG on exhaustive digestion with the above enzymes were 100% of sialic acid; 60% of galactose; 55% of *N*-acetylglucosamine; and 20% of mannose, respectively. The resulting derivatives — i.e., asialo- (N-hCG), asialo-agalacto- (NG-hCG), asialo-agalacto-aglucosamino- (NGA-hCG), and asialo-agalacto-aglucosamino-ammano-hCG (NGAM-hCG) — were used for the evaluation of the contribution of the carbohydrate in biological activities.

A. Role of Carbohydrate in the Immunological Activity of hCG

The effect of removal of specific sugars from hCG on the immunological activity of the hormone was determined by radioimmunoassay (Figure 16). The removal of all of the sialic acid did not cause any significant loss in the immunological activity. Subsequent cleavage of 60% galactose residues, however, resulted in a slight decrease in the activity. In fact, very little change in immunological activity is effected due to the cleavage of each of the monosaccharides. It is likely that the minimal loss of immunological activity that is

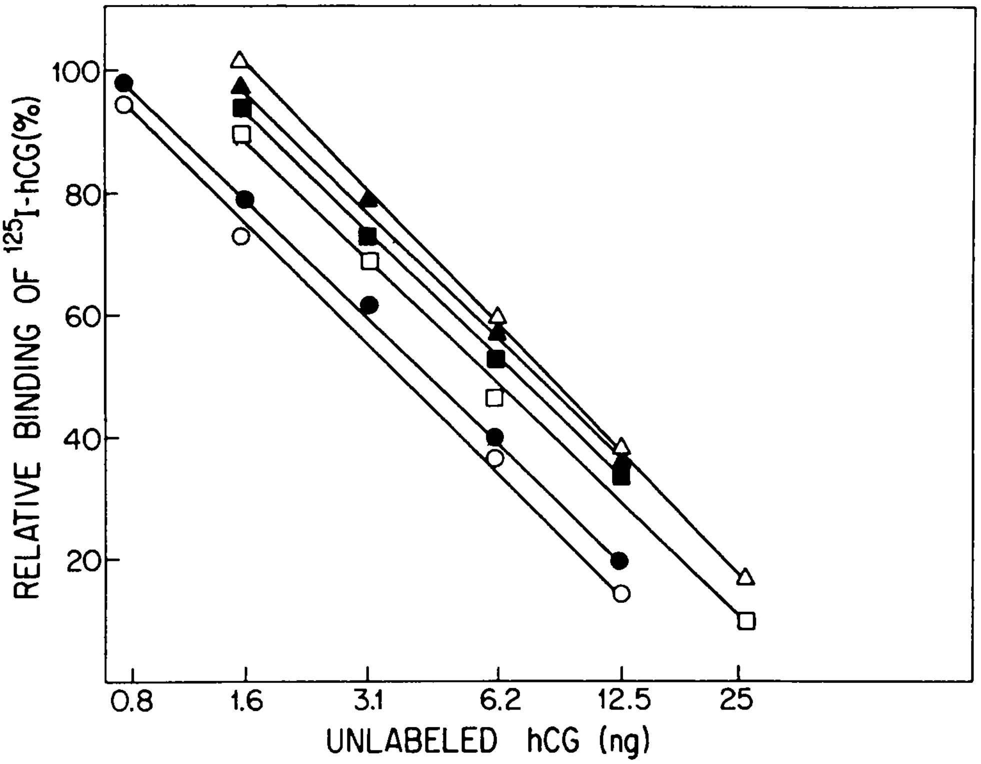


FIGURE 16. Radioimmunoassay of hCG and its derivatives. ○—○, hCG; ●—●, N-hCG; △—△, NG-hCG; □—□, NGA-hCG; ▲—▲, NGAM-hCG-1 and ■—■, NGAM-hCG-2. (Adapted from *Fed. Proc.*, 36, 2122, 1977. With permission.)

observed after the removal of sugars may be due to the conformational change introduced in the molecule as a result of depletion of the carbohydrate, since a sample of NGAM-hCG when stored at—10°C for several weeks resulted in a considerable loss of immunological activity, and when freshly prepared the same derivative was invariably found to be active. Therefore, it seems that the antigenic determinants are not located in the carbohydrate part of the molecule. The role of the fucose residues in the antigenicity has not been evaluated thus far.

B. Binding of hCG to Plasma Membrane Receptors

It has been shown that the plasma membranes of testes and ovaries in several animal species contain specific binding sites for hCG.[296,299,300] The binding is characterized by a high degree of affinity and hormonal specificity, which suggests that the binding sites (receptors) must have highly specific chemical structures. Although the precise chemical nature of the receptors has not yet been elucidated, the binding activity or affinity of the various derivatives of hCG obtained by specific modifications of the protein and the carbohydrate parts of the molecule was evaluated. The method consisted of studying the inhibition of binding of ^{125}I-hCG by the modified forms of hCG to the 2000 × g fraction of the rat testes homogenate.

The individual subunits of hCG have approximately 1% of the binding activity of the intact molecule on equimolar basis. It has been postulated that the biologically active sites of the molecule are derived from both subunits or, if the active sites do reside in the

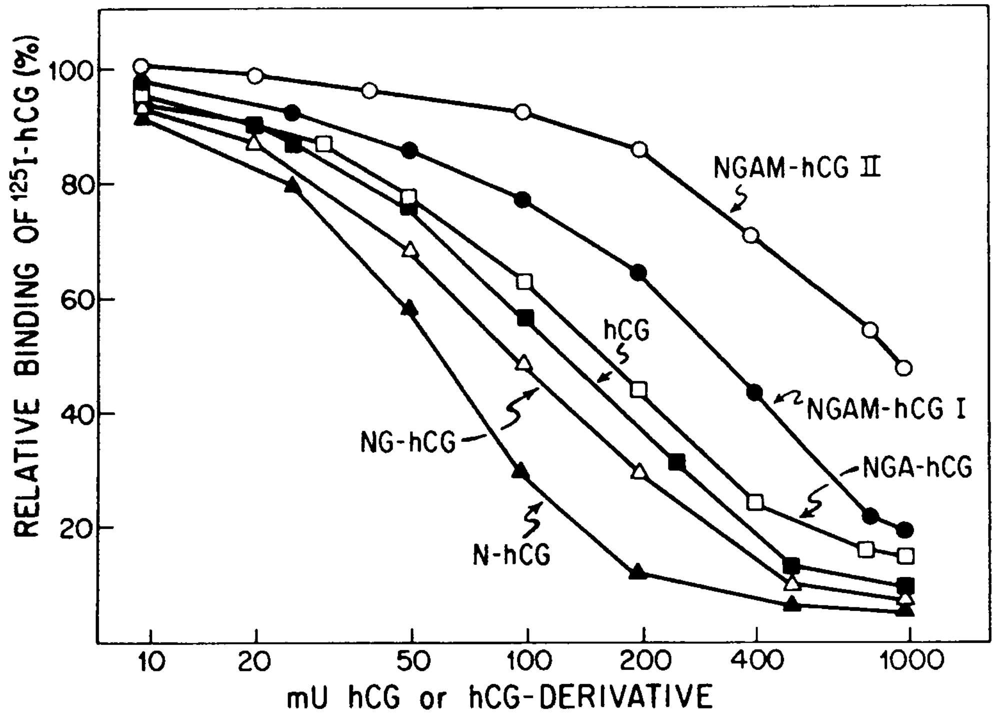


FIGURE 17. Inhibition of binding of ^{125}I-hCG to the 2000 × g pellet of rat testes homogenate by hCG and its derivatives. (Adapted from *Fed. Proc.*, 36, 2122, 1977. With permission.)

individual subunits, they are probably lost due to a drastic conformational change when the subunits are separated from each other. Employing a variety of hCG derivatives in which the protein part of the molecule was modified, it has been found that both the subunits of the hormone are required for the binding activity. However, it is possible to remove some portions of the molecule without loss in the binding activity.[304]

The role of carbohydrate of hCG in the binding to plasma membranes of rat testes (Figure 17) was examined by testing the ability of the modified forms of the hormone to compete for binding to the membrane receptors. The removal of all the sialic acid resulted in a twofold increase in the binding to the plasma membranes, as evidenced by the decreased amount of desialized hCG required to displace 50% of the ^{125}I-hCG from the membranes. In general, glycoproteins, show microheterogeneity in the carbohydrate chains, particularly due to the variation in the sialic acid content. In view of the enhanced binding of the desialyzed hCG to the membranes, it was of interest to find out if any part of the total binding of the intact hCG to the membranes was due to the cell surface sialyltransferases. When labeled intact hCG and desialyzed α_1-acid glycoprotein and fetüin were used in the competition studies there was no effect on binding, indicating that all of the binding was to the specific hCG-receptors on the membranes. The nucleotides CMP, UDP, and GDP did not affect binding of the labeled hCG to the membranes, which again suggested that transferases were not involved. The asialoagalacto-hCG (NG-hCG) showed 40% higher binding that the native hCG. NGA-hCG had 85% binding activity of the hormone. The cleavage of 20% mannose residues for NGAM-hCG (NGAM-hCG-1) resulted in an 85% decrease in the binding activity, as compared to the native molecule. The loss in the activity due to the removal of mannose was dependent on the amount of mannose removed. Thus, while the cleavage of 20% mannose residues yielded a preparation (NGAM-hCG-1) that had only 15% of the activity of hCG, the hydrolysis of 14% mannose residues resulted in a preparation, NGAM-hCG-2, that was 40% as active as the native molecule (Figure 17). Another preparation, NGAM-hCG-3, which was obtained by the sequential degradation of hCG with the glycosidases and had 17% of mannosyl residues hydrolyzed, exhibited 28% of the original binding activity. These observations indicate that the three monosaccharide residues from the nonreducing terminus of the carbohydrate chains are not involved in the binding of the hormone to the receptor. In fact, the removal of sialic acid resulted in an increase in binding. However, the hydrolysis of the mannosyl residues does affect the binding which may be possibly due to a conformational change in hCG induced by the hydrolysis of the carbohydrate. It is also conceivable that the hormone receptor may have a specific site for the mannose residues. As stated earlier, α_1-acid glycoprotein and fetuin, which have carbohydrate structures similar to those present in hCG, failed to inhibit the binding of ^{125}I-labeled hCG to plasma membranes. The data accrued thus far indicates that the carbohydrate has little or no role in the binding. Consequently, it is the polypeptide moiety that appears to play a predominant role in the binding of hCG to the receptor. This is further evidenced by the fact that the receptor binding activity of hCG is destroyed on hydrolysis with trypsin.

C. The Role of Carbohydrate of hCG in the Stimulation of Cyclic-AMP Accumulation and Steroidogenesis

The effect of removal of the carbohydrate on cAMP accumulation and steroidogenesis was studied by using a suspension of rat Leydig cells.[301] Cyclic AMP was measured by protein binding assay, and testosterone was estimated by radioimmunoassay.

When the glycosidase-treated hCG derivatives were examined for their activity to stimulate cAMP accumulation (Figure 18), it was found that none of them was as effective as hCG. In most experiments only the *N*-hCG produced significant cAMP accumulation. The remainder rarely elicited more than 10% of the response seen after treatment with larger concentrations of hCG. From other experiments it was found that the addition of glycosidase-treated derivatives to hCG led to a striking inhibition of cAMP accumulation. The inhibitory effect was ameliorated by increasing the concentration of hCG relative to that of the derivative, suggesting that the inhibition was partly competitive.

The observation that the derivatives were potent inhibitors of hCG-induced cAMP accumulation indicated that removal of the carbohydrate did not interfere with the affinity of the hormone for the cell. It seemed likely that glycosidase treatment interfered with the ability of the hormone to stimulate adenyl cyclase. To test this hypothesis, incubation of the Leydig cells with hCG and its derivatives was carried out in the presence of theophylline, a well-known inhibitor of phosphodiesterase. In the presence of 30 mM theophylline, all of the derivatives stimulated cAMP accumulation, although none were as effective as hCG itself. Sequential removal of the sugar residues led to a progressive loss in the ability of the hormone to stimulate cAMP accumulation.

hCG stimulated the accumulation of cAMP (Figure 18), as well as the synthesis of testosterone (Figure 19). The concentration of the hormone required for the former was considerably greater than that for the latter. Increase in cAMP levels at low concentrations of hCG that stimulated steroidogenesis was not detectable. All of the glycosidase-treated hCG derivatives were found to stimulate steroidogenesis (Figure 19). Sequential removal of sialic acid, galactose, *N*-acetylglucosamine, and mannose led to a progressive loss in the potency of the hormone; however, even NGAM-hCG was capable of eliciting the same maximal steroidogenic response as hCG.

