RECENT DATA ON THE STRUCTURE OF THE CARBOHYDRATE MOIETY OF GLYCOPROTEINS. METABOLIC AND BIOLOGICAL IMPLICATIONS

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ABSTRACT

The author reviews the information acquired in the last four years on the structure of isoglycans (branched glycans) *N*-glycosidically linked to asparagine. Developments of the structural methodology of conjugated carbohydrates are analysed with reference to the following procedures: sugar determination, chemical (partial acid hydrolysis, acetolysis, hydrazinolysis–nitrous deamination), and enzymatic (exo- and endoglycosidases) cleavage of polysaccharide chains, and methylation.

Several structures of glycans are described. It appears that all possess the common pentasaccharidic core of the following mannotriosido-di-*N*-acetylchitobiose

Manα-(1→3)[Manα-(1→6)]Manβ-(1→4)GlcNAcβ-(1→4)GlcNAcβ-(1→)Asn

on which are conjugated either neuraminyl (or fucosyl)-*N*-acetyllactosamine or oligomannoside residues. It is proposed to term these glycan structures, respectively, *N*-acetyllactosaminic type and oligomannosidic type.

The author demonstrates that these structures are in a good agreement with recent data concerning, on the one hand, the biosynthesis and pathological catabolism of glycoproteins and, on the other hand, the biological role of carbohydrate moieties considered as cell recognition signals.

INTRODUCTION

Four years ago, in a lecture given at the Fifth International Symposium on Carbohydrate Chemistry, I reviewed the present state of knowledge concerning the structure of the carbohydrate moiety of the glycoproteins. The conclusion was that about one century after the discovery of these compounds, no structure was known, except those of some linear glycans (*n*-glycans). Our ignorance was due, partly, to the microheterogeneity of glycoproteins and glycans and partly to the lack of precise methods for structural investigation of the branched glycans (iso-glycans). The review[82] concluded by emphasizing the fact that 'an enormous methodological work

remained to be accomplished' and that 'the chances of success were related to application of the following imperative aims:

(1) Isolation of the different variants of glycoproteins and of each of their glycanic residues.
(2) Application of analytical methods to glycopeptides but never to the native glycoproteins.
(3) Finding new methods for specific cleavage of glycosidic bonds.
(4) Improvement of permethylation methods and of analysis of the monosaccharide *O*-methyl derivatives.
(5) Enlarging research in purification methods of exoglycosidases and of the studies of their specific activity. Search for endoglycosidases.'

Now, four years later, we can say that a considerable amount of results has been accumulated and that this field has evolved in a spectacular manner, due to the fact that many scientists have understood the metabolic, biological and pathological importance of glycoconjugates since the discovery that:

—they are cell surface antigens and their structure or position in the membrane may be modified in transformed cells and in cancerous cells;
—they are virus receptor sites;
—they play an important role in intercellular adhesion and recognition;
—they act on the conformation of peptidic chains;
—they protect the protein moiety against proteolytic attack;
—the glycan groups allow the exit of proteins outside the cell and they regulate the catabolism of the circulating proteins by the liver, and the lifetime of proteins and cells.

That is why more and more laboratories have shown interest in ameliorating the methods of investigation of glycan structure and so we describe in the first part of the present review the progress that has been realized within the last few years in the methodology of glycoconjugates and which has been discussed to a large extent at the Symposium on Glycoconjugates organized by the Centre National de la Recherche Scientifique at Villeneuve d'Ascq (France), in June 1973[83].

Then, on the basis of recent reviews*, original papers and results obtained in our laboratory, we shall describe glycan structures to which the application of new and more precise methods have led. The discussion will only include the structure of carbohydrate moieties of *iso*-glycanic type conjugated to the protein by an 'asparaginyl-*N*-acetylglucosamine' linkage.

Finally, we shall decide whether the proposed structures are in good agreement with the recent data concerning the metabolism and the biological role of isoglycans.

I. Referring to glycoprotein metabolism

It has been well established by Roseman, Schachter, Molnar, Bosmann, Louisot and many others that glycoprotein biosynthesis begins in the rough endoplasmic reticulum where a part of the *N*-acetylglucosamine residues and all of the mannose residues are conjugated. Glycan biosynthesis continues

* References: 40, 41, 53, 58, 75–77, 81, 83, 93, 99, 104, 107, 108, 118, 119, 138, 145.

and terminates in the smooth endoplasmic reticulum and Golgi apparatus where the remaining residues of *N*-acetylglucosamine and all of the galactose, fucose and sialic acid residues are incorporated. Thus, the monosaccharide conjugation is sequential and the isoglycan structure should reflect this biosynthetic mechanism. On the other hand, one assumes that glycoprotein catabolism is realized by lysosomal glycosidases.

II. Referring to the biological role of glycoproteins

Some authors believed that the associations of carbohydrate moieties with proteins were metabolic accidents and that the glycans did not play any important biological role. We could term this point of view: '*gadget's hypothesis*'. For example, Gottschalk[40] claimed that glycan biosynthesis took place under the following conditions: (i) presence in the peptidic chains of the amino acid sequences Asn-X-Ser (or Thr) which codes the conjugation of the first *N*-acetylglucosamine residue on asparagine, or Gly-X-Hyl-Gly which codes the conjugation of the first galactose residue on hydroxylysine; (ii) existence of specific glycosyltransferases in the cells; (iii) presence of glycosyl nucleotide precursors. Under these conditions the glycan composition depends directly on the relative concentrations of sugar nucleotides. If this hypothesis is true, the structure of glycans would be random and never well defined.

Then, timidly, timorously, some people suggested that glycans could very well play an important biological role and in this connection, we could mention the following hypotheses:

(1) *Induction of protein conformation hypothesis*, based on glycan–protein and glycan–glycan interactions, creating repulsion forces of ionic type (i.e. rod conformation of mucins under the influence of sialic acid residues) and attraction forces of ionic, hydrophobic or hydrogen bond type.

(2) *Protein protection against proteolytic attack hypothesis*, based on the observation that numerous glycoproteins lose their resistance toward proteinases after treatment with neuraminidase.

(3) *Orientation and local concentration of water molecules hypothesis*, based on the observation that the modification of the carbohydrate moiety (Galβ1 → 4GalNAcα → Thr) of the antarctic fish antifreeze glycoprotein causes the disappearance of the antifreeze property of the glycoprotein[25, 134]. According to this observation, the water concentration and the movement of ions and of low molecular weight molecules along and across the cell membrane would be related to the glycans of the membrane glycoconjugates and, more specifically, to the relative number of hydrophilic sialic acid residues and of hydrophobic fucose residues.

On the other hand, we shall retain the following hypotheses that are directly related to the concept of isoglycan.

(1) *Passport for export of proteins outside the cells hypothesis.* In 1965, Eylar[30] proposed an interesting hypothesis to explain that the majority of extracellular proteins were glycosylated while the intracellular proteins were rarely so treated. According to the author 'the lack of a specific function of the carbohydrate unit in biologically active glycoproteins suggested a more

general role, and it was proposed that the carbohydrate acts as a chemical label which, upon interaction with a membrane receptor or carrier, promotes the transport of the newly synthesized glycoprotein into the extracellular environment'. This hypothesis did not attribute any biological role to the glycan but implied that the structures of carbohydrate moieties of glycoproteins synthesized by the same cell should be identical.

(2) *Recognition signal hypothesis.* According to this concept which only appeared within the last five years, the isoglycans are recognition signals:

(i) intercellular recognition borne by membrane glycoconjugates;
(ii) cell recognition by proteins due to the carbohydrate groups that act like antennae towards cell membranes.

In this last hypothesis proposed by Winterburn and Phelps[137], 'the attached carbohydrate determines the extracellular fate of the protein molecules and the glycoproteins are synthesized *by* cells *for* cells'. Thus 'the sugars are included in protein structures as a means of coding for the topographical location within an organism'. This is the *target cell hypothesis.* A series of experimental data supports this hypothesis:

(a) Erythropoietin[73] and some pituitary hormones[56, 126] become blind after treatment with neuraminidase and are unable to recognize their target cells.

(b) Removal or modification of sialic acid terminal residues from numerous glycoproteins, in particular, from serum glycoproteins, passes the sentence of death on these compounds: following the radioactive evolution of ^{64}Cu-labelled native and modified coeruloplasmin, Ashwell *et al.*[4, 86] have shown that the sialic free-coeruloplasmin, containing terminal galactose residues, disappears in less than half an hour from the plasma of experimental animals, and that in parallel the radioactivity increases in the hepatocytes. The removal of terminal galactose residues by a β-galactosidase reveals *N*-acetylglucosamine residues in a non-reducing terminal position and maintains the level of asialo-agalactocoeruloplasmin in the plasma. The same phenomenon has been observed with many other desialylated glycoproteins: asialomicroglobulin,-thyroglobulin,-haptoglobin,-fetuin,-orosomucoid[86] and transcortin[6]. Thus the galactose terminal residues are the recognition signal of the hepatocyte membranes for these asialoglycoproteins and, moreover, Pricer and Ashwell[103] have demonstrated that these compounds were recognized by the hepatocyte membrane in showing, by incorporation kinetics, that the radioactivity of the [^{125}I]-iodinated sialic free orosomucoid was fixed very quickly on the proteins of hepatocyte membranes.

Thus, it appears more and more evident that numerous glycoproteins play two important roles. The first is of a *physicochemical* order and concerns the protein moiety conformation, the protein protection against proteinases, the water molecules orientation and concentration and the movement of ions and of small molecules in tissues and in the membrane regions. The second is of *biological order*, the basis of which is essentially the notion of recognition signals brought by glycan groups and consequently the notion of specific structure of these groups.

In order to establish relations between specific biological activities and structures, it was necessary to refine the methodology concerning the struc-

tural analysis of glycans. In the following section we shall examine, in relation to each technique, its improvements, its performance and the limits of its applications.

PROGRESS IN GLYCOPROTEIN METHODOLOGY

I. What is new in sugar determination?

Four years ago, the identification and quantitative determination of glycan monosaccharides were mainly realized by colorimetric determinations and paper chromatography. Now, most authors use gas liquid chromatography (g.l.c.) which is more sensitive and the precision and separating power of which have attained perfection in the last few years (see reviews by Clamp *et al.*[22, 23], Dutton[29] and Zanetta and Vincendon[147]).

Table 1. Conditions for g.l.c. of trifluoroacetyl derivatives of methylglycosylated monosaccharides

Methanolysis 0.5 N HCl 100°C 24 h (neutral sugars and sialic acids)
1.5 N HCl 100°C 48 h (hexosamines)
Evaporation to dryness (under nitrogen)
Trifluoroacetylation (trifluoroacetic anhydride/dichloromethane 1:1), 5 min at 150°C (twice)
Gas liquid chromatography (direct injection of the reaction product):
Glass column (0.3 × 300 cm)
OV-210 5% on Varaport 30
Temperature: 110° to 210° (1°/min)
N_2: 7.5 ml/min

In our laboratory, we use the method of Zanetta *et al.*[146, 147] the principle of which is described in *Table 1*. However, we apply two kinds of methanolysis to a given glycopeptide. The first, using half-normal hydrochloric acid at 100°C for 24 hours, allows determination of the neutral monosaccharides and sialic acids. The second, using 1.5 N HCl at 100°C for 48 hours, liberates quantitatively the hexosamines and the neutral sugars, but causes the destruction of a part of the sialic acid residues (*Table 2*). This procedure of double hydrolysis is necessary because of the particular stability of the *N*-acetylglucosamine–asparagine linkages towards methanolysis we observed using asparaginyl-*N*-acetylglucosamine and asparaginyl-*N*-acetylglucosaminyl-β(1 → 6)-*N*-acetylglucosamine samples kindly furnished by I. Yamashina and R. W. Jeanloz, respectively.

Table 2. Carbohydrate molar composition of transferrin glycopeptides obtained in different conditions (Spik and Fournet[113])

Methods	Gal	Man	GlcNAc	NANA
Colorimetric determinations	5		4.2	3
Paper chromatography	2.4	3	—	—
Gas liquid chromatography after methanolysis (100°C; 48 h)				
0.5 N HCl	2.4	3	3.2	3.2
1.5 N HCl	2.5	3	4	1.8

On the other hand, it is important to point out that the most drastic conditions of methanolysis (1.5 N HCl at 100°C for 48 hours) lead to interference of the amino acids in the neutral monosaccharides region of g.l.c. diagrams which become uninterpretable.

II. What is new about methylation?

Four years ago, it was impossible to identify and determine all monosaccharide methyl ethers that are usually present in the isoglycans: those of galactose, mannose, fucose and *N*-acetylglucosamine. At the present time, the identification and determination of these compounds is possible since all the methyl ethers of galactose, mannose and *N*-acetylglucosamine have been prepared or synthesized. By contrast, the association of mass spectrometry and gas liquid chromatography allows an easier identification of the compounds and offers the possibility of work on microquantities.

(A) *Methyl ether preparation*

We refer the reader to the reviews by Clamp *et al.*[23], Dutton[29], Fournet *et al.*[32,33], Haverkamp and Vliegenthart[44], Kochetkov and Chizhov[59], Lindberg[71], Marshall and Neuberger[77] and, concerning the preparation and chromatographic separation of glucosamine methyl ethers, to Hakomori *et al.*[120], thus limiting the presentation to our contribution in this field.