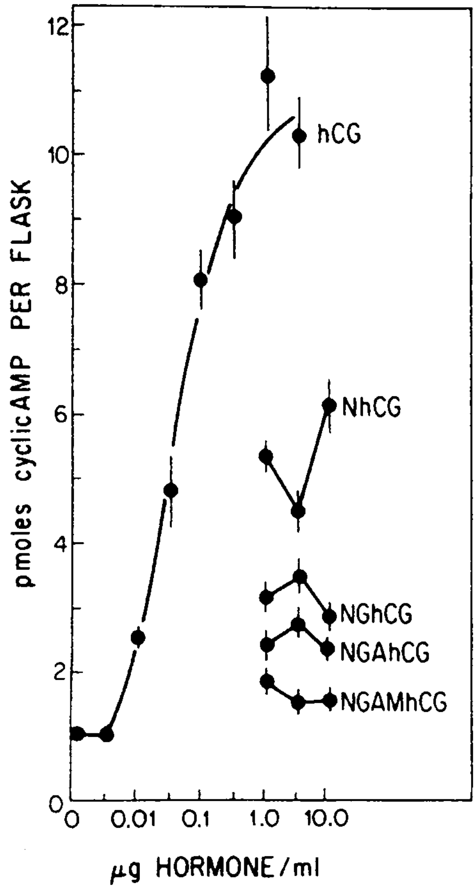


FIGURE 18. Cyclic AMP production in response to hCG and its derivatives. (Adapted from *Fed. Proc.*, 36, 2123, 1977. With permission.)

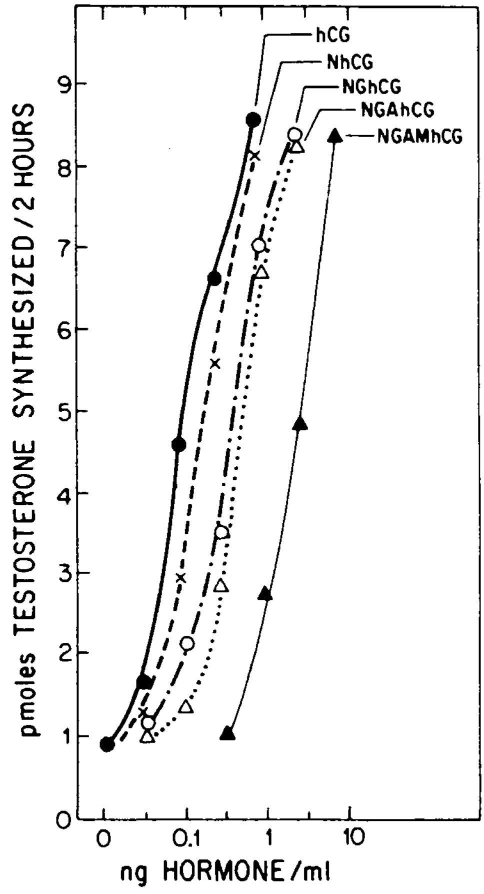


FIGURE 19. Testosterone production in response to hCG and its derivatives. (Adapted from *Fed. Proc.*, 36, 2124, 1977. With permission.)

Since in males hCG stimulates the interstitial cells of the testes to secrete androgens and in females it stimulates the interstitial cells of the preformed follicle converting it into a corpus luteum, experiments were conducted to evaluate the role of carbohydrate of hCG in binding and stimulation of cAMP accumulation and stimulation of progesterone secretion by porcine[302] and monkey granulosa cells.[303] These studies indicated that N-hCG, NG-hCG, NGA-hCG, and NGAM-hCG inhibited the native hCG stimulation of progesterone secretion (Figure 20). The observations support the idea that these hCG derivatives can act as competitive inhibitors at the level of the receptor, with reflections of inhibition at the cAMP levels as well as at the level of progesterone synthesis.

IX. CONCLUDING REMARKS

Considerable progress has been made in our understanding of the chemical structure, biosynthesis, cellular uptake, and degradation of glycoproteins. In fact, the number of carbohydrate structures in glycoproteins elucidated recently has grown exponentially. This growth in structural analyses is due to the refinements in methology, including

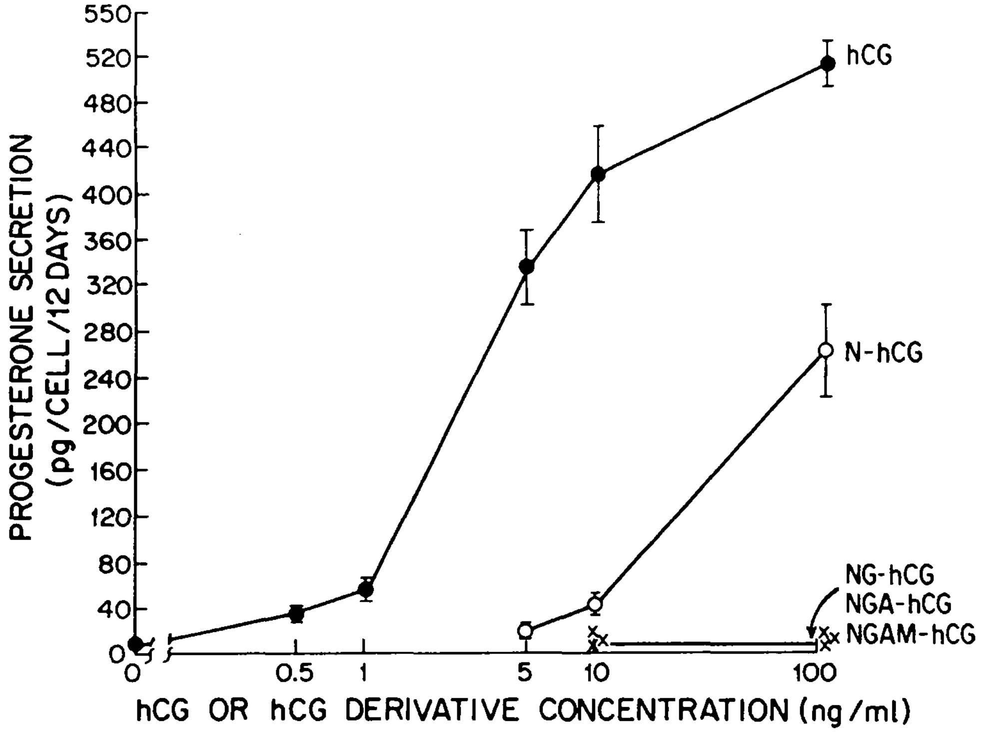


FIGURE 20. Effect of hCG and hCG derivatives upon progesterone secretion by cultures of granulosa cells harvested from two pregnant-mare-serum-gonadotropin-treated monkeys. (Adapted from *Biol. Reprod.,* 17, 710, 1978. With permission.)

improvements in chemical procedures, developments in combined gas chromatography-mas spectrometric techniques, and the availability of a battery of endo- and exoglycosidases. Some of the structures proposed earlier (those of α_1-acid glycoprotein and fetuin) have to be revised on reexamination with the newer methodology. Obviously, some of the other structures which are not consistent with the developing structural patterns have to be reevaluated. Nevertheless, from the available information on the carbohydrate structures of glycopeptides derived from a number of glycoproteins from various sources, a finite number of common patterns of structures have emerged. Barring a few exceptions, as a rule the structure of the core region of asparaginyl glycoproteins has been found to be $\text{Man}\xrightarrow{\alpha 1,6}(\text{Man}\xrightarrow{\alpha 1,3})\text{Man}\xrightarrow{\beta 1,4}\text{GlcNAc}\xrightarrow{\beta 1,4}\text{GlcNAc}$. Like the core, similarities in locations and sequences of the peripheral sugars such as GlcNAc, galactose, fucose, and sialic acid have been observed in a variety of complex carbohydrates. Despite apparent structural similarities so far observed in glycoproteins, one should not rule out the possibility of new structures. For example, recently it has been found for the first time the presence of X-GalNAc attached to α-1,6-linked mannose branch by 1,2 linkage in ovine and bovine LH[94] where the identity of X is yet to be established.

While a great deal is known about the complex type carbohydrate structures, some of the fine structural details are yet to be explored. For instance, in most of the structures there is no unequivocal proof for the identity of the mannose residue to which a specific outer branch is attached. This is particularly true when the structures contain two or more nonidentical outer branches. Such information cannot be derived in some cases by the conventional methods. The use of NMR has been made to elucidate these fine details of the structures. Although a large number of exoglycosidases are available, some of them still lack the essential substrate specificity needed for structure and function studies. For instance, all the α-mannosidases and α-L-fucosidases so far known (with the exception of *A. niger* α-mannosidase and 1,2-α-L-fucosidase from *A. niger* and *C. perfringens)* are capable of preferentially hydrolyzing glycopeptides rather than glycoproteins. Similarly, no endomannosidase which can hydrolyze glycoproteins has been reported thus far. Finally, our knowledge of the conformation of the carbohydrate structures at present is in a state of infancy. Therefore, one would expect an increased activity in this area if one were to understand the function of the carbohydrate in glycoproteins at a cellular and molecular level.

The biosynthesis of the core region of asparaginyl glycopeptides involves the participation of oligosaccharide-lipid intermediates. The sequence of events during biosynthesis is a multiphase process. Instead of stepwise addition of sugars to specific peptidyl asparagine residues, the *N*-glycosylation is affected by the *en bloc* transfer of the oligosaccharide from independently formed oligosaccharide-lipid intermediate. Such intermediate, generally in the form of $(Glu)_{1-3}$-$(Man)_{8-10}$-$(GlcNAc)_2$-P-P-Dolichol, is synthesized by the microsomal enzymes. Its presence has been well documented by chemical characterization. It remains to be seen whether a unique or several dolichol-linked intermediates with varying carbohydrate structures are involved in the synthesis of all asparagine-linked carbohydrates.

The incorporation of terminal glucose(s) into the oligosaccharide-lipid intermediate appears to be an essential factor in the transfer of the oligosaccharide to the protein. It may well be that the terminal glucose(s) serves as a signal for the termination of the synthesis of dolichol-linked oligosaccharide chain and for the enzyme(s) affecting its transfer to the asparagine residue. Thus, glucosylation of the oligosaccharide may be a critical step in the regulation and control of glycoprotein biosynthesis. Much remains to be known concerning the biosynthesis of oligosaccharide-lipid intermediate. It is known that other lipid carriers such as retinol and vitamin K serve as acceptors of monosaccharide residues. The role of these lipid carriers in glycoprotein biosynthesis is yet to be established.

The requirements for the glycosylation of specific asparagine residues within the polypeptide chain are poorly understood. It is known from the complete structures of several glycoproteins and of *N*-glycosidic glycopeptides that the sequence of amino acids in the vicinity of glycosylated asparagine residue is -Asn-X-Ser(Thr), where X is the variable amino acid. The in vitro studies indicate that denatured proteins and a heptapeptide fragment from α-lactalbumin containing this tripeptide sequence, as well as a variety of tripeptides of the type Asn-X-Ser(Thr) in which the amino and carboxy termini are blocked, serve as substrates for the oligosaccharide transferase. Since, in vivo all the asparagine residues with similar tripeptide sequences within the polypeptide chains of glycoproteins are not glycosylated, it can only be inferred that in addition to the tripeptide sequence other factors must be involved in the glycosylation process. The topology of the tripeptide sequence in relation to the overall tertiary structure of the protein and the conformational changes of the molecule as influenced by its microenvironment may be instrinsic factors governing the specificity for glycosylation.