Methods of preparation by g.l.c. of all galactose and mannose methyl derivatives have been performed in our laboratory. The principle of these procedures developed by Fournet[32] is based not on the organic synthesis of each derivative but on the preparative g.l.c. of methyl ethyl mixtures obtained by partial methylation of methyl mannosides and galactosides, according to Kuhn *et al.*[62] or Handa and Montgomery[42]: dimethyl formamide–silver oxide–methyl iodide for eight hours for the di-, tri- and tetramethyl derivatives, and 1.5 h for the mono-, di- and trimethyl derivatives. Under the conditions described in the legends of *Figures 1* to *3*, we are able to isolate in

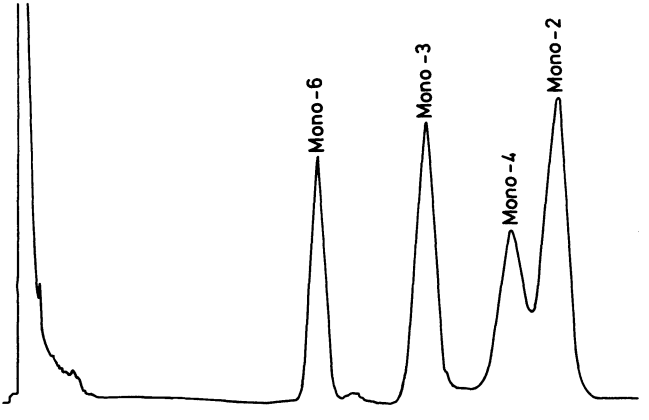


Figure 1. The g.l.c. of *O*-acetyl-methyl-mono-*O*-methyl-α-D-mannosides. Glass column (0.3 × 300 cm). 3 p. 100 ECNSS-M on Chromosorb W (60–80 mesh). Temperature: 170°C. Carrier gas: nitrogen. Gas flowrate: 30 ml/min.

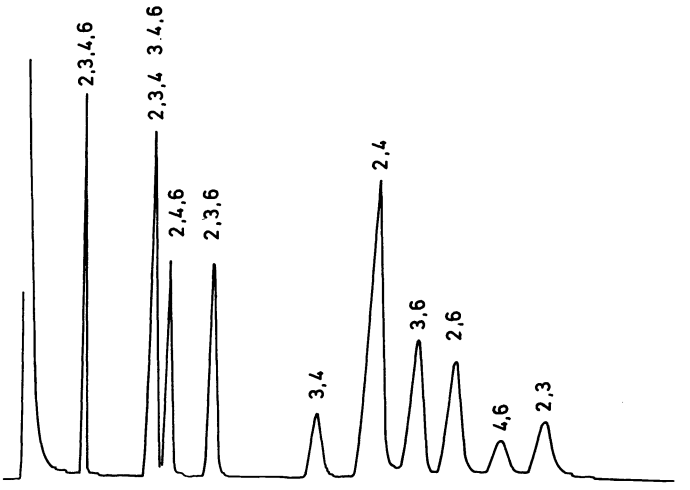


Figure 2. The g.l.c. of methyl-α-D-mannoside di-, tri- and tetra-*O*-methyl derivatives. Glass column (0.3 × 300 cm). 3 p. 100 Carbowax 6000 on Chromosorb W-AW-HMDS (60–80 mesh). Temperature: 170°C. Carrier gas: nitrogen. Gas flowrate: 30 ml/min.

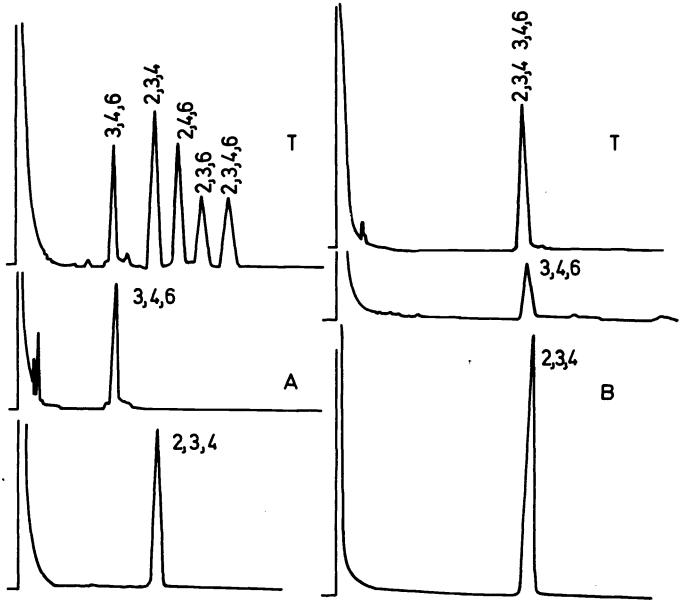


Figure 3. The g.l.c. of methyl-2-TMS-3,4,6-tri-*O*-methyl- and 6-TMS-2,3,4-tri-*O*-methyl-α-D-mannosides (A) and of methyl-3,4,6- and 2,3,4-tri-*O*-methyl-α-D-mannosides (B) obtained from the former TMS derivatives by boiling in a methanol/water mixture (1:1, v/v). Glass column (0.3 × 300 cm). 3 p. 100 Carbowax 6000 on Chromosorb W-AW-HMDS (60–80 mesh). Temperature: 150°C. Carrier gas: nitrogen. Gas flowrate: 30 ml/min.

one step all the mono-, di-, tri- and tetramethylated mannose derivatives, except the 2,3,4- and 2,4,6-tri-*O*-methyl derivatives that can be separated in a second step after trimethylsilylation[32, 33, 35, 36].

On the same principle and under the conditions described in the legend of *Figure 4*, we are able to isolate, in a first step, the methyl derivatives of galactose except the 2- and 3-mono-*O*-methyl derivatives, and the 2,3,6-, 2,4,6- and 3,4,6-tri-*O*-methyl derivatives that are separated, in a second step after *O*-acetylation[34].

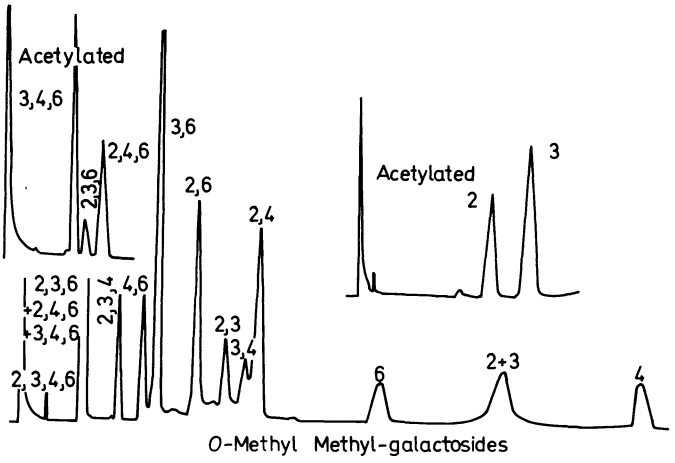


Figure 4. The g.l.c. of *O*-methyl ethers of α-methyl galactoside. Experimental conditions: (i) for *O*-methyl ethers: see *Figure 2*; (ii) for *O*-acetylated *O*-methyl ethers: see *Figure 1*.

All the methyl derivatives we obtained, have been identified by mass spectrography by H. Mayer (Max Planck Institut, Freiburg im Breisgau).

(B) *Identification and determination of monosaccharide methyl esters present in permethylated glycans*

We apply currently in our laboratory the method described by Fournet[32, 33] the principle of which is illustrated in *Figures 5* and *6*. Preliminary fractionation of the electrophoretic 'neutral fraction' by thin layer chromatography (t.l.c.) is generally applied once only at the beginning of the determinations in order to judge the complexity of the mixture of methyl ethers. Then identification and determination of neutral monosaccharide methyl ethers are carried out by direct g.l.c. of the total 'neutral fraction'.

The preparation of pure methyl derivatives allows the determination of molar responses that are, as shown in *Table 3* in reference to di-, tri- and tetramethyl mannose derivatives, very different, varying from 1 to 4 for the extreme values.

(C) *Conclusions*

The question of the analysis of monosaccharide methyl ethers constituting the classical isoglycans is virtually resolved except that of methyl derivatives

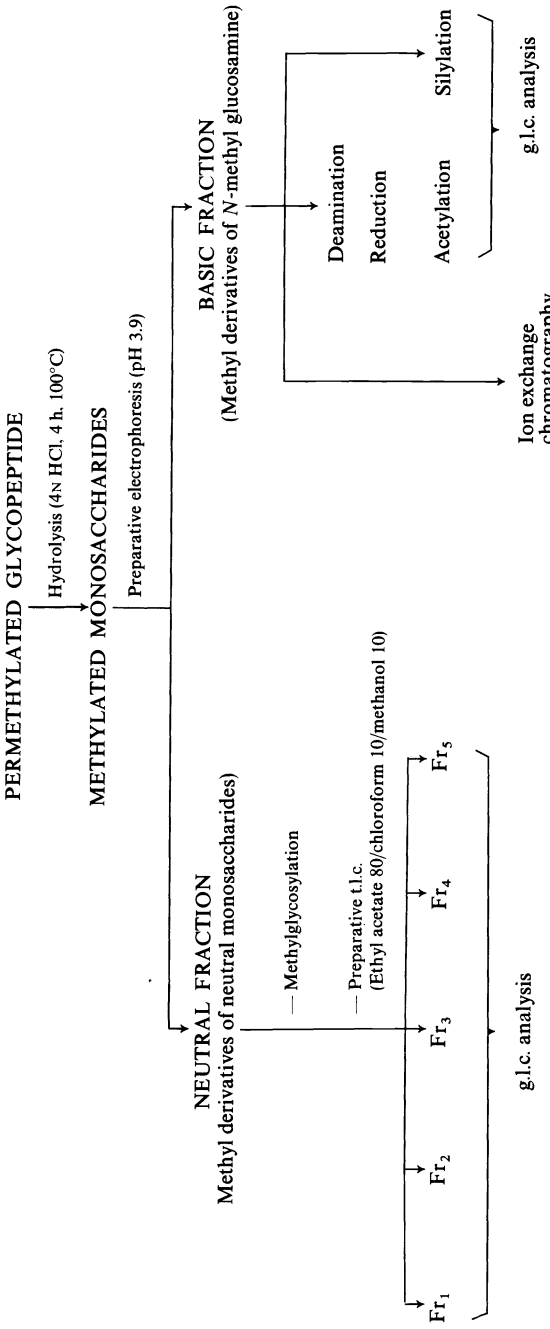


Figure 5. Fractionation of methyl ethers present in the hydrolysates of permethylated glycans or glycopeptides.

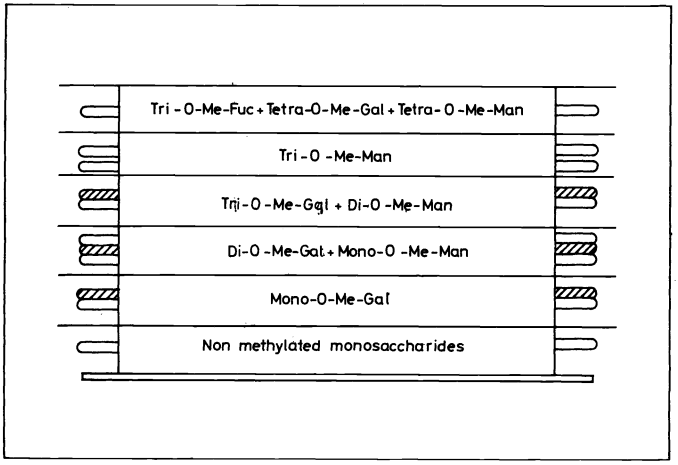


Figure 6. The t.l.c. of a mixture of methyl ethers of fucose, galactose and mannose. Experimental conditions: Silicagel G. Solvent: ethyl acetate/chloroform/methanol: 80:10:10. Revelation by 5% sulphuric acid.

of sialic acid. At the present time, the association of mass spectrometry (m.s.) and g.l.c. should allow us to tackle all the identification problems relating to these compounds and to miniaturize the procedures in order to apply them to very small quantities of substances as is done with membrane glycoconjugates, for instance. There remains, however, a problem when methylation

Table 3. The g.l.c. molar responses of some *O*-methyl ethers of methyl-α-D-mannoside (Fournet[34])

2,3,4,6-tetra-*O*-	1	2,4-di-*O*-	0.80
2,3,4-tri-*O*-	0.75	3,6-di-*O*-	0.57
3,4,6-tri-*O*-	0.39	2,6-di-*O*-	0.54
2,4,6-tri-*O*-	0.50	4,6-di-*O*-	0.49
2,3,6-tri-*O*-	0.90	2,3-di-*O*-	0.27
3,4-di-*O*-	0.53		

takes place on glycoprotides in which the carbohydrate moiety is *O*-glycosidically linked to serine or threonine residues. In this case, the alkaline conditions of the permethylation induce the liberation of the glycan by a β-elimination mechanism and sometimes its degradation by a peeling reaction. So it becomes necessary to liberate and to isolate first the reduced glycan according to Carlson's procedure[18] and to permethylate it afterwards.