Another aspect in the biosynthetic process is the fate of the asparagine-linked oligosaccharide that is transferred from the oligosaccharide-lipid intermediate to a protein. Recent developments in this area clearly indicate that following transfer the oligosaccharide is processed by the action of glycosidases and glycosyltransferases. During processing the terminal glucose residues are cleaved, resulting in a polymannose intermediate. At least in the case of Sindbis virus G glycoprotein biosynthesis, the earlier stage of processing involves the cleavage of glucose and all mannose residues with $\alpha 1 \rightarrow 2$ linkages, leaving a $(Man)_5$-$(GlcNAc)_2$ oligosaccharide attached to asparagine. The next stage of processing involves selective transfer of an *N*-acetylglucosamine residue to a specific mannose residue. This event signals an α-D-mannosidase to cleave two external mannose residues, following which another *N*-acetylglucosamine residue is incorporated at the nonreducing terminus. Once the terminal *N*-acetylglucosamine residues are attached by this processing mechanism, it is considered that the peripheral sugars — i.e., galactose, sialic acid, and fucose — are added stepwise one at a time. The cleavage of glucose(s) (and possibly certain mannose residues during the processing) is an essential step in the formation of a high mannose type carbohydrate. The transformation of the high mannose to the complex type carbohydrate is obviously controlled by specific mannosidase(s) and *N*-acetylglucosaminyl transferases. Whether such a mechanism for the biosynthesis of *N*-glycosidic complex carbohydrate is a universal phenomenon applicable to all glycoproteins or is limited only to certain glycoproteins remains unexplained.

Although it is commonly regarded that the core region of the *N*-glycosidic carbohydrate is synthesized via oligosaccharide-lipid intermediate, little is known concerning the involvement of lipid intermediates in the biosynthesis of other classes of glycoproteins. It has been reported that in yeast mannan only the linkage mannose to serine or threonine is transformed from mannosylphosphoryl dolichol, and the mannose polymerization occurs by the stepwise addition of mannosyl residues from GDP-mannose. Several other problems relating to the assembly of carbohydrates in glycoproteins still remain unanswered. The enzymes involved in the assembly of lipid-linked oligosaccharide intermediates, in the transfer of oligosaccharide(s) to protein and in the processing of the oligosaccharide(s) following its transfer have not been purified. The precise chemical structures of the various intermediates have not been elucidated. Once some of these goals are met it may be possible to synthesize a glycoprotein in a cell-free system employing appropriate mRNA and various glycosylating and hydrolyzing enzymes.

There is little doubt that the physicochemical and biological properties of glycoproteins are somehow related to the carbohydrate prosthetic groups which they contain. The presence of carbohydrate could influence the conformation and the specificity of a glycoprotein by occluding certain regions of the polypeptide chain and exposing others needed for a given activity or function. It is also likely that the carbohydrate component may be involved in the stability of the molecule by virtue of its contribution to the maintenance of a stable conformation. In order to fully understand the role of carbohydrate in the properties of a glycoprotein, it is essential to know the conformation of the carbohydrate in relation to the protein to which it is linked. Sufficient information on the primary structures of carbohydrate units of several glycoproteins and adequate quantities of the oligosaccharides are now available which should facilitate the determination of the conformation of glycoproteins by high-resolution NMR studies.

There is evidence that carbohydrate units of glycoproteins may be involved directly in the expression of the biological function. It is known that the removal of carbohydrate from gonadotropins such as hCG results in the loss of biological activity. The manifest loss of biological activity following removal of carbohydrate implies that carbohydrate is essential for hormonal action.

In spite of the various roles attributed to the saccharides of glycoproteins which have largely remained speculative, rather well-defined functions have been ascribed to the sugar residues of glycoproteins. It has been shown that the terminal sialic acid residues are involved in the survival of glycoproteins during circulation and that the galactose, *N*-acetylglucosamine and mannose residues in them, when exposed, are responsible for their rapid clearance by the liver through the mediation of specific receptors. In addition, the recognitional role of sugars in the uptake of glycoproteins has come from studies with lysosomal glycosidases. The importance of sugars in the uptake of lysosomal enzymes has been further emphasized by an interesting observation by Fischer et al.[305] It has been shown that glycoproteins containing phosphorylated mannose residues are taken up by lysosomes, while those containing mannose residues are processed and secreted.

In summary, the work reviewed here indicates that research in the areas of chemistry and molecular biology of glycoproteins is fast growing, and significant new developments are eagerly awaited.

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REFERENCES

1. **Kornfeld, R. and Kornfeld, S.,** Comparative aspects of glycoprotein structure, in *Annual Review of Biochemistry*, Vol. 45, Snell, E.S., Boyer, P., Meister, A., and Richardson, C.C., Eds., Annual Review Palo Alto, Calif., 1976, 217.
2. **Bellisario, R., Carlsen, R. B., and Bahl, O. P.,** Human chorionic gonadotropin: linear amino acid sequence of the α-subunit, *J. Biol. Chem.*, 248, 6796, 1973.
3. **Carlsen, R. B., Bahl, O. P., and Swaminathan, N.,** Human chorionic gonadotropin: linear amino acid sequence of the β-subunit, *J. Biol. Chem.*, 248, 6810, 1973.
4. **Kessler, M. J., Reddy, M. S., Shah, R., and Bahl, O. P.,** Structures of *N*-glycosidic carbohydrate units of human chorionic gonadotropin, *J. Biol. Chem.*, 254, 7901, 1979.
5. **Kessler, M. J., Mise, T., Ghai, R. D., and Bahl, O. P.,** Structure and location of the *O*-glycosidic carbohydrate units of human chorionic gonadotropin, *J. Biol. Chem.*, 254, 7909, 1979.
6. **Rathnam, P. and Saxena, B. B.,** Primary amino acid sequence of follicle-stimulating hormone from human pituitary glands, *J. Biol. Chem.*, 250, 6735, 1975.
7. **Christakos, S. and Bahl, O. P.,** Pregnant mare serum gonadotropin: purification and physicochemical, biological and immunological characterization, *J. Biol. Chem.*, 254, 4253, 1979.
8. **Liu, W. K., Nahm, H. S., Sweeney, C. M., Lankin, W. M., Baker, H. W., and Ward, D. N.,** The primary structure of ovine luteinizing hormone. I. The amino acid sequence of the reduced and S-aminoethylated S-subunit (LH-α), *J. Biol. Chem.*, 247, 4351, 1972.
9. **Liu, W. K., Nahm, H. S., Sweeney, C. M., Holcomb, G. N., and Ward, D. N.,** The primary structure of ovine luteinizing hormone. II. The amino acid sequence of the reduced, S-carboxymethylated A-subunit (LH-β), *J. Biol. Chem.*, 247, 4365, 1972.
10. **Liao, T. H. and Pierce, J. C.,** The primary structure of bovine thyrotropin. II. The amino acid sequences of the reduced, S-carboxymethyl α and β chains, *J. Biol. Chem.*, 246, 850, 1971.
11. **Jackson, R. L. and Hirs, C. H. W.,** Primary structure of porcine pancreatic ribonuclease. I. Distribution and sites of carbohydrate attachment, *J. Biol. Chem.*, 245, 624, 1970.
12. **Tarentino, A. L., Plummer, T. H., Jr., and Maley, F.,** Studies on the oligosaccharide sequence of ribonuclease B., *J. Biol. Chem.*, 245, 4150, 1970.
13. **DeVries, A. L., Vandenheede, J., and Feeney, R. E.,** Primary structure of freezing point—depressing glycoproteins, *J. Biol. Chem.*, 246, 305, 1971.
14. **Horowitz, M. I. and Pigman, W.,** *The Glycoconjugates*. Academic Press, New York, 1977, chap. 3.
15. **Huang, T. S. and Delange, R. J.,** Egg white avidin. II. Isolation, composition, and amino acid sequences of the tryptic peptides, *J. Biol. Chem.*, 246, 686, 1971.
16. **Delange, R. J. and Huang, T. S.,** Egg white avidin. III. Sequence of the 78-residue middle cyanogen bromide peptide: complete amino acid sequence of the protein subunit, *J. Biol. Chem.*, 246, 698, 1971.
17. **Meyer, K.,** Reflections on "mucopolysaccharides" and their protein complexes, in *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 1, Balazs, E. A., Ed., Academic Press, New York, 1970, 5.

18. **Spiro, R. G.,** Basement membranes and collagens, in *Glycoproteins—their Composition, Structure and Function,* (Part B), Gottschalk, A., Ed., Elsevier, New York, 1972, 964.
19. **Yamada, K. M., Yamada, S. J., and Pastan, I.,** Cell surface protein partially restores mophology, adhesiveness, and contact inhibition of movement to transformed fibroblasts, *Proc. Natl. Acad. Sci. U.S.A.,* 73, 1217, 1976.
20. **Marshall, R. D.,** Glycoproteins, *Annu. Rev. Biochem.,* 41, 673, 1972.
21. **Hughes, R. C.,** Glycoproteins as components of cellular membranes, *Prog. Biophys. Mol. Biol.,* 26, 189, 1973.
22. **Spiro, R. G.,** Glycoproteins, in *Advances in Protein Chemistry,* Vol. 27, Anfinsen, C. B., Edsall, J. T., and Richards, F. M., Eds., Academic Press, New York, 1973, 349.
23. **Montreuil, J.,** Recent data on the structure of the carbohydrate moiety of glycoproteins: metabolic and biological implications, *Pure Appl. Chem.,* 42, 431, 1975.
24. **Waechter, C. J. and Lennarz, W. J.,** The role of polyprenol-linked sugars in glycoproteins syntheses, in *Annual Review of Biochemistry,* Vol. 45, Snell, E. E., Boyer, P. D., Meister, A., and Richardson, C. C., Eds., Annual Review, Palo Alto, Calif., 1976, 95.
25. **Gottschalk, A.,** *Glycoproteins: Their Composition, Structure and Function,* (Parts A and B), Elsevier, New York, 1972.
26. **Sharon, N.,** *Complex Carbohydrates: Their Chemistry, Biosynthesis and Functions,* Addison-Wesley, Reading, Mass., 1975.
27. **Horowitz, M. I. and Pigman, W.,** *The Glycoconjugates,* Vol. 2, Academic Press, New York, 1978.
28. **Spiro, R. G. and Bhoyroo, V. D.,** Studies on the carbohydrate of collagens: Characterization of a glucuronic acid-mannose disaccharide unit from Nereis cuticle collagen, *J. Biol. Chem.,* 255, 5347, 1980.
29. **Burke, D. J. and Keegstra, K.,** Purification and composition of the proteins from sindbis virus grown in chick and BHK cells, *J. Virol.* 20, 676, 1976.
30. **Reading, C. L., Penchoet, E., and Ballou, C.,** Carbohydrate structure of vesicular stomatitis virus glycoprotein, *J. Biol. Chem.,* 253, 5600, 1978.
31. **Yamaguchi, N., Ikenaka, T., and Matsushima, Y.,** The complete sequence of a glycopeptide obtained from Taka-amylase A, *J. Biochem. (Tokyo),* 70, 587, 1971.
32. **Nakajima, T. and Ballou, C. E.,** Structure of the linkage region between the polysaccharide and protein parts of *Saccharomyces cerevisiae* mannan, *J. Biol. Chem.,* 249, 7685, 1974.
33. **Yasuda, Y., Takahashi, N., and Murachi, T.,** Composition and structure of carbohydrate moiety of stem bromelain, *Biochemistry,* 9, 25, 1970.
34. **Lis, H. and Sharon, N.,** Soybean agglutinin—a plant glycoprotein: structure of the carbohydrate unit, *J. Biol. Chem.,* 253, 3468, 1978.
35. **Huang, C. C., Mayer, M. E., Jr., and Montgomery, R.,** Microheterogeneity and paucidispersity of glycoproteins, *Carbohydr. Res.,* 13, 127, 1970.
36. **Shainkin, R. and Perlmann, G. E.,** Phosvitin, a phosphoglycoprotein: composition and partial structure of carbohydrate moiety, *Arch. Biochem. Biophys.,* 145, 693, 1971.
37. **Williams, S.,** Comparison of glycopeptides from the ovotransferrin and serum transferrin of the hen, *Biochem. J.,* 108, 57, 1968.
38. **Monsigny, M., Adam-Classon, A., and Montrieul, J.,** Etude sur les glycoproteins. XXII. Determination de la nature du point d'attache glycanne-protein, dans les preparations d'ovomucoide de poule, *Bull. Soc. Chim. Biol.,* 50, 857, 1968.
39. **Kabasawa, I. and Hirs, C. H. W.,** Structural studies with the glycopeptides from porcine pancreatic ribonuclease, *J. Biochem.,* 247, 1610, 1972.
40. **Toyoshima, S., Fukuda, M., and Osawa, T.,** The presence of β-mannosidic linkage in acidic glycopeptide from porcine thyroglobulin, *Biochem. Biophys. Res. Commun.,* 51, 945, 1973.
41. **Plummer, T. H., Jr. and Sarda, L.,** Isolation and characterization of the glycopeptides of porcine pancreatic lipases L_A and L_B, *J. Biol. Chem.,* 248, 7865, 1973.
42. **Catley, B. J.,** Observations on the carbohydrate moiety of bovine pancreatic deoxyribonuclease A, *Arch. Biochem. Biophys.,* 159, 214, 1973.
43. **Sukeno, T., Tarentino, A. L., Plummer, T. H., Jr., and Maley, F.,** On the nature of α-mannosidase-resistant linkages in glycoproteins, *Biochem. Biophys. Res. Commun.,* 45, 219, 1971.
44. **Spiro, R. G.,** Periodate oxidation of the glycoprotein fetuin, *J. Biol. Chem.,* 239, 567, 1964.
45. **Tarentino, A. L., Plummer, T. H., Jr., and Maley, F.,** A β-mannosidic linkage in the unit A oligosaccharide of bovine thyroglobulin, *J. Biol. Chem.,* 248, 5547, 1973.
46. **Spiro, R. G., Studies on the renal glomerular basement membrane,** *J. Biol. Chem.,* 242, 1923, 1967.
47. **Tai, T., Ito, S., Yamashita, K., Muramatsu, T., and Kobata, A.,** Asparagine-linked oligosaccharide chains of IgG: a revised structure, *Biochem. Biophys. Res. Commun.,* 65, 968, 1975.
48. **Heller, H. and Lawrence, M.A.,** Structure of the glycopeptide from bovine visual pigment 500, *Biochemistry,* 9, 864, 1970.

49. **Baker, J. R., Cifonelli, J. A., and Roden, L.**, The linkage of corneal keratosulphate to protein, *Biochem. J.*, 115, 119, 1969.
50. **Wagh, P. V., Bornstein, I., and Winzler, R. J.**, The structure of a glycopeptide from human orosomucoid (α_1-acid glycoprotein), *J. Biol. Chem.*, 244, 658, 1969.
51. **Dunn, J. T. and Spiro, R. G.**, The α_2-macroglobulin of human plasma. II. Studies on the carbohydrate units, *J. Biol. Chem.*, 242, 5556, 1967.
52. **Thomas, D. B. and Winzler, R. J.**, Structure of glycoproteins of human erythrocytes, *Biochem. J.*, 124, 55, 1971.
53. **Tomita, M. and Marchesi, V. T.**, Amino acid sequence and oligosaccharide attachment sites of human erythrocyte glycophorin, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 2964, 1975.
54. **Spik, G., Bayard, B., Fournet, B., Stecker, G., Bouquelet, S., and Montreuil, J.**, Studies on glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferrin, *FEBS Lett.*, 50, 296, 1975.
55. **Spik, G., Vandersyppe, R., Fournet, B., Bayard, B., Charet, P., Bouguelet, S., Strecker, G., and Montreuil, J.**, Structure of glycopeptides isolated from human serum transferrin and lactotransferrin, *Methodologie de la Structure et du Metabolism des Glycoconjugues*, Vol. 1, C.N.R.S., Paris, 1974, 383.
56. **Ryden, L. and Eaker, D.**, The amino acid sequence of three tryptic glycopeptides from human ceruloplasmin, *Eur. J. Biochem.*, 44, 171, 1974.
57. **Arima, T. and Spiro, R. G.**, Studies of the carbohydrate units of thyroglobulin: structure of the mannose-*N*-acetylglucosamine unit (unit A) of the human and calf proteins, *J. Biol. Chem.*, 247, 1836, 1972.
58. **Shome, B. and Parlow, A. F.**, Human TSH subunits: radioimmunoassay and primary structure of the beta-subunit, Program 55th Annu. Meet. Endocr. Soc. Chicago, 1973, A-60.
59. **Baig, M. M. and Aminoff, D.**, Glycoproteins and blood group activity. I. Oligosaccharides of serologically inactive hog submaxillary glycoproteins, *J. Biol. Chem.*, 247, 6111, 1972.
60. **Lombardt, C. G. and Winzler, R. J.**, Isolation and characterization of oligosaccharide from canine submaxillary mucin, *Eur. J. Biochem.*, 49, 77, 1974.
61. **Ozeki, T. and Yosizawa, Z.**, Glycopeptide isolated from bovine submaxillary mucin, *Arch. Biochem. Biophys.*, 142, 177, 1971.
62. **Bertolini, M. and Pigman, W.**, The existence of oligosaccharide in bovine and ovine submaxillary mucins, *Carbohydr. Res.*, 14, 53, 1970.
63. **Reddy, M. S., Taro, K. K., and Bahl, O. P.**, Structural elucidation of neutral and sialylated carbohydrates of normal and fibrocystic mucins, *J. Biol. Chem.*, submitted.
64. **Feizi, T., Kabat, E. A., Vicari, G., Anderson, B., and Marsh, W. L.**, Immunochemical studies on blood groups. XLIX. I antigens complex: Specificity differences among anti-I serums revealed by quantitative precipitin studies. Partial structure of the I determinant specific for one anti-I serum, *J. Immunol.*, 106, 1578, 1971.
65. **Bray, B. A., Lieberman, R., and Meyer, K.**, Structure of human skeletal keratosulfate: the linkage region, *J. Biol. Chem.*, 242, 3373, 1967.
66. **Spiro, R. G. and Bhoyroo, V. D.**, Structure of the *O*-glycosidically linked carbohydrate units of fetuin, *J. Biol. Chem.*, 249, 5704, 1974.
67. **Codington, J. F., Linsley, K. B., Jeanloz, R. W., Irimura, T., and Osawa, T.**, Immunochemical and chemical investigation of the structure of glycoprotein fragment obtained from epiglycanin, a glycoprotein at the surface of the TA3-Ha cancer cell, *Carbohydr. Res.*, 40, 171, 1975.
68. **Thomas, D. B. and Winzler, R. J.**, Structural studies of human erythrocyte glycoprotein, *J. Biol. Chem.*, 244, 5943, 1969.
69. **Lindahl, U. and Roden, L.**, Carbohydrate-peptide linkages in proteoglycans of animal, plant and bacterial origin, in *Glycoproteins: Their Composition, Structure and Function* (Part A), Gottschalk, A., Ed., Elsevier, Amsterdam, 1972, 491.
70. **Lineback, D. R.**, The carbohydrate polypeptide linkage in a fungal glucoamylase, *Carbohydr. Res.*, 7, 106, 1968.
71. **Pazur, J. H., Knull, M. R., and Simpson, D. L.**, A note on the role for the carbohydrate moietes, *Biochem. Biophys. Res. Commun.*, 40, 110, 1970.
72. **Nakajima, T. and Ballou, C. E.**, Characterization of the carbohydrate fragments obtained from *Saccharomyces cerevisiae* mannan by alkaline degradation. *J. Biol. Chem.*, 249, 7679, 1974.
73. **Raizada, M. K., Schutzbach, J. S., and Ankel, H.**, *Cryptococcus laurentii* cell envelope glycoprotein. Evidence for separate oligosaccharide side chains of different composition and structure, *J. Biol. Chem.*, 250, 3310, 1975.
74. **Rosenthal, A. L. and Nordin, J. H.**, Enzymes that hydrolyze fungal cell wall polysaccharides. *J. Biol. Chem.*, 250, 5295, 1975.

75. **Sentandreu, R. and Northcote, D. H.,** The characterization of oligosaccharides attached to threonine and serine in a mannan glycopeptide obtained from the cell wall of yeast, *Carbohydr. Res.*, 10, 584, 1969.
76. **Muir, L. and Lee, Y. C.,** Structures of the D-galactose oligosaccharides from earthworm cutide collagen, *J. Biol. Chem.*, 244, 2343, 1969.
77. **Hallgren, P., Lundblad, A., and Svensson, S.,** A new type of carbohydrate protein linkage in a glycopeptide from normal human urine, *J. Biol. Chem.*, 250, 5312, 1972.
78. **Steiner, S., Via, D., Larriba, G., Sramek, S., and Laine, R.,** Role of glycoconjugates in expression of the transformed phenotype, in *Glycoproteins and Glycolipids in Disease Processes*, American Chemical Society Symposium Series 80, 1978, 378.
79. **Butler, W. T. and Cunningham, L. W.,** An evidence for the linkage of a disaccharide to hydroxylysine in tropocollagen, *J. Biol. Chem.*, 241, 3882, 1966.
80. **Isemura, M., Ikenaka, T., and Matsushima, Y.,** Comparative study of carbohydrate-protein complexes. I. The structures of glycopeptide derived from cuttlefish skin collagen, *J. Biochem. (Tokyo)*, 74, 11, 1973.
81. **Spiro, R. G.,** The structure of the disaccharide unit of the renal glomerular basement membrane, *J. Biol. Chem.*, 242, 4813, 1967.
82. **Beisswenger, P. J. and Spiro, R. G.,** Human glomerular basement membrane: chemical alteration in diabetes mellitus, *Science*, 168, 596, 1970.
83. **Spiro, R. G. and Finkunsin, S.,** The lens capsule: studies on the carbohydrate units, *J. Biol. Chem.*, 244, 2049, 1969.
84. **Lamport, D. T. A.,** The isolation and partial characterization of hydroxy proline-rich glycopeptides obtained by enzyme degradation of primary cell walls, *Biochemistry*, 8, 1155, 1969.
85. **Miller, D. H., Lamport, D. T. A., and Miller, M.,** Hydroxyproline heterooligosaccharide in *Chlamydomonas*, *Science*, 176, 918, 1972.
86. **Lote, C. J. and Weiss, J. B.,** Identification of digalactosylcystine in a glycopeptide isolated from urine by a new preparative technique, *FEBS Lett.*, 16, 81, 1971.
87. **Weiss, J. B., Lote, C. J., and Bobinski, M.,** New low molecular weight glycopeptide containing triglucosylcysteine in human erythrocyte membrane, *Nature (London)*, 234, 25, 1971.
88. **Yamashita, K., Tachibena, Y., and Kobata, A.,** The structures of the galactose containing sugar chains of ovalbumin, *J. Biol. Chem.*, 253, 3862, 1978.
89. **Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Binett, P., and Schmid, K.,** Determination of the primary structures of 16 asialo-carbohydrate units derived from human plasma α_1-acid glycoprotein by 360-MH$_z^1$ H NMR spectroscopy and permethylation analysis, *Biochemistry*, 17, 5206, 1978.
90. **Kondo, T., Fukuda, M., and Osawa, T.,** The structure of unit β-type glycopeptides from porcine thyroglobulin, *Carbohydr. Res.*, 58, 405, 1977.
91. **Mizuochi, T., Yonemasu, K., Yamashita, K., and Kobata, A.,** The asparagine-linked sugar chains of subcomponent Clq of the first component of human complement, *J. Biol. Chem.*, 253, 7404, 1978.
92. **Baenziger, J.U. and Fiete, D.,** Structure of the complex oligosaccharides of fetuin, *J. Biol. Chem.*, 254, 789, 1979.
93. **Hara, K., Rathnam, P., and Saxena, B. B.,** Structure of the carbohydrate moieties of α-subunits of human follitropin, lutropin, and thyrotropin, *J. Biol. Chem.*, 253, 1582, 1978.
94. **Bahl, O. P., Bedi, G. S., Reddy, M. S., and Wash, P. V.,** A novel carbohydrate structure in bovine and ovine luteinizing hormones, *Fed. Proc.*, 39, 2080, 1980.
95. **Neiderhiser, D. M., Plantner, J. J., and Carlson, D. M.,** The purification and properties of glycoproteins of pig gall bladder bile, *Arch. Biochem. Biophys.*, 145, 155, 1971.
96. **Plantner, J. J. and Carlson, D. M.,** Assay for olefinic amino acids: products of the β-elimination reaction in glycoproteins, in *Methods in Enzymology*, Vol. 28B, Ginsburg, V., Ed., Academic Press, New York, 1972, 46.
97. **Plantner, J. J. and Carlson, D. M.,** Studies of mucin-type glycoproteins: olefinic amino acid products of the β-elimination reaction, *Anal. Biochem.*, 65, 153, 1975.
98. **Tanaka, K. and Pigman, W.,** Improvements in hydrogenation procedure for demonstration of *O*-threonine glycosidic linkage in bovine submaxillary mucin, *J. Biol. Chem.*, 240, PC1487, 1965.
99. **Spiro, R. G.,** Study of the carbohydrates of glycoproteins, in *Methods of Enzymology*, Vol. 28B, Ginsburg, V., Ed., Academic Press, New York, 1972, 3.
100. **Simpson, D. E., Hranisavljevic, J. and Davidson, E. A.,** β-elimination and sulfite addition as a means of localization and identification of substituted seryl and threonyl residues in proteins and proteoglycans, *Biochemistry*, 11, 1849, 1972.
101. **Oates, M. D. G., Rosbottom, A. C., and Schrager, J.,** Further investigations into the structure of human gastic mucin: the structural configuration of the oligosaccharide chains, *Carbohydr. Res.*, 34, 115, 1974.