III. What is new about the chemical cleavage of glycosidic linkages?

(A) *Partial hydrolysis and acetolysis*

Partial acid hydrolysis by sulphonated polystyrene resins, and acetolysis of glycoprotides have been standardized in our laboratory by Bayard[8, 9, 12] who performed a rapid, sensitive and reproducible method of chromatographic mapping[12] derived from Ingram's finger printing procedure. This

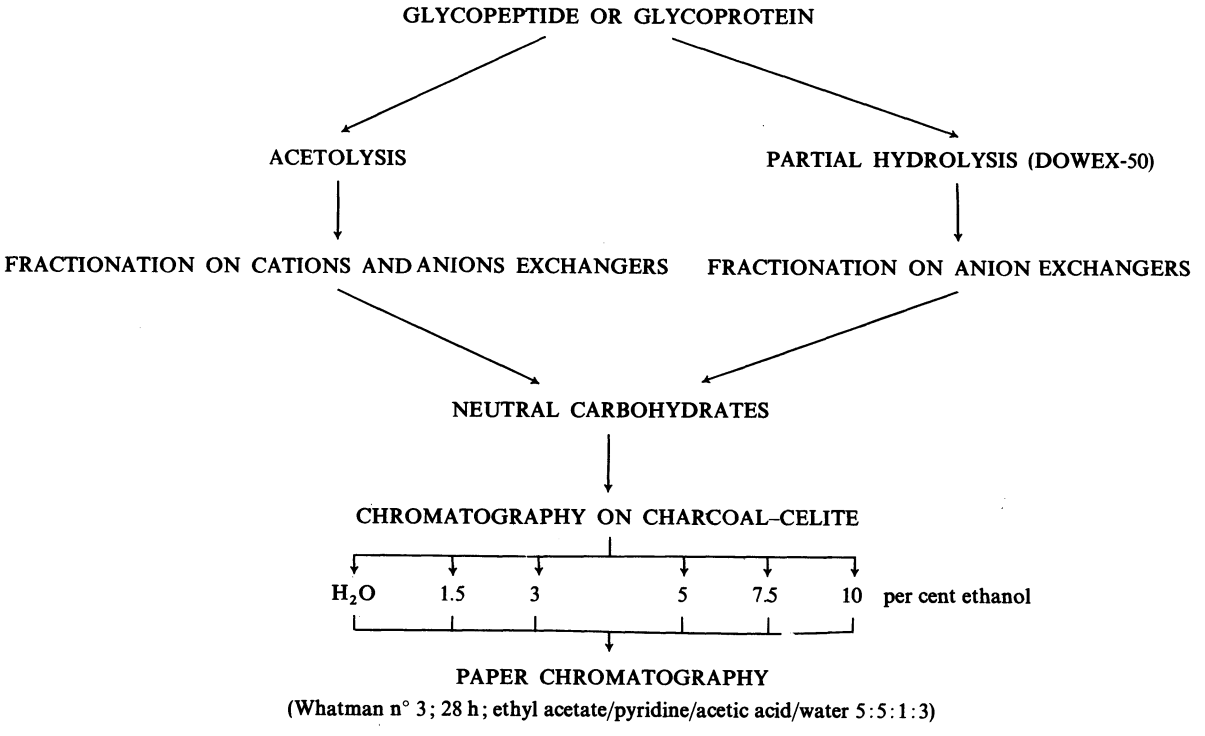


Figure 7. Procedure for chromatographic maps of neutral oligosaccharides obtained by acetolysis or partial hydrolysis of glycoprotids.

method associates charcoal column chromatography according to Whistler and Durso[136] with one-dimensional paper chromatography. It provides information on the monosaccharide sequences in the glycans and allows us, as an alternative, to compare the structures of glycoproteins of different origins.

The principle of this technique is illustrated in *Figure 7* which concerns the neutral oligosaccharides liberated by partial hydrolysis using Dowex-50 and by acetolysis. This procedure requires quantities of about 5 to 10 mg glycan.

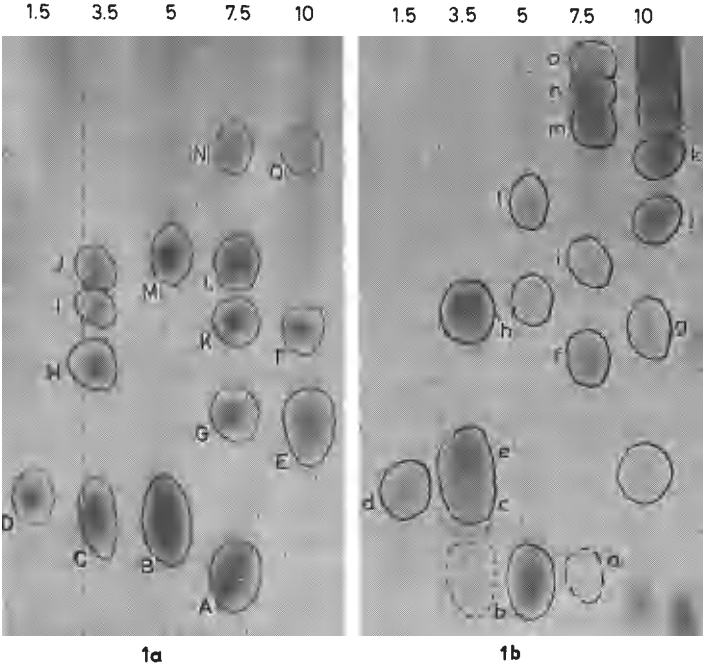


Figure 8. Chromatographic maps of oligosaccharides present in acetolysates (1a) and in partial hydrolysates (1b) of asialo-ovomucoid 1. Chromatography on Whatman paper n° 3 for 28 h. Solvent system: ethyl acetate/pyridine/acetic acid/water (5:5:1:3). Chromatograms are revealed with aniline oxalate reagent. 0.5, 1.5, 3, 5 and 10: ethanol concentration of charcoal column eluates. Letters refer to the oligosaccharides the structure of which is described in *Figures 9* and *10*.

Figure 8 shows the chromatographic maps of ovomucoid acetolysate and partial hydrolysate (see the structure of ovomucoid glycan in *Figure 32*). One can see that about 15 spots are well defined on the paper chromatogram and strictly localized in the different ethanolic fractions. One observes, on the other hand, that the chromatographic maps of hydrolysate and acetolysate are not superimposable but are complementary. Indeed, the structures of the 14 oligosaccharides present in the acetolysate (*Figure 9*) and the 12 oligosaccharides present in the partial hydrolysate (*Figure 10*) have been determined. They are quite different, except four of them.

In this way, information about monosaccharide sequences can be easily and rapidly obtained as it can with haemoglobin peptide sequences and tRNA nucleotidic sequences. Thus, this procedure is *analytical* and one can verify, in this connection, that the oligosaccharides obtained from ovomucoid are related to the structure of the glycan of this glycoprotein (*Figure 32*).

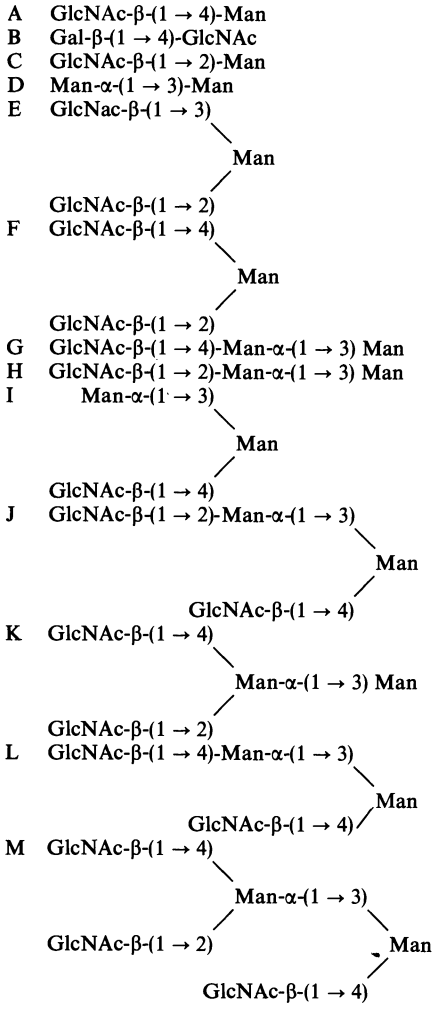


Figure 9. Structure of neutral oligosaccharides isolated from ovomucoid acetolysates.

By contrast, elution of oligosaccharides from charcoal being quantitative in each of the ethanolic fractions, the method becomes *comparative* and reveals the profound (*Figure 11*) or slight (*Figure 12*) differences that exist between the structures of glycans from various sources. *Figure 12* shows[51], for example, that the glycans of the ovomucoid variants 1 and 2 possess very similar structures and that the only difference concerns an oligosaccharide

a GlcNAc-β-(1 → 4)-Man
b Gal-β-(1 → 4)-GlcNAc
c_1 Man-β-(1 → 4)-GlcNAc
c_2 GlcNAc-β-(1 → 2)-Man
d Man-α-(1 → 3)-Man
e Man-α-(1 → 6)-Man
f Man-α-(1 → 3)-Man-β-(1 → 4)-GlcNAc
g Man-α-(1 → 6)-Man-β-(1 → 4)-GlcNAc
h Man-α-(1 → 3)
Man
Man-α-(1 → 6)
i Man-α-(1 → 3)
Man-β-(1 → 4)-GlcNAc
Man-α-(1 → 6)
j GlcNAc-β-(1 → 2)-Man-α-(1 → 3)
Man
GlcNAc-β-(1 → 2)-Man-α-(1 → 6)
k GlcNAc-β-(1 → 2)-Man-α-(1 → 3)
Man-β-(1 → 4)-GlcNAc
Man-α-(1 → 6)

Figure 10. Structure of neutral oligosaccharides isolated from ovomucoid partially hydrolysed by Dowex-50.

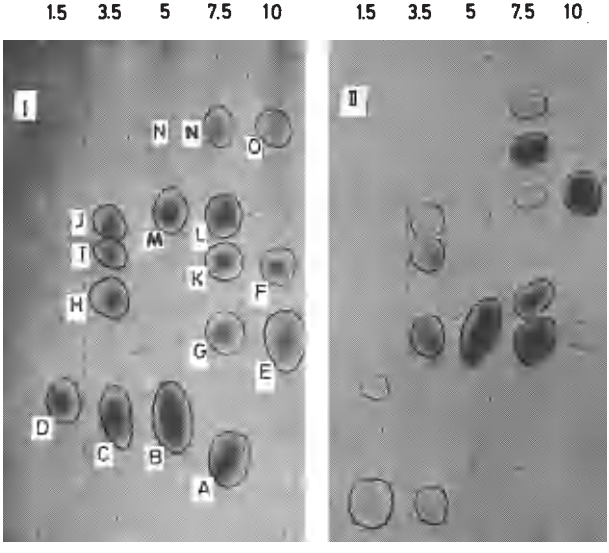


Figure 11. Comparative chromatographic maps of oligosaccharides present in acetolysates of asialo-ovomucoid 1 (I) and human serotransferrin (II) glycopeptides. For experimental conditions, see *Figure 8.*

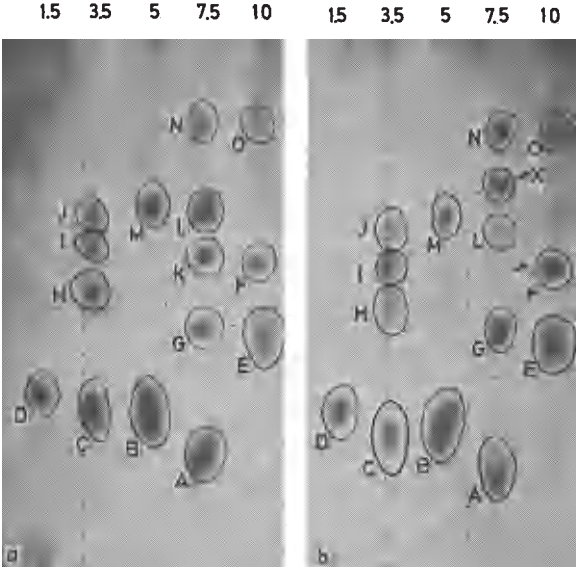


Figure 12. Comparative chromatographic maps of oligosaccharides present in acetolysates of asialo-ovomucoids 1(a) and 2(b). For experimental conditions, see *Figure 8.*

sequence: oligosaccharide K is present in variant 1 and oligosaccharide X. the structure of which is still unknown, in variant 2.

(B) *Hydrazinolysis–nitrous deamination of glycans*

Four years ago, I evoked[82] an interesting procedure of specific cleavage of *N*-acetylglucosaminyl and -galactosaminyl bonds proposed by Matsushima and Fujii[78] in 1957 and applied by these authors[38, 78] and by others[47, 140, 144] to glycosaminoglycan studies. The principle of this procedure which is based on Akabori's method for the determination of C-terminal amino acids is the following: the *N*-acetylhexosamine residues are first deacetylated by anhydrous hydrazine (or by 2.5 M sodium hydroxide at 90° for 2.5 h according to Isemura and Schmid[50], then deaminated by nitrous acid; a series of molecular rearrangements provokes spontaneous cleavage of hexosaminyl bonds with replacement of hexosamine residues by 2,5-anhydro-hexose residues[27, 38, 48, 78]. Finally the following compounds are obtained: (i) free 2,5-anhydro-hexose residues coming from the *N*-acetylhexosamine residues located at the non-reducible end of glycan moieties or (and) in oligo-*N*-acetylhexosaminyl sequences; (ii) oligosaccharides that possess a 2,5-anhydro-hexose residue at the reducible terminal position. These compounds are easily revealed on chromatograms by urea or resorcinol reagents. *Figure 13* illustrates the principle of the procedure applied to a 'classical' asialoglycan linked to the peptidic moiety by an asparaginyl-*N*-acetylglucosamine bond.

Some authors have studied the reaction mechanism and conditions on oligosaccharide models. To our knowledge, the procedure has been applied only to the orosomucoid by K. Schmid *et al.*[50]. In our laboratory, Bayard

has entirely resolved the problem and we have been using this method regularly for the last two years.