102. **Carlson, D.**, Structures and immunochemical properties of oligosaccharides isolated from pig submaxillary mucins, *J. Biol. Chem.*, 243, 616, 1968.
103. **Slomiany, A. and Slomiany, B. L.**, Structures of the acidic oligosaccharides isolated from rat sublingual glycoproteins, *J. Biol. Chem.*, 253, 7301, 1978.
104. **Salnikow, J., Moore, S., and Stein, W. H.**, Comparison of the multiple forms of bovine pancreatic deoxyribonuclease, *J. Biol. Chem.*, 245, 5685, 1970.
105. **Salnikow, J. and Murphy, D.**, Bovine pancreatic deoxyribonucleases A and C: a proline for histidine substitution in deoxyribonuclease C, *J. Biol. Chem.*, 248, 1499, 1973.
106. **Liao, T. H.**, Bovine pancreatic ribonuclease D, *J. Biol. Chem.*, 249, 2354, 1974.
107. **Plummer, T. H., Jr. and Hirs, C. H. W.**, The structure of bovine pancreatic ribonuclease B: isolation of a glycopeptide, *J. Biol. Chem.*, 239, 2530, 1964.
108. **Plummer, T. H., Jr.**, Glycoproteins of bovine pancreatic juice: isolation of ribonucleases C and D, *J. Biol. Chem.*, 243, 5961, 1968.
109. **Isemura, S. and Ikenaka, T.**, The amino acid sequences of glycopeptides obtained from Take-amylase A with trypsin and chymotrypsin, *J. Biochem. (Tokyo)*, 74, 1, 1973.
110. **Shipolini, R. A., Callewaert, G. L., Cottrell, R. C., and Vernon, C. A.**, The amino acid sequence and carbohydrate content of phospholipase A_2 from bee venom, *Eur. J. Biochem.*, 48, 465, 1974.
111. **Shome, B. and Parlow, A. F.**, Human follicle stimulating hormone (hFSH): first proposal for the amino acid sequence of the α-subunit (LFSHα) and first demonstration of its identity with the α-subunit of human luteinizing hormone (hLHα), *J. Clin. Endocrinol. Metab.*, 39, 199, 1974.
112. **Sairam, M. R., Papkoff, H., and Li, C. H.**, Human pituitary interstitial cell stimulating hormone: primary structure of the α-subunit, *Biochem. Biophys. Res. Commun.*, 48, 530, 1972.
113. **Closset, J. and Hennen, G.**, Human luteinizing hormone. The amino acid sequence of the β-subunit, *FEBS Lett.*, 29, 97, 1973.
114. **Sairam, M. R., Samy, T. J. A., Papkoff, H., and Li, C. H.**, Primary structure of ovine interstitial cell-stimulating hormone II, the β-subunit, *Arch. Biochem. Biophys.*, 153, 577, 1972.
115. **Shome, B. and Parlow, A. F.**, Human follicle-stimulating hormone. First proposal for the amino acid sequence of the hormone specific β subunit (FSHβ), *J. Clin. Endocrinol. Metab.*, 39, 203, 1974.
116. **Edelman, G. M., Cunnigham, B. A., Gall, W. E., Gottlieb, P. D., Rutishauser, U., and Waxdal, M. J.**, The covalent structure of an entire γG immunoglobulin molecule, *Proc. Natl. Acad. Sci. U.S.A.*, 63, 78, 1969.
117. **Nolan, C. and Smith, E. L.**, Glycopeptides. II. Isolation and properties of glycopeptides from rabbit γ-globulins, *J. Biol. Chem.*, 237, 446, 1962.
118. **Melchers, F.**, The attachment site of carbohydrate in a mouse immunoglobulin light chains, *Biochemistry*, 8, 938, 1969.
119. **Shimizu, A., Putnam, F. W., Paul, C., Clamp, J. R., and Johnson, I.**, Structure and role of the five glycopeptides of human IgM immunoglobulin, *Nature (London) New Biol.*, 231, 73, 1971.
120. **Schmid, K., Ishiguro, M., Emura, J., Isemura, S., Kaufman, H., and Motoyama, T.**, The amino acid sequence of two large glycopeptides derived from the carbohydrate carrying region of α_1-acid glycoprotein, *Biochem. Biophys. Res. Commun.*, 42, 280, 1971.
121. **Sox, H. C., Jr. and Hood, L.**, Attachment of carbohydrate to the variable region of myeloma immunoglobulin light chain, *Proc. Natl. Acad. Sci. U.S.A.*, 66, 975, 1970.
122. **Iwanaga, S., Blomback, B., Grondahl, N.J., Hessel, B., and Wallen, P.**, Amino acid sequence of the *N*-terminal part of α-chain in human fibrinogen, *Biochem. Biophys. Acta*, 160, 280, 1968.
123. **Johansen, P. G., Marshall, R. D., and Neuberger, A.**, Carbohydrates in protein. III. The preparation and some of the properties of a glycopeptide from hen's egg albumin, *Biochem. J.*, 78, 518, 1961.
124. **Rawitch, A. B., Liao, T. H., and Pierce, J. G.**, The amino acid sequences of a tryptic glycopeptide from thyroglobulin, *Biochem. Biophys. Acta*, 160, 360, 1968.
125. **Dayhoff, M. D.**, *Atlas of Protein Sequence and Structure*, Vol. 5, National Biomedical Research Foundation, Silver Springs, Md., 1972.
126. **Sinohara, H. and Maruyama, T.**, Evolution of glycoproteins as judged by the frequency of occurrence of the tripeptides Asn-X-Ser and Asn-X-Thr in proteins, *J. Mol. Evol.*, 2, 117, 1973.
127. **Pless, D. D. and Lennarz, W. J.**, Enzymatic conversion of proteins to glycoproteins, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 134, 1977.
128. **Kiely, M. L., McKnight, G. S., and Schimke, R. T.**, Studies on the attachment of carbohydrate to ovalbumin nascent in hen oviduct, *J. Biol. Chem.*, 251, 5490, 1976.
129. **Smith, D. S. and Utsumi, S.**, Structure at the hinge region in rabbit immunoglobulin-G, *Nature (London)*, 216, 332, 1967.
130. **Hill, M. D., Schwyzer, M., Steinman, H. M., and Hill, R. L.**, The submaxillary mucin: primary structure and peptide substrates of UDP-*N*-acetylgalactosamine; mucin transferase, *J. Biol. Chem.*, 1252, 3799, 1977.

131. **Miller, C. J.,** Biochemical characteristics and biological significance of the genetically distinct collagens, *Mol. Cell. Biochem.,* 13, 165, 1976.
132. **Butler, W. T.,** Carbohydrate moieties of the collagen and collagen-like proteins, in health and disease, in *Glycoproteins and Glycolipids in Disease Processes,* ACS Symposium, Ser. 80, American Chemical Society, Washington, D.C., 1978, 213.
133. **Schwarzwann, G. O. H., Hatcher, V. B., and Jeanloz, R. W.,** Purification and structural elucidation of several carbohydrate side chains from α-acid glycoproteins, *J. Biol. Chem.,* 253, 6983, 1978.
134. **Zinn, A. B., Marshall, T. S., and Carlson, D. M.,** Preparation of glycopeptides and oligosaccharides from thyroxine binding globulin, *J. Biol. Chem.,* 253, 6761, 1978.
135. **Moschera, J. and Pigman, W.,** The isolation and characterization of rat sublingual mucus-glycoprotein, *Carbohydr. Res.,* 40, 53, 1975.
136. **Francois, C., Marshall, R. D., and Neuberger, A.,** Carbohydrates in protein. IV. The determination of mannose in hen's egg albumin by radioisotope dilution, *Biochem. J.,* 83, 335, 1962.
137. **Graham, E. R. B. and Neuberger, A.,** The estimation of galactose, mannose and fucose in glycoprotein in radioisotope dilution, *Biochem. J.,* 106, 593, 1968.
138. **Sloneker, J. M.,** Gas chromatography of carbohydrates, in *Biomedical Application of Gas Chromatography,* Vol. 2, Szymanski, N. A., Ed., Plenum Press, New York, 1968, 87.
139. **Clamp, J. R., Bhatti, T., and Chambers, R. E.,** Determination of carbohydrate in biological materials by gas-liquid chromatography, *Methods Biochem. Anal.,* 19, 229, 1971.
140. **Sawardekar, J. S., Sloneker, J. H., and Jeans, A.,** Quantitative determination of monosaccharides and their acetate by gas liquid chromatography, *Anal. Chem.,* 37, 1602, 1965.
141. **Kim, J. H., Shome, B., Liao, T. H., and Pierce, J. G.,** Analysis of neutral sugars by gas liquid chromatography of alditol acetates. Application to thyrotropic hormone and other glycoproteins, *Anal. Biochem.,* 20, 258, 1967.
142. **Lehndardt, W. F. and Winzler, R. J.,** Determination of neutral sugars in glyco-proteins by gas liquid chromatography, *J. Chromatogr.,* 34, 475, 1968.
143. **Neidermeier, W.,** Gas chromatography of neutral and amino sugar in glycoproteins, *Anal. Biochem.,* 40, 465, 1971.
144. **Lee, Y. C.,** Analysis of sugars by auto liquid chromatography in *Methods in Enzymology,* Vol. 28B, Ginsburg, V., Ed., Academic Press, New York, 1972, 63.
145. **Cheng, P. and Boat, T. F.,** An improved method for the determination of galactosaminitol, glucosaminitol, glucosamine, and galactosamine on an amino acid analyzer, *Anal. Biochem.,* 85, 276, 1978.
146. **Warren, L.,** Thiobarbituric acid assay of sialic acids. *J. Biol. Chem.,* 234, 1971, 1959.
147. **Hammond, K. S. and Papermaster, D. S.,** Fluorometric assay of sialic acid in the picomole range: a modification of the thiobarbituric acid assay, *Anal. Biochem.,* 74, 292, 1976.
147a. **Jourdian, G. W., Dean, L., and Roseman, S.,** The Sialic Acids. XI. The periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides, *J. Biol. Chem.,* 246, 430, 1971.
148. **Murayama, J., Tomita, M., Tsuji, A., and Hamada, A.,** Fluorimetric assay of sialic acid, *Anal. Biochem.,* 73, 535, 1976.
149. **Craven, D. and Gehrke, C.,** Quantitative determination of *N*-acetylneuraminic acid by gas liquid chromatography, *J. Chromatogr.,* 37, 414, 1968.
150. **Casals-Stenzel, J., Buscher, M., and Schauer, E.,** Gas liquid chromatography of *N*- and *O*-acetylated neuraminic acids, *Anal. Biochem.,* 65, 507, 1975.
151. **Kamerling, J. and Vliegenhart, J.,** Identification of *O*-acetylated *N*-acylneuraminic acid by mass spectrometry, *Carbohydr. Res.,* 41, 7, 1975.
152. **Monohen, I. and Karkkaren, J.,** Quantitative determination of neuraminic acid by gas chromatography-mass spectrometry, *FEBS Lett.,* 59, 190, 1975.
153. **Roboz, J., Suzuki, R., and Bekesi, J. G.,** Determination of neuraminidase susceptible and total *N*-acetylneuraminic acid in cells by selected ion monitoring, *Anal. Biochem.,* 87, 195, 1978.
154. **Goldstein, I. J., Hay, G. W., Lewis, B. A., and Smith, F.,** Controlled degradation of polysaccharides by periodate oxidation, reduction and hydrolysis, *Methods Carbohydr. Chem.,* 5, 361, 1965.
155. **Painter, T. and Larsen, B.,** Formation of hemiacetals between neighboring hexuronic acid residues during periodate oxidation of alginate, *Acta Chem. Scand.,* 24, 813, 1970.
156. **Painter, T. and Larsen, B.,** Kinetic evidence for inter-residue hemiacetal formation during oxidation of amylase by periodate ion, *Acta Chem. Scand.,* 24, 2724, 1970.
157. **Dixon, J. S. and Lipkin, D.,** Spectrophotometric determination of vicinal glycols: application to the determination of ribofuronosides, *Anal. Chem.,* 26, 1092, 1954.
158. **Hughes, K. W. and Clamp, J. R.,** Use of gas chromatography in periodate oxidation studies of glycopeptides and related materials, *Biochim., Biophys. Acta,* 264, 418, 1972.
159. **Avigad, G.,** Rapid sensitive determination of periodate, *Carbohydr. Res.,* 11, 119, 1969.
160. **Hughes, R. C. and Jeanloz, R. W.,** Sequential periodate oxidation of the α_1-acid glycoprotein of human plasma, *Biochemistry,* 5, 253, 1966.