In a first step, Bayard[8, 10] defined the conditions of a quantitative deacetylation of *N*-acetylhexosamine residues by using the methyl *N*-acetyl-β-D-glucosaminide : anhydrous hydrazine/glycoprotein ratio : 1 : 10, 100°C for 30 h.

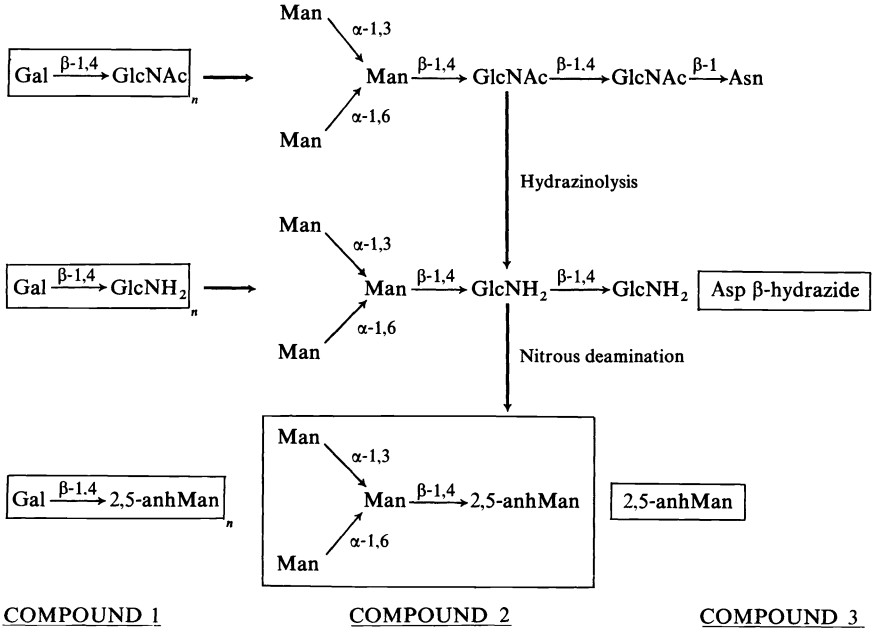


Figure 13. Hydrazinolysis–nitrous deamination of a 'classical' asparaginyl-asialoglycan. Compound 1: mannotriosido-2,5-anhydromannose. Compound 2: galactosido-2,5-anhydromannose. Compound 3: 2,5-anhydromannose.

In these conditions, hydrazinolysis liberates, under *N*-deacetylated form, the glycans that are conjugated to the proteins by an asparaginyl-*N*-acetylglucosamine linkage (*Figure 13*)[8, 10, 57, 144]. On the contrary, because the *O*-glycosidic bonds are stable towards hydrazine, the *O*-glycosidically conjugated glycans remain, under *N*-deacetylated form, attached to an amino acid hydrazine residue[8, 10]. Thus the method allows us to distinguish the glycans *O*- and *N*-glycosidically linked to the peptidic chains.

In a second step, Bayard applied the method to different glycoproteins and performed the following techniques[3, 10]: *N*-deacetylated glycan purification; nitrous deamination; fractionation, identification and quantitative determination of cleavage products by paper chromatography. Applied to the sialic acid free-orosomucoid the procedure leads to the formation of 2,5-anhydromannose, galactosido-2,5-anhydromannose and mannotriosido-2,5-anhydromannose in 1 : 4 : 1 ratio. These results confirm the existence of four *N*-acetyllactosamine residues in orosomucoid (see *Figure 31*).

Finally, as the hydrazinolysis–nitrous deamination is quantitative, it is possible to obtain comparative chromatographic maps (*Figure 14*) which

show the presence of the following oligosaccharide sequences common to numerous glycoproteins: di-*N*-acetylchitobiose, *N*-acetyllactosamine and mannotriosido-*N*-acetylglucosamine.

The hydrazinolysis–nitrous deamination procedure appears to be a very valuable method in the study of glycan structures. It can be applied equally

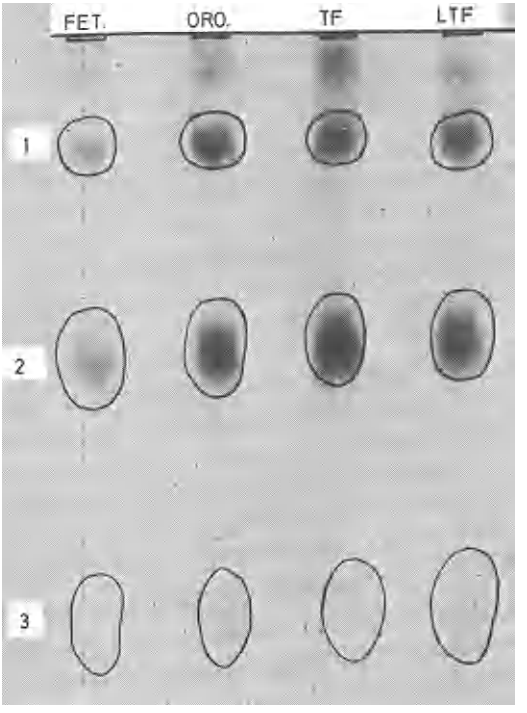


Figure 14. Comparative paper chromatography of the compounds obtained by hydrazinolysis–nitrous deamination of fetuin (FET), orosomucoid (ORO), serotransferrin (TF) and lactotransferrin (LTF). Experimental conditions: Whatman 3 paper; pyridine/ethyl acetate/acetic acid/water (5:5:1:3); 10 h; phosphoric acid–urea reagent. 1: mannotriosido-β-1,4-anhMan; 2: galactosido-β-1,4-anhMan; 3: 2,5-anhydromannose(anhMan).

to glyco-amino acids, to glycopeptides or to glycoproteins. It is very sensitive since it needs only a few milligrams of compound. Contrary to Isemura and Schmid's alkaline cleavage procedure, it liberates completely the non-degraded and quantitatively *N*-deacetylated glycans (*Table 4*). Lastly, it allows us to obtain quickly the tetrasaccharidic core of glycoproteins, mannotriosido-2,5-anhydromannose, and to demonstrate by the successive action of α-, then of β-mannosidases the presence of a β-mannosidic linkage.

(C) *Action of alkalis*

It is well known since the works of Meyer and Pigman, that alkaline solutions cleave the *O*-glycosidic bonds of serine and threonine by a β-elimination process. Several research programmes have been undertaken since 1963

Table 4. Lability of *N*- and *O*-glycosidic linkages and of the carbohydrate moiety towards alkaline solutions and hydrazine

	ACTION OF ALKALINE SOLUTIONS				HYDRAZINOLYSIS
	0.1 M NaOH 0.3 M $NaBH_4$ 4° or 20°C; 1–7 days	0.05 N KOH 1.0 M KBH_4 45°C; 15–24 h	0.2 M NaOH 1.0 M $NaBH_4$ 100°C; 10–20 h	2.5 M NaOH 90°C; 2–5 h	NH_2—NH_2 100°C; 30 h
	Meyer *et al.* (1963) Pigman *et al.* (1964)	D. Carlson (1968)	Marshall (1973)	Isemura and Schmid (1971)	Bayard and Montreuil (1972)
Carbohydrate → Asn	?	10%	90–100%	< 100%	100%
Carbohydrate → Ser (Thr)	90–100%	95–100%	95–100%	?	0
Carbohydrate → Hyl (Hyp)	0	0	0	?	0
Carbohydrate moiety	Peeling reaction	No peeling reaction	Peeling reaction	66% *N*-deacetylated. Peeling reaction possible	N-deacetylated. No peeling reaction

to understand the mechanism of the reaction, on the one hand, and to avoid the peeling-reaction of the liberated glycans on the other hand (see reviews by Aminoff *et al.*[1], Carlson[18], Neuberger *et al.*[92, 93] and Pigman *et al.*[100]). This work has led Carlson[18] to propose a procedure (*Table 4*) that allows us to obtain reduced glycans protected from subsequent degradation by the presence of a polyol residue in the terminal position. One cannot, however, avoid the cleavage of some asparaginyl-*N*-acetylglucosamine bonds that are unstable in an alkaline medium at elevated temperatures[5].

(IV) What is new about glycosidases?

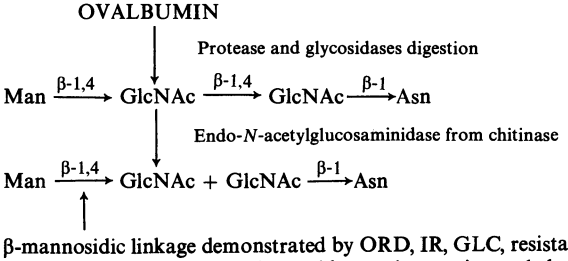
(A) *Exoglycosidases*

Four years ago the sources of glycosidases were rare and it was moreover difficult to foresee the activity and the specificity of exoglycosidase action that might vary with the origin of the enzymes and with the structure of the carbohydrate moiety to which the terminal non-reducing monosaccharide residue is attached.

At the present time, active preparations are commercialized and the various parameters of their activity and specificity are well defined (see recent reviews in ref. 90).

It is important, however, to avoid extrapolating, generalizing and postulating, for instance that galactose residues are always β-glycosidically linked and that the mannose or fucose residues are always α-glycosidically conjugated. The results obtained by some authors require prudence: (i) Nelsestuen and Sutties[91] have characterized α-galactose residues in bovine prothrombin (α/β-galactose ratio:1:1); (ii) Buddecke *et al.*[135] have demonstrated that there exists about two per cent α-galactosidic bonds and six per cent α-glucosidic bonds in the G_{M1}-ganglioside from cattle brain.

In this connection, the demonstration by Tarantino *et al.*[124, 129, 131] and by many other authors[69, 122, 133] that β-mannosidic bonds exist in numerous glycoproteins is of capital importance from structural, metabolic and biological points of view. This fact has been proved for the first time on the ribonuclease B and on the ovalbumin[124] by an experimentation which is summarized in *Figure 15* and which is susceptible to be extended to numerous glycans. Moreover, the fact can be rapidly demonstrated by using the hydrazinolysis–deamination procedure (see above) or by applying to the



β-mannosidic linkage demonstrated by ORD, IR, GLC, resistance to α-mannosidase and cleavage by β-mannosidase from hen oviduct, urine or pineapple bromelin devoid of α-mannosidase activity.

Figure 15. Demonstration by Tarentino *et al.* that mannose is β-glycosidically linked to the di-*N*-acetylchitobiose residue in hen ovalbumin.

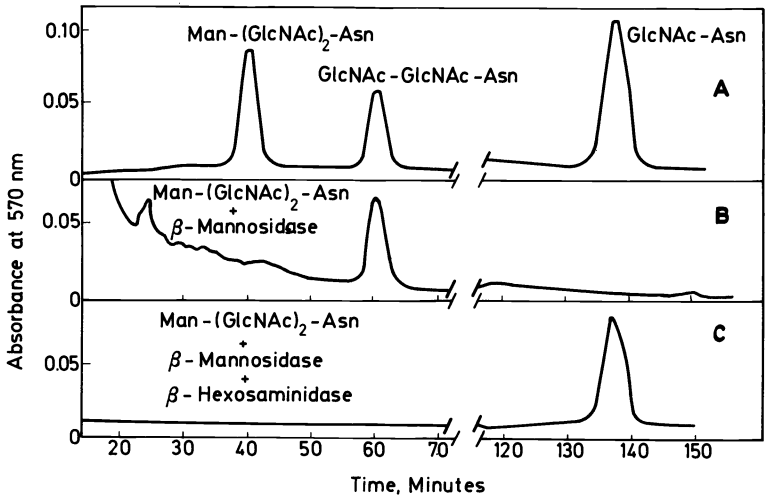
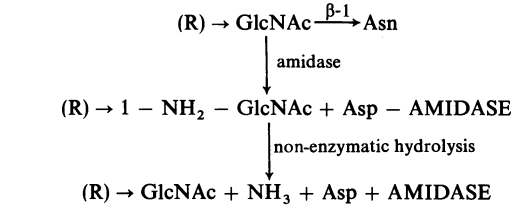


Figure 16. Identification of the glycosidic digestion products from ovalbumin glycopeptide Man → GlcNAc → GlcNAc → Asn. Beckman 120 C amino acid analyser with column of PA-28 cation exchange of resin.

Manβ-(→ 4)GlcNAcβ-(1 → 4)GlcNAcβ-(1 →)Asn sequence an elegant chromatography procedure on cation exchangers described by Li *et al.*[69] and illustrated in *Figure 16*. Snail[123], human urine[70], pineapple bromelain[68], hen oviduct[125], *Turbo cornutus* and *Charonia lampas*[90], are good sources of β-mannosidases.

(B) *Endoglycosidases*

Four years ago, I regretted the lack of endoglycosidases that put us in an inferior position towards the protein structure specialists who possessed numerous endopeptidases. At the present time, two types of endoglycosidases have been described that are active on the isoglycans linked by an asparaginyl-*N*-acetylglucosamine bond. One liberates the complete glycan and the other



Sources of amidase: Ram epididymis (Eylar, 1966)
Hen oviduct (Tarentino, 1969)
Rat liver (Conchie, 1969)
Hog serum and kidney (Yamashina, 1972)
Snails (Kaverzneva, 1973)

Figure 17. Mechanism of 4-L-aspartylglycosylamine amido hydrolase (glycoaspartamidase) action and sources of enzyme.

Table 5.