161. **Yamaguchi, H., Ikenaka, T., and Matsushima, Y.,** Humogeneity and sequential periodate oxidation of a glycopeptide from Taka-amylase A, *J. Biol. Chem. (Tokyo)*, 65, 793, 1969.
162. **Lindberg, B.,** Methylation analysis of polysaccharides, in *Methods in Enzymology*, Vol. 28B, Ginsburg V., Ed., Academic Press, New York, 1972, 178.
163. **Lindberg, B. and Lonngren, J.,** Methylation analysis of complex carbohydrates: general procedure and application for sequence analysis, in *Methods in Enzymology*, Vol. 50C, Ginsburg, V., Ed., Academic Press, New York, 1978, 3.
164. **Hirst, E. L. and Percival, E.,** Methylation of polysaccharides and fractionation of the methylated products, in *Methods in Carbohydrate Chemistry*, Vol. 5, Whistler, R. L., Ed., Stein Hall and Co., New York, 1965, 287.
165. **Hakomori, S.,** Rapid permethylation of glycolipids and polysaccharides catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide, *J. Biochem. (Tokyo)*, 55, 205, 1964.
166. **Stoffyn, A., Stoffyn, P. J., and Orr, J. C.,** Methylation of 2 acetamido-2-deoxy-D-hexoses, *Carbohydr. Res.*, 23, 251, 1972.
167. **Stellner, K., Saito, K., and Hakomori, S.,** Determination of amino sugar linkage in glycolipids by methylation: amino sugar linkage of ceramide pentasaccharides of rabbit erthrocytes and of Forssman antigen, *Arch. Biochem. Biophys.*, 155, 464, 1973.
168. **Dutton, C. G. S.,** Application of gas liquid chromatography to carbohydrates: part I, in *Advances in Carbohydrate Chemistry and Biochemistry*, Vol. 28, Tipson, R. S. and Horton, D., Eds., Academic Press, New York, 1973, 16.
169. **Dutton, C. G. S.,** Application of gas liquid chromatography to carbohydrates: part II, in *Advances in Carbohydrate Chemistry and Biochemistry*, Vol. 30, Tipson, R. S. and Horton, D., Eds., Academic Press, New York, 1974, 9.
170. **Lonngren, J. and Svensson, S.,** Mass spectrometry in structural analysis of natural carbohydrates, in *Advances in Carbohydrate Chemistry and Biochemistry*, Vol. 29, Tipson, R. S. and Horton, D., Eds., Academic Press, New York, 1974, 41.
171. **Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B., and Lonngren, J.,** A practical guide to the methylation analysis of carbohydrates, *Chem. Commun. Univ. Stockholm*, 8, 1976.
172. **Sweet, D. P., Shapiro, R. H., and Albersheim, P.,** Quantitative analysis by various glc response factor theories for partially methylated and partially ethylated alditol acetates, *Carbohydr. Res.*, 40, 217, 1975.
173. **Zinn, A. B., Marshall, J. S., and Carlson, D. M.,** Carbohydrate structures of thyroxine-binding globulin and their effects on hepatocyte membrane binding, *J. Biol. Chem.*, 253, 6768, 1978.
174. **Bjorndal, H., Lindberg, B., Piloti, A., and Svensson, S.,** Mass spectra of partially methylated alditol acetates. II. Deuterium labeling experiments, *Carbohydr. Res.*, 15, 339, 1970.
175. **Boren, H. B., Goregg, P. J., Lindberg, B., and Svensson, S.,** Mass spectra of partially methylated alditol acetates, II. Labeling experiments in the mass spectrometry of partially methylated deoxyalditol acetates, *Acta Chem. Scand.*, 25, 3299, 1971.
176. **Ginsburg, V., Ed.,** *Methods in Enzymology*, Vol. 28B, Academic Press, New York, 1972.
177. **Ada, G., French, E., and Lind, P.,** Purification and properties of neuraminidase from *Vibrio cholerae*, *J. Gen. Microbiol.*, 24, 409, 1961.
178. **Cassidy, J. T., Jourdian, G. W., and Roseman, S.,** The sialic acids. VI. Purification and properties of sialidase from *Clostridium perfringens*, *J. Biol. Chem.*, 240, 3501, 1965.
179. **Hughes, R. C. and Jeanloz, R. W.,** The extra-cellular glycosidases of *Diplococcus pneumoniae*. I. Purification and properties of a neuraminidase and a β-galactosidase. Action on the α_1-acid glycoprotein of human plasma, *Biochemistry*, 3, 1535, 1964.
180. **Graham, E. R. B. and Gottschalk, A.,** Studies on mucoproteins. I. The structure of the prosthetic group of ovine submaxillary gland mucoprotein, *Biochim. Biophys. Acta*, 38, 513, 1960.
181. **Spiro, R. G.,** Studies on the monosaccharide sequence of the serum glycoprotein fetuin, *J. Biol. Chem.*, 237, 646, 1962.
182. **Kathan, R. H. and Winzler, R. J.,** Structure studies on the myxovirus hemagglutination inhibitor of human erythrocytes, *J. Biol. Chem.*, 238, 21, 1963.
183. **Spiro, R. G. and Spiro, M. J.,** The carbohydrate composition of the thyroglobulins from several species, *J. Biol. Chem.*, 240, 997, 1965.
184. **Bahl, O. P.,** Human chorionic gonadotropin. II. The nature of the carbohydrate units, *J. Biol. Chem.*, 244, 575, 1969.
185. **Roberts, B. I. and Wagh, P. V.,** Further studies on a highly purified glycoprotein from the intimal region of porcine aorta, *Biochim. Biophys. Acta*, 439, 26, 1976.
186. **Sodetz, J. M., Paulson, J. C., Pizzo, S. V., and McKee, P. A.,** Carbohydrate on human factor VIII/von Willebrand factor: impairment of function by removal of specific galactose residues, *J. Biol.* Chem., 253, 7202, 1978.

187. **Spiro, R. G.,** Studies on the renal glomerular basement membrane: preparation and chemical properties, *J. Biol. Chem.*, 242, 1915, 1967.

188. **Bahl, O. P.,** Glycosidases of *Aspergillus niger*. II. Purification and general properties of 1,2-α-*L*-fucosidase, *J. Biol. Chem.*, 245, 299, 1970.

189. **Aminoff, D. and Furukawa, K.,** Enzymes that destroy blood group specificity. I. Purification and properties of α-*L*-fucosidase from *Clostridium perfringens*, *J. Biol. Chem.*, 245, 1659, 1970.

190. **Carlsen, R. B. and Pierce, J. G.,** Purification and properties of an α-*L*-fucosidase from rat epididymis, *J. Biol. Chem.*, 247, 23, 1972.

191. **Tanaka, K., Nakano, T., Noguchi, S., and Pigman, W.,** Purification of α-*L*-fucosidase of abalone liver, *Arch. Biochem. Biophys.*, 126, 624, 1968.

192. **Ogata-Arakawa, M., Muramatsu, T., and Kobata, A.,** α-*L*-fucosidases from almond emulsin: characterization of the two enzymes with different specificities, *Arch. Biochem. Biophys.*, 181, 353, 1977.

193. **Bahl, O. P. and Agrawal, K. M. C.,** Glycosidases of *Aspergillus niger*. I. Purification and characterization of α- and β-galactosidases and β-*N*-acetylglucosaminidase, *J. Biol. Chem.*, 244, 2970, 1969.

194. **Chipowsky, S. and McGuire, E. J.,** Hydrolysis of glycosides in glycoproteins by purified exoglycosidases, *Fed. Proc.*, 28, 606, 1969.

195. **Hu, A. S. L., Wolfe, R. G., and Reithel, F. J.,** The preparation and purification of β-galactosidase from *Escherichia coli*, ML 308, *Arch. Biochem. Biophys.*, 81, 500, 1959.

196. **Agrawal, K. M. L. and Bahl, O. P.,** Glycosidases of *Phaseolus vulgaris*. II. Isolation and general properties, *J. Biol. Chem.*, 243, 103, 1968.

197. **Li, S. C., Mazzota, M. Y., Chien, S. F., and Li, Y. T.,** Isolation and characterization of jack bean β-galactosidase, *J. Biol. Chem.*, 250, 6786, 1975.

198. **Wagh, P. V.,** Purification of jack bean meal β-*D*-galactosidase by a new affinity adsorbent, *Biochim. Biophys. Acta*, 522, 515, 1978.

199. **Hughes, R. C. and Jeanloz, R. W.,** The extra-cellular glycosidases of *Diplococcus pneumoniae*. II. Purification and properties of a βN-acetylglucosaminidase. Action on a derivative of the α_1-acid glycoprotein of human plasma, *Biochemistry*, 3, 1543, 1964.

200. **Kornfeld, R. and Kornfeld, S.,** The structure of a phytohemaglutinin receptor site from human erythrocyte, *J. Biol. Chem.*, 245, 2536, 1970.

201. **Kornfeld, R., Ketler, J., Baenziger, J., and Kornfeld, S.,** The structure of the glycopeptide of human γG myeloma proteins, *J. Biol. Chem.*, 246, 3259, 1971.

202. **Li, Y. T.,** Studies on the glycosidases in jack bean meal. I. Isolation and properties of α-mannosidase, *J. Biol. Chem.*, 242, 5474, 1967.

203. **Wagh, P. V.,** Simple procedure for the isolation of jack bean α-*D*-mannosidase by benzidine-sepharose chromatography, *J. Chromatogr.*, 152, 565, 1978.

204. **Matta, K. L. and Bahl, O. P.,** Glycosidases of *Aspergillus niger*. IV. Purification and characterization of α-mannosidase, *J. Biol. Chem.*, 247, 1780, 1972.

205. **Muramatsu, T. and Egami, F.,** α-Mannosidase and β-mannosidase from the liver of *Turbo cornutus*: purification, properties and application in carbohydrate research, *J. Biochem. (Tokyo)*, 62, 700, 1967.