GlcNAc $\xrightarrow{\beta\text{-}1,4}$ GlcNAc $\xrightarrow{\beta\text{-}1}$ Asn

This sequence is present in the following glycoproteins:

α-Amylase (*A. orizae*)
Bromelin
Deoxyribonuclease A (bovine)
Fetuin
IgG (human and bovine)
IgM, IgE (human)
Invertase (*S. cerevisiae*)
Lactotransferrin (human)
Orosomucoid
Ovalbumin
Ovomucoid
Prothrombin (bovine)
Ribonuclease B (bovine and porcine)
Serotransferrin (human)
Taka amylase A
Thyroglobulin (human, calf, porcine)

the glycan shorn of the *N*-acetylglucosamine residue conjugated to the asparagine residue.

(1) The first enzyme is a glyco-aspartamidase (see Yamashina's review[143]). It cleaves the *N*-acetylglucosamine–asparagine amide bond provided that the latter is not conjugated to any amino acid. The 4′-L-aspartyl-*N*-acetylglucosylamine amide hydrolase sources and the mechanism of its action, elucidated by Tanaka *et al.*[127], are described in *Figure 17.*

(2) The second type of endoglycosidases concerns enzymes of *Diplococcus*

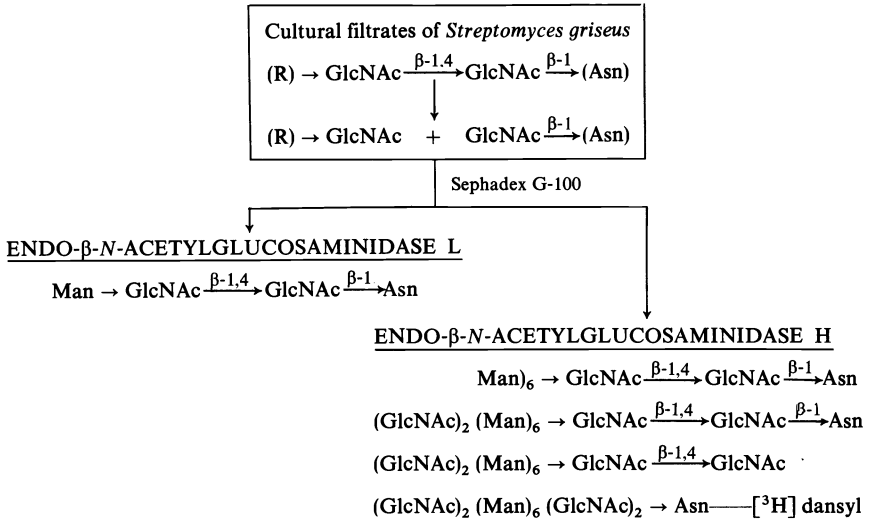


Figure 18. Properties of the endo-β-*N*-acetylglucosaminidases L and H from *Streptomyces griseus.*

pneumoniae[60, 71, 89] and *Streptomyces griseus*[128, 131, 132] that act as endo-β-*N*-acetylglucosaminidases on β-1,4-*N*-acetylglucosaminide bond of the di-*N*-acetylchitobiose residue present in numerous isoglycans (*Table 5*). These preparations can be fractionated (*Figure 18*) by Sephadex G-100 chromatography into an endo-β-*N*-acetylglucosaminidase L which is active only on asparaginyl oligosaccharides of low molecular weight, and into an endo-β-*N*-acetylglucosaminidase H which is active on compounds of high molecular weight. This last form acts on the [^{3}H]-dansylaspartyl oligosaccharides, a property which allows its characterization and the determination of its activity[128]. A similar enzyme had also been characterized in chitinase preparations[130] and more recently in porcine and rat liver[94].

INFORMATION ON ISOGLYCAN STRUCTURES

Four years ago, no structure of isoglycan was established with certainty. Numerous schemes of structure were, however, proposed, concerning in particular orosomucoid, ovalbumin and ovomucoid, but all were based on the application of two or three methods of structure determination, generally periodic oxidation and the use of glycosidases. Any of them survived to the application of the precise methods performed within the last few years. At the present time, the structures of numerous isoglycans have been determined and may be considered as definitive, in so far as one can be sure that a scientific problem is ever definitively resolved.

Before reviewing the present state of knowledge of the structure of isoglycans, I should like to present first the results we obtained in our laboratory concerning serotransferrin, the entire structure of both glycans of which we have determined by application of the following procedures: acetolysis, partial acid hydrolysis, hydrazinolysis–nitrous deamination, methylation and enzymatic hydrolysis. Then, on the basis of the structure of the serotransferrin glycans, I shall describe the isoglycan structure of other glycoproteins recently proposed.

(I) Structure of serotransferrin isoglycans

Serotransferrin (STF) is a glycoprotein the protein fraction of which is formed of a single peptidic chain with valine on the N-terminal position and half-cystine on the C-terminal position. Two isoglycans[52, 115] whose monosaccharide composition is given in *Table 2* are conjugated to the protein by an asparaginyl-*N*-acetylglucosamine linkage[110, 115]. Serotransferrin fixes reversibly two iron atoms and it has been demonstrated[31, 54, 87, 88] that the ferriprotein is able to associate with the reticulocyte membrane proteins from which it separates after ceding the iron it transported. Although the STF fixation mechanism on the membranes is not yet known, one can imagine that the glycans carry the recognition signal for these membranes.

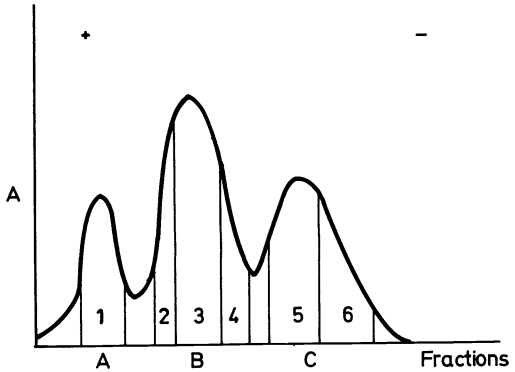
In our laboratory, in order to avoid an eventual error due to structural differences between the two glycans, we have, in a first step, isolated them in the form of glycopeptides and we have, in a second step, determined their structure[112, 114, 116, 117]. We shall call Glycan I and Glycan II the carbohydrate moieties conjugated toward the N-terminus and the C-terminus, respectively.

(A) *Glycopeptide isolation*

In a first series of papers[110, 115], Spik *et al.* have shown that asialo-STF, hydrolysed by pronase, yielded two glycodipeptides in which the glycan was linked by an asparaginyl-*N*-acetylglucosamine bond. The structure of the peptide fractions is Asn-Lys for the one, and Ser-Asn for the other. More recently[116], Spik *et al.* hydrolysed the native STF with pronase and applied the free flow electrophoresis technique to the fractionation of hydrolysates.

APO-SEROTRANSFERRIN

Hydrolysis by pronase
Purification on cation and anion exchangers
Free flow preparative electrophoresis pH 2.4
Detection of carbohydrates with phenol–H_2SO_4 reagent



	A 100 mg Ser–Asn	B 400 mg Ser–Asn	C 420 mg Asn–Lys
NANA	3	2	2
Gal	3	2	2
Man	3	3	3
GlcNAc	5	4	4

Figure 19. Fractionation by free flow preparative electrophoresis (Elphor Vap II apparatus) and composition of the glycopeptides obtained by pronase digestion of human serotransferrin (Spik *et al.*, 1974).

In *Figure 19* is described the procedure and the characteristics of the isolated glycopeptides. The results obtained can be summarized as follows:

(1) The free flow electrophoresis yields three glycopeptides that are electrophoretically homogeneous.

(2) Glycopeptides B and C differ in their amino acid composition but are identical in their carbohydrate composition. Glycopeptides A and B possess the same amino acid composition but are different in their carbohydrate composition: Glycopeptide A possesses one *N*-acetyl-neuraminic acid, one galactose and one *N*-acetylglucosamine residue more than glycopeptides B and C. These differences in amino acid and sialic acid composition of glycopeptides A, B and C explain their electrophoretic behaviour.

(3) By associating these results with some data previously obtained[19, 20] about the peptidic structure of two glycopeptides isolated from tryptic and

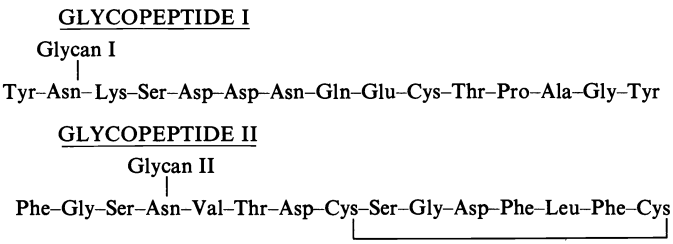


Figure 20. Peptidic sequences of glycopeptides I and II isolated from hydrolysates of human serotransferrin.

chymotryptic hydrolysates of STF (*Figure 20*), we can identify and replace in the peptide chain the glycopeptides A, B and C. Indeed, by considering as markers the serine and lysine residues on the one hand, and the half-cystine residue on the other hand, we can conclude (i) that glycopeptide C comes from the 'inner' fraction of the peptidic chain and that its glycan is Glycan I; (ii) that glycopeptides A and B are located on the C-terminal part of the peptidic chain and that their glycans are Glycans II (*Figure 21*).

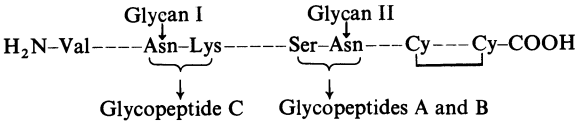


Figure 21. Position of the glycans I and II in the polypeptide chain of human serotransferrin.

(B) *Structure of STF glycans*

(1) *Glycopeptides B and C glycans.* Application of the above-mentioned procedures to the glycans of glycopeptides B and C leads to the conclusion that they are identical and possess the structural pattern described in *Figure 22*.

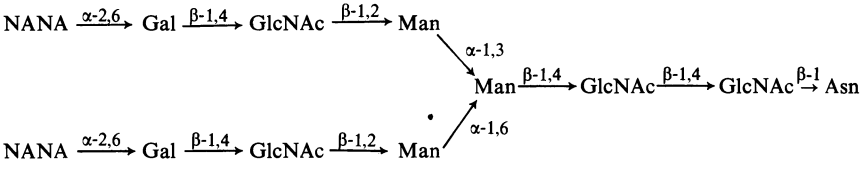


Figure 22. Structure of glycans I and II of glycopeptides C and B, respectively (see *Figures 21* and *23*).

This structure has been established on the basis of the following experimental results;

(a) Mild acid hydrolysis carried out in different conditions liberates all sialic acid residues and, among numerous other oligosaccharides, *N*-acetyllactosamine, the mannotriose fragment and aspariginyl-*N*-acetylglucosamine.

(b) Acetolysis gives the following interesting oligosaccharides: Galβ-(1 → 4) GlcNAc; GlcNAcβ-(1 → 2)Man; Manβ-(1 → 4)GlcNAc; NANAα-

(2 → 6)Galβ-(1 → 4)GlcNAc; NANAα-(2 → 6)Galβ-(1 → 4)GlcNAcβ-(1 → 2)-Manα(1 → 3)Man.

(c) Hydrazinolysis–nitrous deamination of asialo-glycopeptides B and C leads to the formation of three components (*Figure 4*) which were isolated by preparative paper chromatography and identified as 2,5-anhydromannose (anhMan), Galβ-(1 → 4) anhMan and Manα(1 → 3) [Manα-(1 → 6)] Manβ-(1 → 4)anhMan, in a 1:2:1 molar ratio. As no *N*-acetylglucosamine residue was found in an external position by methylation and by enzymatic hydrolysis, the presence of free 2,5-anhydromannose demonstrates that the sequence GlcNAc → GlcNAc is present in both glycans. The β-mannosidic linkage has been proved by the use of a β-mannosidase from pineapple bromelain devoid of α-mannosidase activity, after removal of α-mannose residues by α-mannosidase.

(d) Exhaustive methylation of sialo and asialo-glycopeptides B and C shows (*Figure 23* and *Table 6*) that (i) the same methylated neutral sugars in identical molar ratios are found in both glycopeptides; (ii) the galactose residues are substituted in 6-position by sialic acid residues; (iii) only 3,6-di-*O*-methyl-*N*-methylglucosamine has been identified.

(e) The anomery of glycosidic bonds was determined with glycosidases acting either on native glycopeptides or on oligosaccharides obtained by

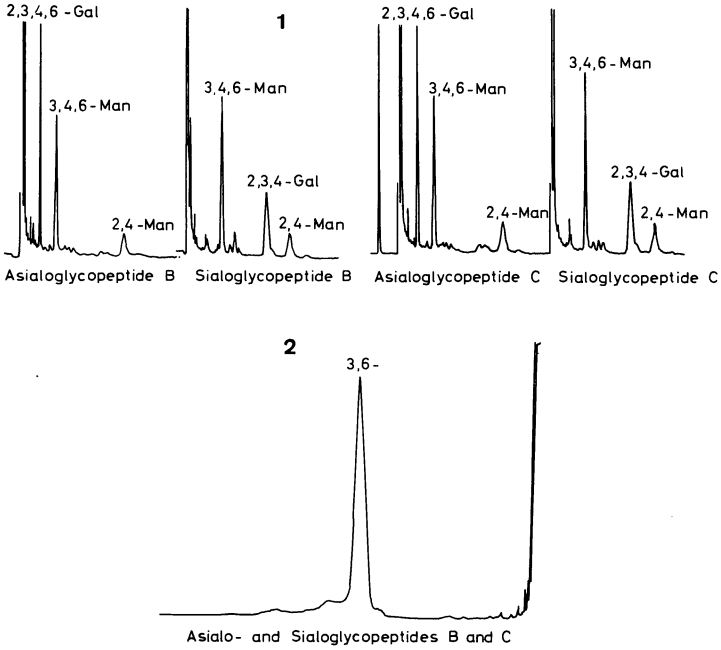


Figure 23. The g.l.c. of methylated neutral sugars (1) and *N*–CH_3 silylated glucosamine (2) from serotransferrin glycopeptide B (Ser–Asn–CHO) and glycopeptide C (CHO–Asn–Lys). Experimental conditions: (1) Glass column (0.3 × 300 cm). 3 p. 100 Carbowax 6000 on Chromosorb W–AW–HMDS (60–80 mesh); temperature: 170°C. (2) Glass column (0.3 × 180 cm). 3 p. 100 OV-17 on Chromosorb W–AW–HMDS (100–120 mesh); temperature: 120°C.