206. **Okumura, T. and Yamashina, I.,** Purification of α-mannosidase from hog kidney and its action on glycopeptides, *J. Biochem. (Tokyo)*, 68, 561, 1970.

207. **Li, Y. T.,** Presence of α-*D*-mannosidic linkage in glycoproteins. Liberation of *D*-mannose from various glycoproteins by α-mannosidase isolated from jack bean meal, *J. Biol. Chem.*, 241, 1010, 1966.

208. **Kornfeld, S., Li, E., and Tabas, I.,** The synthesis of complex-type oligosaccharides. II. Characterization of the processing intermediates in the synthesis of the complex oligosaccharide units of the vesicular stomatitis virus G protein, *J. Biol. Chem.*, 253, 7771, 1978.

209. **Sugahara, K., Okumura, T., and Yamashina, I.,** Purification of β-mannosidase from a snail, *Achatina fulica*, and its action on glycoproteins, *Biochim. Biophys, Acta*, 268, 488, 1972.

210. **Findlay, J. and Levvy, G. A.,** Purification of β-*N*-acetylglucosamindase from the pig epididymis, *Biochem. J.*, 77, 170, 1960.

211. **Li, Y. T. and Li, S. C.,** Studies on the glycosidases of jack bean meal. III. Crystallization and properties of β-*N*-acetylglucosaminidase, *J. Biol. Chem.*, 245, 5153, 1970.

212. **Spiro, R. G.,** Characterization of carbohydrate units of glycoproteins in complex carbohydrates, in *Methods in Enzymology*, Vol. 8, Neufeld, F. and Ginsburg, V., Eds., Academic Press, New York, 1966, 26.

213. **Clamp, J. R. and Putnam, F. W.,** The carbohydrate prosthetic group of human γ-globulin, *J. Biol. Chem.*, 239, 3233, 1964.

214. **Jamieson, G. A., Jett, M., and DeBernardo, S. L.,** The carbohydrate sequence of the glycopeptide chains of human transferrin, *J. Biol. Chem.*, 246, 3686, 1971.

215. **Bahl, O. P. and Agrawal, K. M. L.,** α-Galactosidase, β-galactosidase and β-*N*-acetylglucosaminidase from *Aspergillus niger*, *Methods Enzymol.*, 28, 728, 1972.

216. **McDonald, M. J. and Bahl, O. P.,** α-*N*-acetylgalactosesaminidase from *Aspergillus niger*, in *Methods in Enzymology*, Vol. 28B, Ginsburg, V., Ed., Academic Press, New York, 1972, 734.
217. **Buddecke, E., Schauer, H., Werries, E., and Gottschalk, A.,** Characterization of *O*-seryl-*N*-acetylgalactosaminide glycohydrolase as an α-*N*-acetylgalactosaminidase, *Biochem. Biophys. Res. Commun.*, 34, 517, 1969.
218. **Weissman, B. and Hinrichsen, D. F.,** Mammalian α-acetylgalactosaminidase: occurrence, partial purification, and action on linkages in submaxillary mucins, *Biochemistry*, 8, 2034, 1968.
219. **Koide, N. and Muramatsu, T.,** Endo-β-*N*-acetylglucosaminidase acting on carbohydrate moieties of glycoproteins: purification and properties of the enzymes from *Diplococcus pneumoniae*, *J. Biol. Chem.*, 249, 4897, 1974.
220. **Tarentino, A. L. and Maley, F.,** Purification and properties of an endo-β-*N*-acetylglucosaminidase from *Streptomyces griseus*, *J. Biol. Chem.*, 249, 811, 1974.
221. **Maley, F., Tarentino, A. L., and Trimble, R. L.,** *Glycoproteins and Glycolipids in Disease Processes*, Symposium Series 80, Walborg, E. F., Jr., Ed., American Chemical Society, Washington, D.C., 1978, 86.
222. **Ito, S., Muramatsu, T., and Kobata, A.,** Endo-β-*N*-acetylglucosaminidases acting on carbohydrate moieties of glycoproteins: purification and properties of the two enzymes with different specificities from *Clostridium perfringens*, *Arch. Biochem. Biophys.*, 171, 78, 1975.
223. **Fukuda, M. N. and Matsumura, G.,** Endo-β-galactosidase of *Escherichia freundii*: purification and endoglycosidic action on Keratan sulfate, oligosaccharides and blood group active glycoprotein, *J. Biol. Chem.*, 251, 6218, 1976.
224. **Unemoto, J., Bhavanandan, V. P., and Davidson, E. J.,** Purification and properties of an endo-α-*N*-acetyl-*O*-galactosaminidase from *Diplococcus pneumoniae*, *J. Biol. Chem.*, 252, 8609, 1977.
225. **Takashi, N.,** Demonstration of a new amidase acting on glycopeptides, *Biochem. Biophys. Res. Commun.*, 76, 1194, 1977.
226. **Trimble, R. B., Tarentino, A. L., Plummer, T. H., Jr., and Maley, F.,** Asparaginyl glycopeptides with a low mannose content are hydrolyzed by endo-β-*N*-acetylglucosaminidase H, *J. Biol. Chem.*, 253, 4508, 1978.
227. **Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y., and Kobata, A.,** Structural studies of two ovalbumin glycopeptides in relation to the endo-β-*N*-acetylglucosaminidase specificity, *J. Biol. Chem.*, 250, 8569, 1975.
228. **Ginsburg, V., Ed.,** *Methods in Enzymology*, Vol. 50C, Academic Press, New York, 1978.
229. **Trimble, R. B. and Maley, F.,** The use of endo-β-*N*-acetylglucosaminidase, H in characterizing the structure and function of glycoproteins, *Biochem. Biophys. Res. Commun.*, 78, 935, 1977.
230. **Robbins, P. W., Hubbard, S. C., Turco, S. J., and Wirth, D. F.,** Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins, *Cell*, 12, 893, 1977.
231. **Yosizawa, Z., Sato, T., and Schmid, K.,** Hydrazinolysis of α_1-acid glycoprotein, *Biochim. Biophys. Acta*, 121, 417, 1966.
232. **Bayard, B. and Montreuil, J.,** Specific cleavage of *N*-acetylglucosaminic linkages, in *Methodologie de le Structure et du Metabolism des Glycoconjugues*, Vol. 1, C.N.R.S., Paris, 1974, 209.
233. **Neuberger, A., Gottschalk, A., Marshall, R. D., and Spiro, R. G.,** Carbohydrate-peptide linkages in glycoproteins and methods for their elucidation, in *Glycoproteins—their Composition, Structure and Function*, (Part A), Gottschalk, A., Ed., Elsevier, Amsterdam, 1972, 450.
234. **Lee, Y C. and Scocca, Jr.,** A common structural unit in asparagine-oligosaccharides of several glycoproteins from different sources, *J. Biol. Chem.*, 247, 5753, 1972.
235. **Hase, S. and Matsushima, Y.,** Amino sugar analysis by gas-liquid chromatography, *J. Biochem. (Tokyo)*, 66, 57, 1969.
236. **Isemura, M. and Schmid, K.,** Studies on the carbohydrate moiety of α_1-acid glycoprotein (orosomucoid) by using alkaline hydrolysis and deamination by nitrous acid, *Biochem. J.*, 124, 591, 1971.
237. **Mort, A. J. and Lamport, D. T. A.,** Anhydrous hydrogen fluoride deglycosylates glycoprotein, *Anal. Biochem.*, 82, 289, 1977.
238. **Strominger, J. L., Higashi, Y., Sandermann, H., Stone, K. J., and Willoughby, E.,** The role of polyisoprenol alcohols in the biosynthesis of the peptidoglycan of bacterial cell walls and other complex polysaccharides, in *Biochemistry of Glycosidic linkages, An integrated View*, Piras, R. and Pontis, H. G., Eds., Academic Press, New York, 1972, 135.

238a. **Heifetz, A. and Elbein, A. D.,** Solubilization and properties of mannose and *N*-acetylglucosamine transferases involved in the formation of polyprenyl-sugar intermediates, *J. Biol. Chem.*, 252, 3057, 1977.

238b. **Kang, M. S., Spencer, J. P., and Elbein, A. D.,** Amphomycin inhibition of mannose and GlcNac incorporation into lipid-linked saccharides, *J. Biol. Chem.*, 253, 8860, 1978.

238c. **Spencer, J. P. and Elbein, A. D.,** Synthesis of heptasaccharide-lipid by soluble mannosyl transferase, *Fed. Proc.*, 39, 1671, 1980.

238d. **Kornfeld, S., Chapman, A., and Fujimoto, K.**, Studies of the synthesis of lipid-linked oligosaccharides, *Fed. Proc.*, 255, 1671, 1980.

238e. **Das, R. C. and Heath, E. C.**, Dolichyl pyrophosphoryl oligosaccharide-protein oligosaccharyl transferase: solubilization, purification and properties, *Fed. Proc.*, 255, 1672, 1980.

239. **Li, E., Tabas, I., and Kornfeld, S.**, The synthesis of complex-type oligosaccharides. I. Structure of the lipid-linked oligosaccharide precursor of the complex-type oligosaccharides of the vesicular stomatitis virus G protein, *J. Biol. Chem.*, 253, 7762, 1978.

240. **Spiro, R. G., Spiro, M. J., and Bhoyroo, V. D.**, Lipid-saccharide intermediates in glycoprotein biosynthesis. III. Studies on the structure of an oligosaccharide-lipid from thyroid, *J. Biochem.*, 251, 6409, 1976.

241. **Chambers, J., Forsee, W. T., and Elbein, A. D.**, Enzymatic transfer of mannose from mannosylphosphoryl polyprenol to lipid-linked oligosaccharides by pig aorta, *J. Biol. Chem.*, 252, 2498, 1977.

242. **Lucas, J. J., Waechter, C. J., and Lennarz, W. S.**, The participation of lipid-linked oligosaccharide in synthesis of membrane glycoproteins, *J. Biol. Chem.*, 250, 1992, 1975.

243. **Hsu, A. F., Baynes, J. W., and Heath, E. C.**, The role of a dolichol-oligosaccharide as an intermediate in glycoprotein biosynthesis, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 2391, 1974.

244. **Struck, D. K. and Lennarz, W. J.**, Evidence for the participation of saccharide-lipids in the synthesis of the oligosaccharide chain of ovalbumin, *J. Biol. Chem.*, 252, 1007, 1977.

245. **Hart, G., Elting, J., Chen, W., Brew, K., and Lennarz, W.**, Synthesis and modification of the oligosaccharide chains of *N*-linked glycoprotein, *Fed. Proc.*, 38, 291, 1979.

246. **Tabas, I., Schlesinger, S., and Kornfeld, S.**, Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain, *J. Biol. Chem.*, 253, 716, 1978.

247. **Grinna, L. S. and Robbins, P. W.**, Processing of glycoproteins: glucosidases which process oligosaccharides, *Fed. Proc.*, 38, 291, 1979.

248. **Liu, T., Stetson, B., Turco, S. J., Hubbard, S. C., and Robbins, P. W.**, Arrangement of glucose residues in the lipid-linked oligosaccharide precursor or asparaginyl oligosaccharides, *J. Biol. Chem.*, 254, 4554, 1979.

249. **Schacter, M., Narasimhan, S., and Wilson, J. R.**, Biosynthesis and catabolism of glycoproteins, in *Glycoproteins and Glycolipids in Disease Processes*, Symposium Series 80, Walborg, E. F., Jr., Ed., American Chemical Society, Washington, D.C., 1978, 21.

250. **Tabas, I. and Kornfeld, S.**, The synthesis of complex-type oligosaccharides. III. Identification of an α-D-mannosidase activity involved in a late stage of processing of complex-type oligosaccharides, *J. Biol. Chem.*, 253, 7779, 1978.

251. **Narasimhan, S., Stanley, P., and Schacter, H.**, Control of glycoprotein synthesis: lectin-resistant mutant containing only one of two distinct *N*-acetylglucosaminyl transferase activities present in wild type chinese hamster ovary cells, *J. Biol. Chem.*, 252, 3926, 1977.

252. **Harpaz, N. and Schacter, H.**, Processing of Asn-linked oligosaccharides by an *N*-acetylglucosaminyl transferase I (Gn-T I)-dependent α-D-mannosidase in rat liver Golgi-rich membranes, Abstr. No. 17, Society for Complex Carbohydrates Annual Meeting, Toronto, July 1979.

253. **Schacter, H.**, Glycoprotein biosynthesis, in *The Glycoconjugates*, Vol. 2, Horowitz, M. I. and Pigman, W., Eds., Academic Press, New York, 1977, 87.

254. **Jeanloz, R. W. and Classe, A.**, The linkage between sialic acid and galactose in the α_1-acid glycoprotein of human plasma, *Fed. Proc.*, 22, 538, 1963.

255. **Sato, T., Yosizawa, Z., Masabuchi, M., and Yamauchi, F.**, Structure of the carbohydrate moiety of the α_1-acid glycoprotein of human plasma, *Carbohydr. Res.*, 5, 387, 1967.

256. **Bartholomew, B. A., Jourdian, G. W., and Roseman, S.**, The sialic-acids. XV. Transfer of sialic acid to glycoproteins by a sialyltransferase from colostrum, *J. Biol. Chem.*, 248, 5751, 1973.

257. **Hudgin, R. L. and Schacter, H.**, Evidence for two CMP-*N*-acetylneuraminic acid: lactose sialyltransferases in rat, porcine, bovine and human liver, *Can. J. Biochem.*, 50, 1024, 1972.

258. **Paulson, J. C., Beranet, W. E., and Hill, R. L.**, Purification of a sialyltransferase from bovine colostrum by affinity chromatography on CDP-agarose, *J. Biol. Chem.*, 252, 2356, 1977.

259. **Paulson, J. C., Rearick, J. I., and Hill, R. C.**, Enzymatic properties of β-D-galactosidase α2-6 sialyltransferase from bovine colostrum, *J. Biol. Chem.*, 252, 2363, 1977.

260. **Wirth, D. F., Katz, F., Small, B., and Lodish, H. F.**, How a single Sindbis virus on RNA directs the synthesis of one soluble protein and two integral membrane glycoproteins, *Cell*, 10, 253, 1977.

261. **Blobel, G. and Dobberstein, B.**, Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma, *J. Cell. Biol.*, 67, 835, 1975.

262. **Blobel, G. and Dobberstein, B. J.**, Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components, *J. Cell. Biol.*, 67, 852, 1975.

263. **Rothman, J. E. and Lodish, H. F.**, Synchronized transmembrane insertion and glycosylation of a nascent membrane protein, *Nature (London)*, 269, 775, 1977.
264. **Watkins, W. M.**, Blood group substances: their nature and genetics, in *The Red Blood Cell*, Vol. 1, 2nd ed., Surgenor, D. M., Ed., Academic Press, New York, 1974, 293.
265. **McGuire, E. J.**, Biosynthesis of submaxillary mucins, in *Blood and Tissue Antigens*, Aminoff, D., Ed., Academic Press, New York, 1970, 461.
266. **Schwyzer, M. and Hill, R. L.**, Porcine A blood group-specific *N*-acetylgalactosaminyl transferase. I. Purification from porcine submaxillary glands, *J. Biol. Chem.*, 252, 2338, 1977.
267. **Schwyzer, M. and Hill, R. L.**, Porcine A blood group-specific *N*-acetyl-galactosaminyl transferase. II. Enzymatic properties, *J. Biol. Chem.*, 252, 2346, 1977.
268. **Rearick, J. I., Sadler, J. E., Paulson, J. C., and Hill, R. L.**, Enzymatic characterization of β-D-galactoside $\alpha 2 \rightarrow 3$ sialyltransferase from porcine submaxillary gland, *J. Biol. Chem.*, 254, 4444, 1979.
269. **Sadler, J. E., Rearick, J. I., and Hill, R. L.**, Purification to homogeneity and enzymatic characterization of an α-*N*-acetylglucosaminide $\alpha 2 \rightarrow 6$ sialyltransferase from porcine submaxillary glands, *J. Biol. Chem.*, 254, 5934, 1979.
270. **Roden, L.**, Biosynthesis of acidic glycosaminoglycans (mucopolysaccharides), in *Metabolic Conjugation and Metabolic Hydrolysis*, Vol. 2, Fishman, W. H., Ed., Academic Press, New York, 1970, 345.
271. **Helting, T.**, Biosynthesis of heparin. Solubilization and partial purification of uridine diphosphate glucuronic acid: acceptor glucuronosyltransferase from mouse mastocytoma, *J. Biol. Chem.*, 247, 4327, 1972.
272. **O'Brien, P. J. and Neufeld E. F.**, Biosynthesis of glycoproteins—glycosyl transferases, in *Glycoproteins—Their Composition, Structure and Function*. Part B, Gottschalk, A., Ed., Elsevier, Amsterdam, 1972, 1170.
273. **Babczinski, P. and Tanner, W.**, Involvement of dolicholmonophosphate in the formation of specific mannosyl-linkages in yeast glycoproteins, *Biochem. Biophys. Res. Commun.*, 54, 1119, 1973.
274. **Sharma, C. B., Babczinski, P., Lehle, L., and Tanner, W.**, The role of dolicholmonophosphate in glycoprotein biosynthesis in *Saccharomyces cerevisiae*, *Eur. J. Biochem.*, 46, 35, 1974.
275. **Bretthauer, R. K. and Wu, S.**, Synthesis of the mannosyl-*O*-serine(threonine) linkage of glycoproteins from polyisoprenylphosphate mannose in yeast *(Hansenula holstii)*, *Arch. Biochem. Biophys.*, 167, 151, 1975.
276. **Lehle, L. and Tanner, W.**, Glycosyl transfer from dolichyl phosphate sugars to endogenous and exogenous glycoprotein acceptors in yeast, *Eur. J. Biochem.*, 83, 563, 1978.
277. **Plummer, T. H., Jr. and Hirs, C. H. W.**, The isolation of ribonuclease B, a glycoprotein, from bovine pancreatic juice, *J. Biol. Chem.*, 238, 1396, 1963.
278. **Becker, R. R., Halbrook, J. L., and Hirs, C. H. W.**, Isolation and characterization of ovine ribonuclease A, B, and C from pancreatic secretion, *J. Biol. Chem.*, 248, 7826, 1973.
279. **Ashwell, G. and Morell, A. G.**, The role of sulfate carbohydrates in the hepatic recognition and transport of circulating glycoproteins, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1974, 41, 99.
280. **Krantz, M. J., Holtzman, N. A., Stowell, C. P., and Lee, Y. C.**, Attachment of thioglycosidases to proteins: enhancement of liver membrane binding, *Biochemistry*, 15, 3963, 1976.
281. **Stockert, R. J., Morell, A. G., and Scheinberg, I. H.**, The existence of a second route for the transfer of certain glycoproteins from the circulation into the liver, *Biochem. Biophys. Res. Commun.*, 68, 988, 1976.
282. **Lunney, J. and Ashwell, G.**, A hepatic receptor of ovarian origin capable of binding specifically modified glycoproteins, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 341, 1976.
283. **Baynes, J. W. and Wold, F.**, Effect of glycosylation on the *in vivo* circulating half-life of ribonuclease, *J. Biol. Chem.*, 251, 6016, 1976.
284. **Winkelhase, J. L. and Nicolson, G. L.**, A glycosylantibody: effect of exoglycosidase treatments on autochthonous antibody survival time in the circulation, *J. Biol. Chem.*, 251, 1074, 1976.
285. **Achord, D. T., Brot, F. E., and Sly, W. E.**, Inhibition of the rat clearance system for agalacto-orosomucoid by yeast mannans and by mannose, *Biochem. Biophys. Res. Commun.*, 77, 409, 1977.
286. **Stahl, P., Rodman, J. S., Miller, M. J., and Schlesinger, P.**, Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 1399, 1978.
287. **Schlesinger, P. M., Doebber, T. W., Mandell, B. F., White, R., DeSchryver, C., Rodman, J. S., Miller, M. J., and Stahl, P.**, Plasma clearance of glycoproteins with terminal mannose and *N*-acetylglucosamine by liver non-parenchymal cells: studies with β-glucosidase, *N*-glucosaminidase, ribonuclease B and agalacto-orosomucoid, *Biochem. J.*, 176, 103, 1978.
288. **Kaplan, A., Achord, D. T., and Sly, W. S.**, Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 2026, 1977.

289. **Kaplan, A., Fischer, D., Achord, D., and Sly, W.,** Phosphohexosyl recognition is a general characteristic of pinocytosis of lysosomal glycosidases by human fibroblasts, *J. Clin. Invest.*, 60, 1088, 1977.
290. **Haney, D. N. and Bunn, H. F.,** Glycosylation of hemoglobulin *in vitro*: affinity labeling of hemoglobin by glucose-6-phosphate, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 3534, 1976.
291. **Davis, L. G., Costello, A. J. R., Javaid, J. I., and Brunngraber, E. G.,** Nuclear magnetic resonance studies on the phosphoglycopeptides obtained from rat brain glycoproteins. *FEBS Lett.*, 65, 35, 1976.
292. **Hieber, V., Diffler, J., Myerowitz, R., Schmickel, R. D., and Jourdian, G. W.,** The role of glycosidically bound mannose in the assimilation of β-galactosidase by generalized gangliosidosis fibroblasts, *Biochem. Biophys. Res. Commun.*, 73, 710, 1976.
293. **Distler, J., Hieber, V., Schmickel, R., and Jourdian, G. W.,** Glycopeptide inhibition of the assimilation of β-galactosidase by fibroblasts, *Fed. Proc.*, 36, 653, 1977.
294. **Bahl, O. P., März, L., and Moyle, W. R.,** The role of carbohydrate in the biological function of human chorionic gonadotropin, in *Hormone Binding and Target Cell Activation in the Testis,* Dafau, M. L. and Means, A. R., Eds., Plenum Press, New York, 1974, 125.
295. **Bahl, O. P.,** Human chorionic gonadotropin, its receptors and mechanism of action, *Fed. Proc.*, 36, 2219, 1977.
296. **Rao, Ch. W. and Saxena, B. B.,** Gonadotropin receptors in the plasma membranes of rat luteal cells, *Biochem. Biophys. Acta,* 313, 372, 1973.
297. **Dafau, M. L., Catt, K. J., and Tsuruhara, T.,** A sensitive gonadotropin responsive system; radioimmunoassay of testosterone production by the rat testes, *in vitro, Endocrinology,* 90, 1032, 1972.
298. **Dafau, M. L.,** Gonadotropin stimulation of testosterone production by the rat testis, *in vitro, Biochim. Biophys. Acta,* 252, 574, 1971.
299. **Catt, K. J., Tsuruhara, T., and Dafau, M. L.,** Gonadotropin binding sites of the rat testis, *Biochim. Biophys. Acta,* 279, 194, 1972.
300. **Danzo, B. J.,** Characterization of a receptor for human chorionic gonadotropin in luteinized rat ovaries, *Biochim. Biophys. Acta,* 304, 560, 1973.
301. **Moyle, W. R., Bahl, O. P., and März, L.,** Role of the carbohydrate of human chorionic gonadotropin in the mechanism of hormone action, *J. Biol. Chem.*, 250, 9163, 1975.
302. **Channing, C. P., Sakai, C. N., and Bahl, O. P.,** Role of the carbohydrate residues of human chorionic gonadotropin in binding and stimulation of adenosine 3′, 5′-monophosphate accumulation by porcine granulosa cells, *Endocrinology,* 103, 341, 1978.
303. **Channing, C. P. and Bahl, O. P.,** Role of carbohydrate residues of human chorionic gonadrotropin in stimulation of progesterone secretion by cultures of monkey granulosa cells, *Biol. Reprod.*, 17, 707, 1978.
304. **Bahl, O. P.,** unpublished data.
305. **Fischer, H. D., Gonsalez-Noriega, A., Sly, W. S., and Morre, D. J.,** Phosphomannosyl-enzyme receptors in rat liver. Subcellular distribution and role in intracellular transport of lysosomal enzymes, *J. Biol. Chem.*, 255, 9608, 1980.