Table 6. Identification and molar ratios of monosaccharide methyl ethers present in the hydrolysates of sialo- and asialoglycopeptides B and C of serotransferrin

Methyl ethers	Glycopeptide B		Glycopeptide C	
	Sialo-	Asialo-	Sialo-	Asialo-
2,3,4,6-Gal	—	1.85	—	1.90
2,3,4-Gal	2.04	—	2.09	—
3,4,6-Man	1.70	1.85	2.24	1.62
2,4-Man	1.00	1.00	1.00	1.00

partial acid hydrolysis, by acetolysis or by hydrazinolysis–nitrous deamination. The exoglycosidases we used were the following: neuraminidase from *C. perfringens*, β-galactosidase and *N*-acetyl-β-hexosaminidase from ox spleen, α-mannosidase from jack bean meal and β-mannosidase from crude pineapple bromelain.

(2) *Glycopeptide A glycan.* Methylation studies and glycosidase action demonstrate that the glycopeptide A glycan, as compared to glycopeptide B

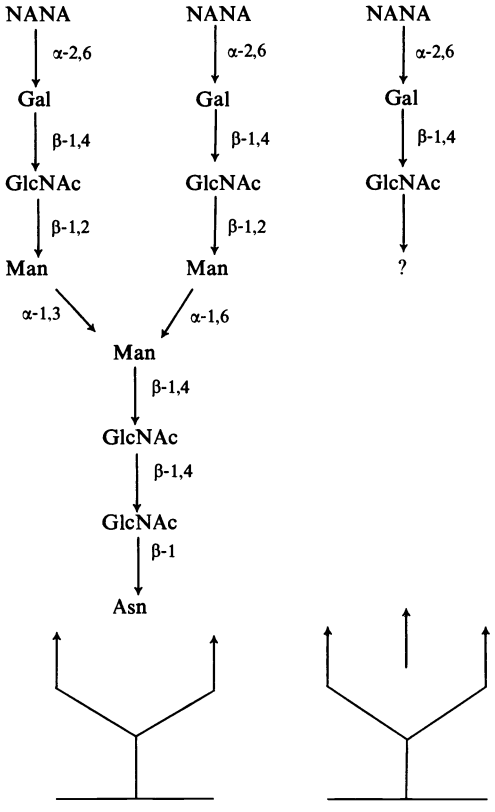


Figure 24. Bi- and tri-antennary glycans of human serotransferrin.

and C glycans, possesses a third sequence NANAα-(2 → 6)Galβ-(1 → 4)-GlcNAc but we still ignore the monosaccharide residue on which this trisaccharide is conjugated.

(C) *Conclusion*

On the basis of these results we can conclude that:

(1) Human serotransferrin possesses two types of glycans (*Figure 24*). The first is 'biantennary' and results from the substitution of the mannotriosido-di-*N*-acetylchitobiose core by two *N*-acetylneuraminyl-α(2 → 6)-*N*-acetyllactosamine residues. The second is 'triantennary' and results from the conjugation of three *N*-acetylneuraminyl-α(2 → 6)-*N*-acetyllactosamine residues to the same core.

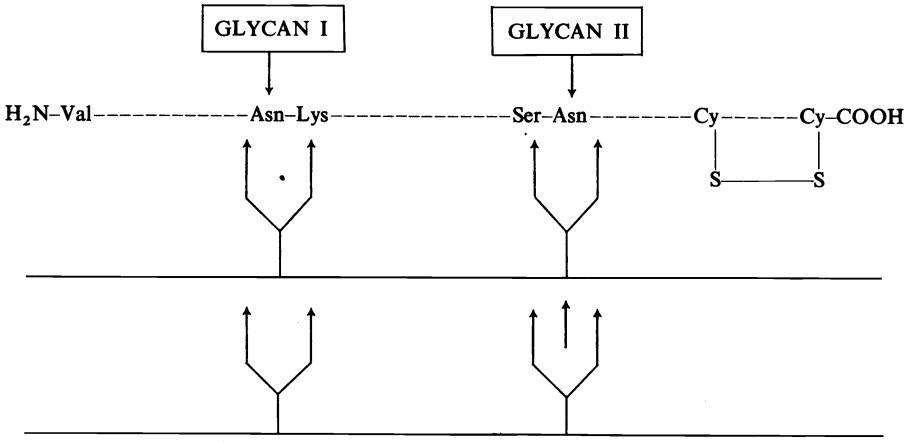


Figure 25. Two hypothetical 'isomers' of human serotransferrin.

(2) Only one biantennary glycan is linked to the serotransferrin peptide chain in 'position I', while either a bi- or triantennary glycan can be fixed in 'position II' (*Figure 25*). We postulate, but it is pure speculation and imagination, that there are two human serotransferrin 'isomers', the one probably recognizing the reticulocyte membranes and the other recognizing other cell membranes.

(II) Structure of other glycoprotein isoglycans

Applying the method of glycan isolation in the form of glycopeptides to lactotransferrin, a glycoprotein from human milk[84, 111] that like serotransferrin reversibly fixes two iron atoms, we have isolated the two glycans of that glycoprotein in the form of glycopeptides (*Figure 26*)[117] and determined their structure (*Figure 27*)[114, 116, 117]. These glycans possess identical structures and the only difference between them and the structure of the serotransferrin biantennary glycans consists of the supplementary fucose residue linked by an α-1,6 bond to one of the four *N*-acetylglucosamine residues of each glycan.

GLYCOPEPTIDE I

Glycan
↓
Pro–Phe–Leu–Asn–Gln–Thr–Gly–Pro–Pro–Pro–Ile–Gln–Ala–Val–Ala–Trp–Arg

GLYCOPEPTIDE II

Glycan
↓
Leu–Phe–Asn–Gln–Thr–Gly–Ser–Cy–Trp–Lys

Figure 26. Amino acid sequences of the two glycopeptides isolated from tryptic hydrolysates of human lactotransferrin.

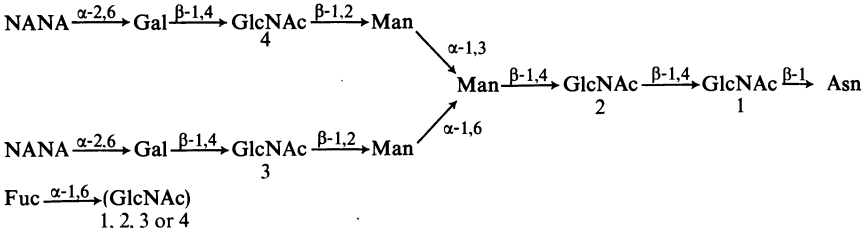


Figure 27. Structure of the two glycans of human lactotransferrin.

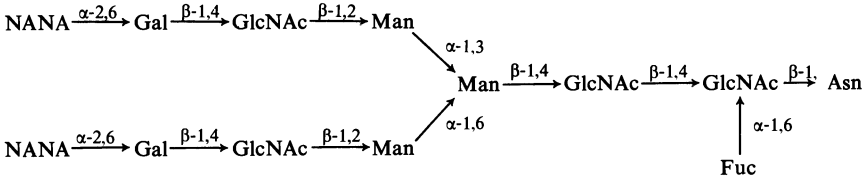


Figure 28. Structure of B-glycopeptide of myeloma IgE glycan.

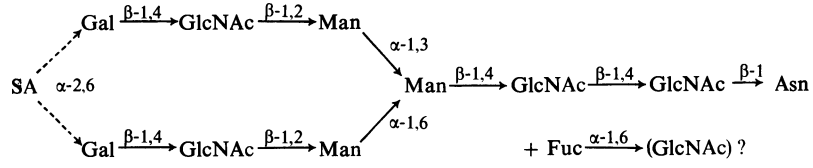


Figure 29. Structure of cow colostrum IgG glycan.

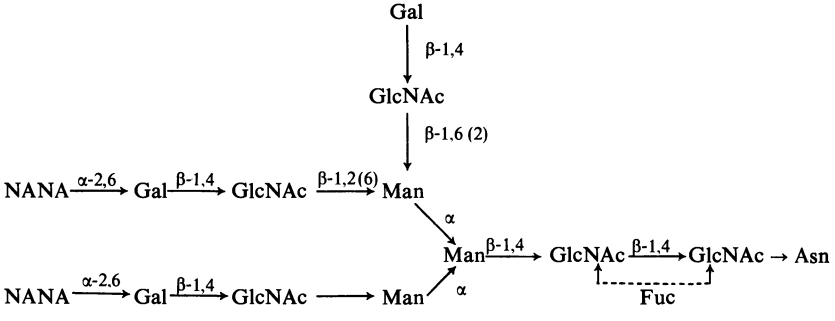


Figure 30. Structure of porcine thyroglobulin glycan.

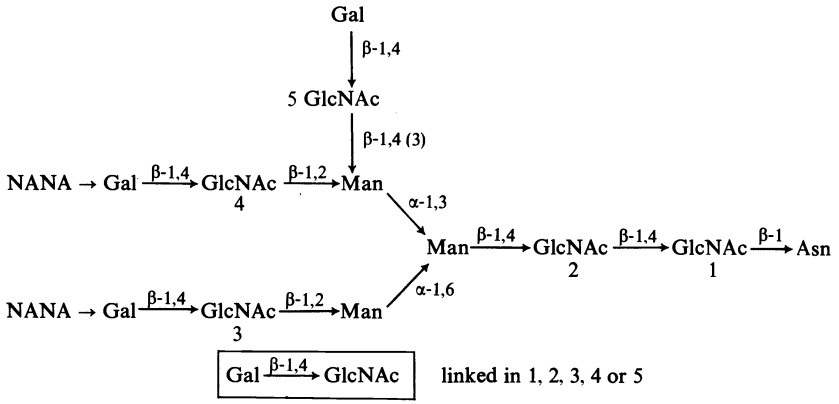


Figure 31. Structure of A, B and C glycans of orosomucoid.

In *Figures 28* to *37* are depicted isoglycan structures of the following glycoproteins: myeloma IgE (*Figure 28*)[7], cow colostrum IgG (*Figure 29*)[21], porcine thyroglobulin (*Figure 30*)[39, 133], orosomucoid A, B and C glycans (*Figure 31*)[43, 109], asialo-ovomucoids 1 and 2[51] (*Figure 22*)[11], fetuin (*Figure 33*)[8, 119], ovalbumin (*Figure 34*)[49], bovine ribonuclease B (*Figure 35*)[129], Taka-amylase A (*Figure 36*)[142], human and calf thyroglobulin (*Figure 37*)[2].

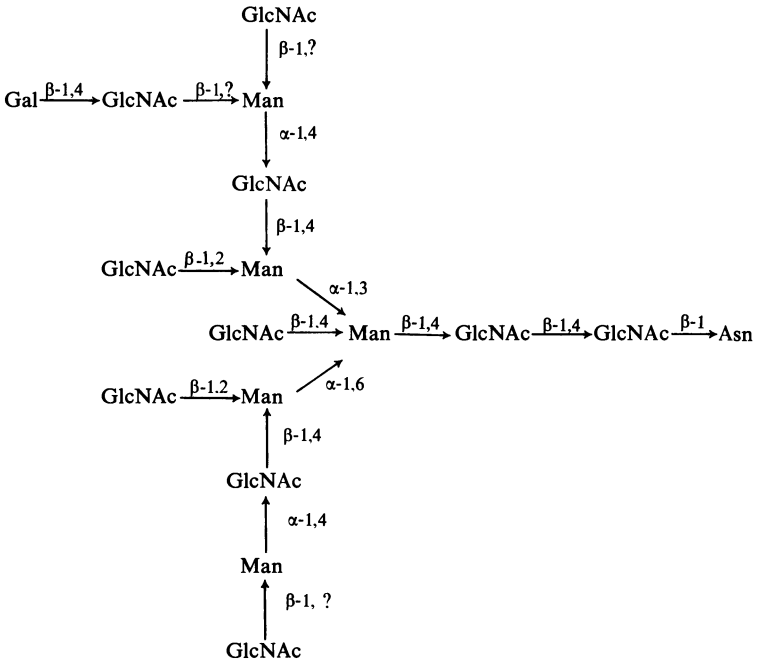


Figure 32. Structure of glycans of asialo-ovomucoids with 1 and 2.

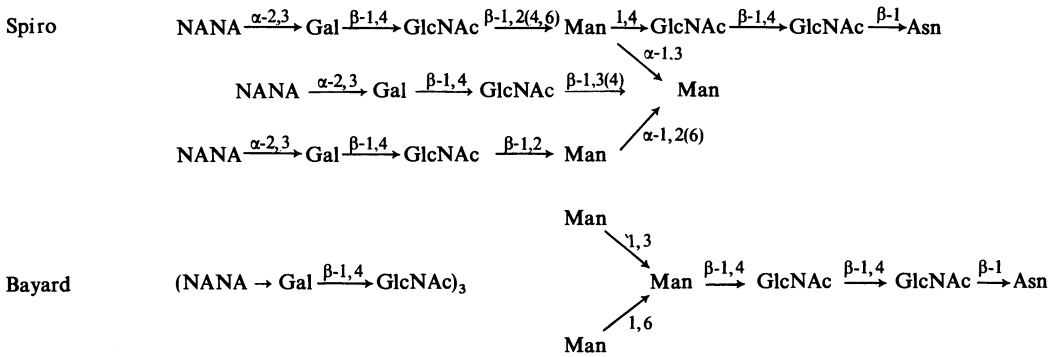


Figure 33. Structure of fetuin glycan according to Spiro[119] with a detail of structure given by Bayard[8] applying the hydrazinolysis–nitrous deamination method.

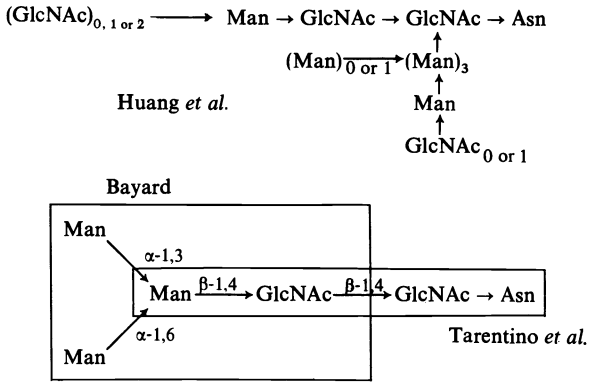


Figure 34. Structure of ovalbumin glycan according to Montgomery[49] modified by Bayard[8] applying the hydrazinolysis–nitrous deamination method and by Tarentino[130].

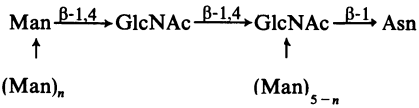


Figure 35. Structure of bovine ribonuclease B glycan.

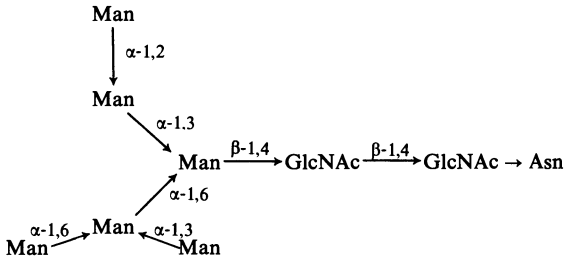


Figure 36. Structure of Taka-amylase A glycan.

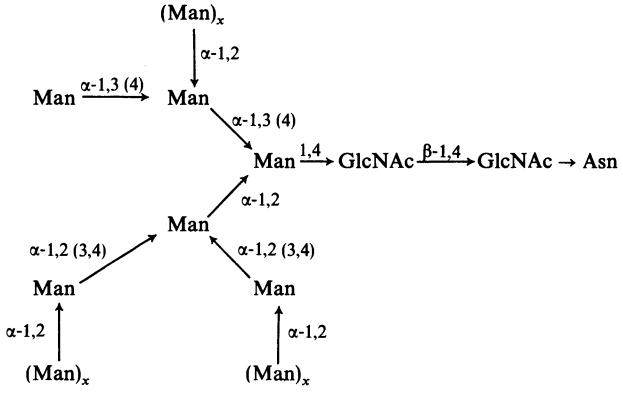
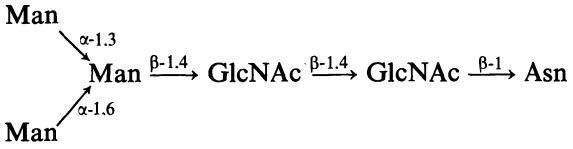


Figure 37. Structure of human and calf thyroglobulin glycan.

(III) Conclusions

The recently acquired knowledge concerning the structure of isoglycans leads to the following observations and conclusions:

(1) Most of the structures described in *Figures 22* and *27* to *37* present the common structural pattern of the mannotriosido-di-*N*-acetylchitobiose linked to an asparagine residue:



In a first family of isoglycans (*Figures 22* and *27* to *33*), this pentasaccharidic core is substituted by a variable number of *N*-acetyllactosamine (Galβ1 → 4GlcNAc) to which sialic acid or fucose residues might be conjugated. In a second family of mannose-rich isoglycans (*Figures 34* to *37*), the pentasaccharidic core is only or essentially substituted by oligomannosidic residues. We propose that these glycoproteins are said to be of '*N*-acetyllactosamine type' and of 'oligomannoside type', respectively. These conceptions are illustrated by the schemes of *Figure 38*.

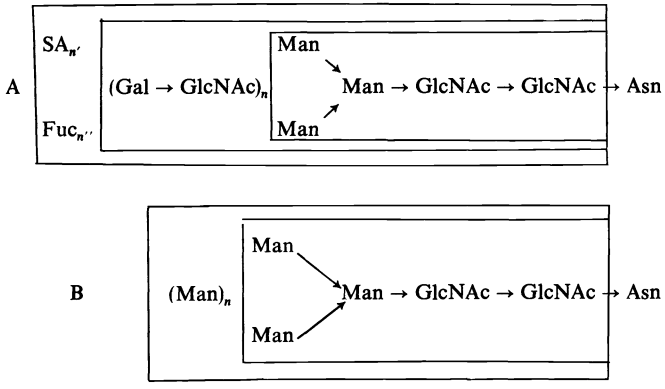


Figure 38. General structural scheme of glycoproteins of *N*-acetyllactosamine type (A) and of oligomannosidic type (B).

(2) Several structural patterns are common to numerous glycoproteins. They are reviewed in *Tables 5* and *7* to *11*.

(3) Thus we reach the conclusion that isoglycans present a certain unity in their structures. May I recall, in this connection, that we expressed this hypothesis 12 years ago[85] on the basis of results we obtained by studying the kinetics of numerous glycoprotein hydrolyses and the nature of the liberated products. In every case, sialic acid and fucose were entirely liberated at the beginning of the hydrolysis, followed by galactose, *N*-acetyllactosamine and an important amount of (*N*-acetyl)glucosamine; mannose appeared later on and moreover we were able to isolate a non-dialysable fraction containing

Table 7.

Man $\xrightarrow{\beta\text{-}1,4}$ GlcNAc $\xrightarrow{\beta\text{-}1,4}$ GlcNAc $\xrightarrow{\beta\text{-}1}$ Asn

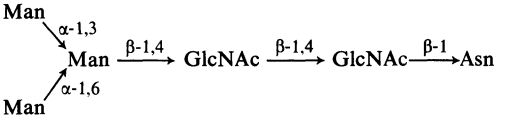
This sequence is present in the following glycoproteins:

α-Amylase (*A. orizae*)
Bromelain
Fetuin
IgG (human and bovine)
IgM, IgE (human)
Lactotransferrin (human)
Orosomucoid
Ovalbumin
Ovomucoid
Prothrombin (bovine)
Ribonuclease B (bovine and porcine)
Serotransferrin (human)
Taka amylase A
Thyroglobulin (human, calf, porcine)

mannose and glucosamine. Among the liberated oligosaccharides we always characterized, in addition to *N*-acetyllactosamine, two mannobiose isomers, later identified by Fournet[37] as α-1,3 and α-1,6 isomers, and a mannotriose.

(4) We must, however, take care in generalizing and relate *a priori* all isoglycan structures to the two fundamental schemes of *Figure 38*. Indeed, as shown in *Figures 39* to *41*, several isoglycan structures do not correspond to these two patterns. One must, however, consider that they are relatively ancient, that they were generally determined on the basis of using two or three analytical methods and that they probably might not resist the application of techniques refined and improved since the time of their elaboration.

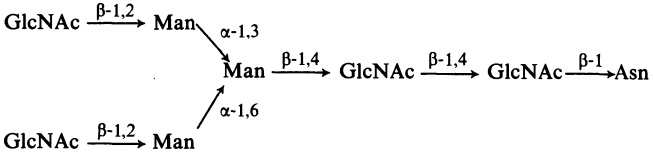
Table 8.



This sequence is present in the following glycoproteins:

Fetuin
IgE, IgM (human)
IgG (human and bovine)
Lactotransferrin (human)
Orosomucoid
Ovalbumin
Ovomucoid
Prothrombin (bovine)
Serotransferrin
Thyroglobulin (human, calf, porcine)
Taka amylase A

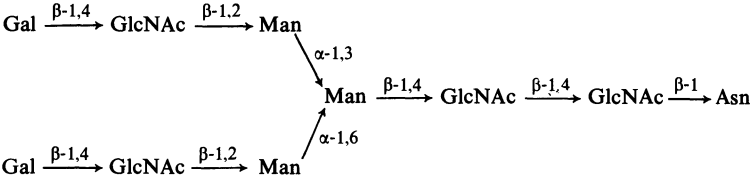
Table 9.



This sequence is present in the following glycoproteins:

IgE, IgM (human)
IgG (human and bovine)
Lactotransferrin (human)
Orosomucoid
Ovomucoid
Serotransferrin
Thyroglobulin (porcine)

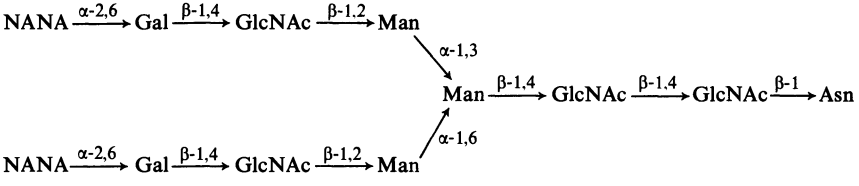
Table 10.



This sequence is present in the following glycoproteins:

IgE (human)
IgG (bovine)
Lactotransferrin (human)
Orosomucoid
Serotransferrin
Thyroglobulin (porcine)

Table 11.



This sequence is present in the following glycoproteins:

Myeloma human IgE
Cow colostrum IgG
Human serotransferrin
Human lactotransferrin

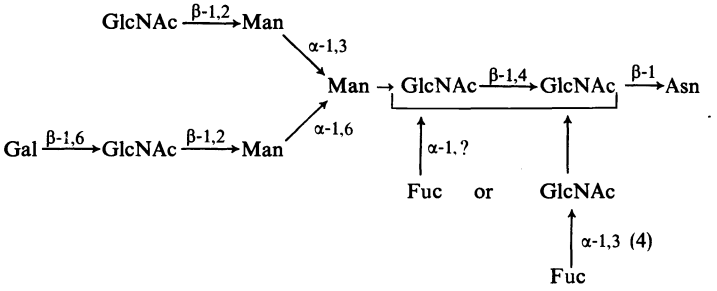


Figure 39. Structure of human myeloma IgG glycan[61].

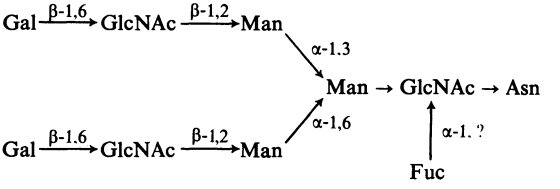


Figure 40. Structure of human myeloma IgM glycan[79].

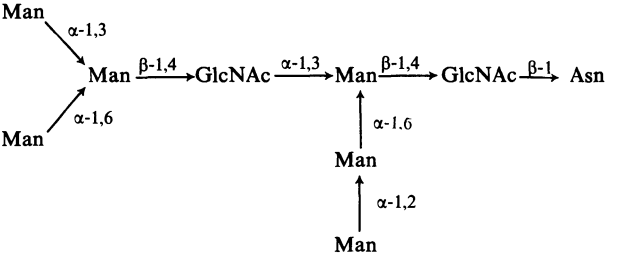


Figure 41. Structure of C-1 glycopeptide from human myeloma IgE[7].

The example of the ovalbumin structure is, in this connection, quite demonstrative (*Figure 34*): the use of new enzymes by Tarentino *et al.*[130] and the application of the hydrazinolysis–nitrous deamination by Bayard[8, 10] has related Montgomery's structure to the mannotriosido-di-*N*-acetyl-chitobiose core.

METABOLIC AND BIOLOGICAL IMPLICATIONS

Are the recent data concerning the isoglycan structures and, in particular, the structural similarities compatible with their metabolism and with the biological role of cell recognition signals they play?

(I) Structure and metabolism

(A) *Glycoprotein biosynthesis*

It is now well established (see Clauser *et al.*[24], O'Brien and Neufeld[95],

Louisot[72], Piras and Pontis[101], Bosmann[14], Bosmann *et al.*[15], Molnar[80], Roseman[105], Schachter[106] for reviews) that the glycans are synthesized by the transfer of monosaccharides from sugar nucleotides on acceptors. This transfer is catalysed by specific glycosyltransferases according to the following general reaction

$$\text{Monosaccharide} \rightarrow \text{nucleotide} + \text{Acceptor} \xrightarrow[\text{Glycosyltransferase}]{M^{2+}} \text{Monosaccharide} \rightarrow \text{Acceptor} + \text{nucleotide}$$

It has also been shown that glycan biosynthesis is sequential: a part of the *N*-acetylglucosamine residues and all the mannose residues are transferred into the rough reticulum and the remaining monosaccharides into the smooth reticulum.

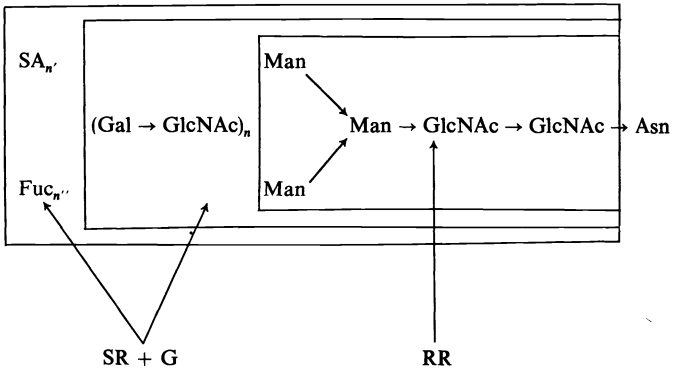


Figure 42. Biosynthesis by the rough reticulum (RR), smooth reticulum (SR) and Golgi apparatus (G) of the different segments of a glycan molecule.

The structures we have described are in good agreement with this conception since the monosaccharide sequence in the glycans follows the sequence of metabolic events (*Figure 42*). On the other hand, since the glycan structures are well defined and never random, we can conclude that their biosynthesis is strictly controlled either by the glycosyltransferase specificity or by regulation systems.

Finally, the question is how to determine whether the schemes of structure we have described are compatible with the most recent conceptions of glycoprotein biosynthesis. In the ancient concept, it was thought that the monosaccharides were conjugated one after the other and that they were directly transferred from the sugar nucleotides to the peptide or carbohydrate acceptors. Since the works of Horecker, Jeanloz, Leloir, Lennartz, Osborn, Robbins, Strominger and many others (see reviews of Heath[45], Leloir[63], Lennarz[64, 65], Lennarz and Scher[66], Osborn[97], Wright and Kanegasaki[141]) it is well established that there must exist between the precursors and the acceptors some phospholipid intermediates with isoprenic structures, as for example, the dolichol phosphate (*Figure 43*) and that these compounds are capable of conjugating to the acceptors not only

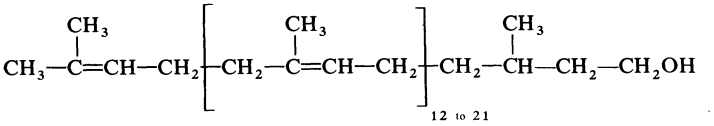


Figure 43. General structure of dolichols.

monosaccharides but also oligosaccharides. This conception is illustrated by the *Figure 44*[13].

The schemes of isoglycan structure we know at the present time are absolutely compatible with the concept of sugar phospholipid carriers. In this connection, and in a purely speculative manner, we propose in *Figure 45*

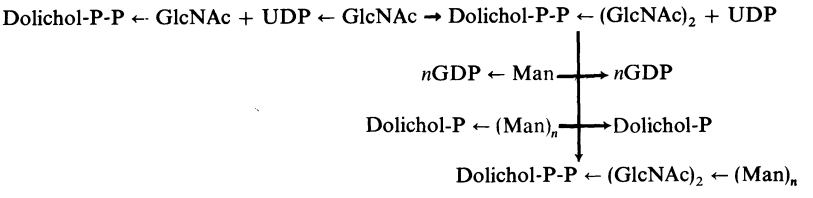


Figure 44. Glycoprotein biosynthesis according to Behrens *et al.*

a modification of Behrens's mechanism which concerns essentially the conjugation of the first *N*-acetylglucosamine residue to the peptidic chain and the transfer as a whole of the mannotriosido-*N*-acetylglucosamine tetrasaccharide to the first *N*-acetylglucosamine residue. In this way, the mannotriosido-di-*N*-acetylchitobiose core common to numerous isoglycans is synthesized in the rough reticulum.

(B) *Glycoprotein catabolism and pathology*

At the present time, the research on glycan pathology which results essentially from lysosomal diseases, is developing in a prodigious manner. We do not intend to describe in detail the various diseases that result from exoglycosidase deficits, such as fucosidases and mannosidases, but we refer the reader to the excellent reviews by Aronson[3], Brunngraber[16], Dingle and Fell[26], Durand[28], Hers and Van Hoof[46], Öckermann *et al.*[96], and Patel and Tappel[98]. We shall limit ourselves to the description of three particular pathological cases which demonstrate indirectly the existence of cellular endoglycosidases.

The first concerns galactose-rich glycopeptide accumulation in cerebral gray matter[17] and oligosaccharides accumulating in liver[139] of G_{M1}-gangliosidosis type I, an inherited glycolipid storage disease. Two oligosaccharides (*Figure 46*) have been isolated that, according to the authors, 'likely derive from erythrocyte stromal glycoproteins whose normal breakdown is impaired during development by the deficient β-galactosidase

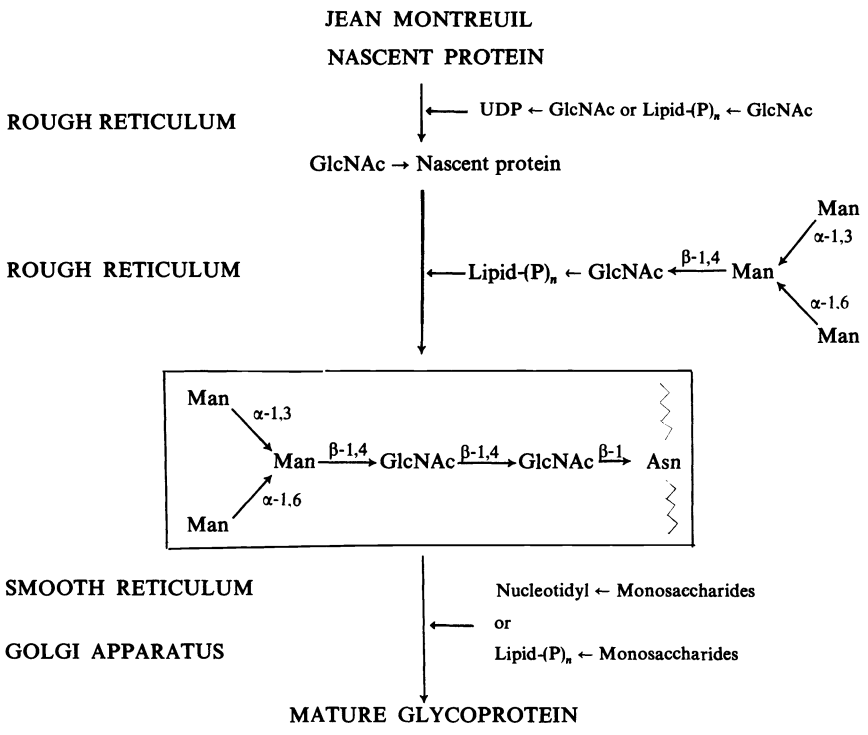


Figure 45. Proposed mechanism of glycoprotein biosynthesis.

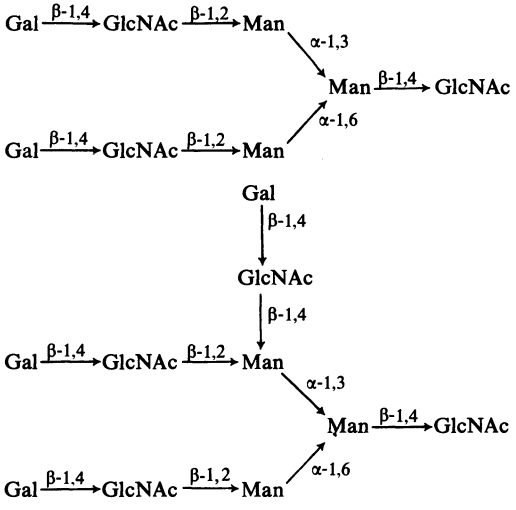


Figure 46. Structure of major oligosaccharides isolated from the liver of G_{M1}-gangliosidosis (Wolfe *et al.*[139]).

activity present in this disease'. Their structure consists of entire isoglycan fragments such as they exist in orosomucoid, transferrins or IgE. A possible explanation for the presence of these oligosaccharides possessing only one *N*-acetylglucosamine residue attached to the mannotriose moiety in a reducing position is that the di-*N*-acetylchitobiose residue is cleaved normally by an endo-β-*N*-acetylglucosaminidase H, as well as the *N*-acetylneuraminic acid residues by a neuraminidase but the deficit in β-galactosidase blocks the later catabolism of the oligosaccharides.

Table 12. Monosaccharide molar composition of six oligosaccharides isolated from urine of I-cell disease

Oligosaccharides	Gal	Man	GlcNAc	NANA
1	1	2	2	1
2	1	3	2	1
3	2	3	3	1
4	3	3	4	1
6	2	3	3	2
7	3	3	4	2

Terminal reducing monosaccharide: GlcNAc

Oligosaccharide daily elimination:

Healthy children (8 months): 15–30 mg
I-cell disease child (8 months): 135 mg

The second case relates to the separation by Strecker[121], in our laboratory, of an oligosaccharide group isolated from the urine of a child with a Leroy's I-cell disease[67] or mucolipidosis type II, an inherited disease likely to be due to a deficit of neuraminidase[121]. We have reported in *Table 12* the principal properties of six of these oligosaccharides, the structures of which are still unknown: (i) the six oligosaccharides are sialosides; (ii) all possess a *N*-acetylglucosamine residue in a reducing terminal position; (iii) the number of *N*-acetylglucosamine residues exceeds the number of galactose residues by one unit only and five oligosaccharides possess three mannose residues. We can thus postulate that oligosaccharides n^{os} 2 to 7 possess the mannotriosido-*N*-acetylglucosamine tetrasaccharide to which a variable number of *N*-acetyllactosamine residues are conjugated more or less substituted by *N*-acetylneuraminic acid residues: one in oligosaccharide n° 2, two in oligosaccharide n^{os} 3 and 6, three in oligosaccharides n^{os} 4 and 7. If these structures were to be verified in the future, the mechanism of formation of these oligosaccharides would be analogous to the previous one: (1) a primary action of an endo-β-*N*-acetylglucosaminidase H; (2) a secondary action of exoglycosidases reduced owing to the deficit in neuraminidase.

The third case[74] concerns the separation and the structure determination of oligosaccharides excreted in the urine of patients with mannosidosis, an inherited disease characterized by a deficit in mannosidase. *Figure 47* shows that oligosaccharides contain the following sequence common to numerous isoglycans

$$\text{Man}\alpha\text{-}(1 \rightarrow 3)\text{Man}\beta\text{-}(1 \rightarrow 4)\text{GlcNAc}$$

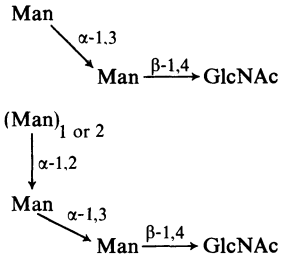


Figure 47. Structure of oligosaccharides isolated from urine of patients with mannosidosis (Lundblad *et al.*[74]).

on which a variable number of mannose residues are conjugated as in the thyroglobulin glycan (*Figure 37*), for example.

In the case of gangliosidosis type I and of mucolipidosis type II, the metabolic abnormality concerned the glycoproteins of '*N*-acetyllactosamine type'; in the case of mannosidosis it affected the glycoproteins of 'oligomannoside type'. We can thus postulate, according to the results acquired in the pathological field, that glycoprotein catabolism proceeds in two steps: (i) the first is marked by the intervention of endo-*N*-acetyl-β-D-glucosaminidases H that liberate the isoglycans possessing an *N*-acetylglucosamine residue in the terminal reducing position and shorn of one *N*-acetylglucosamine residue which remains attached to the peptide chain. That is why, in diseases characterized by a deficit[102] in β-aspartylglucosylamine amidohydrolase (*Figure 17*), a lysosomal enzyme, the asparaginyl-*N*-acetylglucosamine formed after the exhaustive action of cellular or extracellular proteases accumulates in the urine of patients with mental retardation and connective tissue lesions[55]; (ii) in the latter part of isoglycan catabolism, exoglycosidases stepwise hydrolyse the detached oligosaccharides.

(II) Structure and biological role

The information we have at the present time about the structure of glycans reveals the poor imagination of the glycosyltransferases which lead to the production of a restricted number of structural patterns. Is this fact compatible with the biological role of glycans, and, in particular, with their role as recognition signals?

Figure 48 shows the disposition in space of the serotransferrin biantennary glycan. It is clear that an isoglycan is composed of two parts: (1) a compact zone, close to the protein and comprising the mannotriosido-di-*N*-acetylchitobiose core. This part of the isoglycan molecules is invariable. It could be termed the *inv* part as in the case of the invariable peptidic fraction of immunoglobulins; (2) an 'aerial' zone which is constituted by 'antennae' planted on the core. This part of the molecule—the *var* part—is variable and supports the glycan specificity.

On the basis of this conformation, we can imagine that, although the glycans possess similar, analogous or sometimes identical structures, the biological specificities of isoglycans can be supported by the following variable patterns of structure:

(1) The number of antennae constituted by sialyl-*N*-acetyllactosamine as well as oligomannoside residues conjugated to the pentasaccharidic core can vary from one glycan to another: two antennae in the serotransferrin glycans B and C, three in fetuin, four in orosomucoid, for example.

(2) If a basic structure, like that described in *Table 10*, is common to several glycoproteins, the specificity can be held by the nature and the number of terminal monosaccharide residues. We can imagine, for example, (a) that each antenna terminates with a sialic acid residue in a given glycoprotein, or with a fucose residue in another one; (b) fucose and sialic

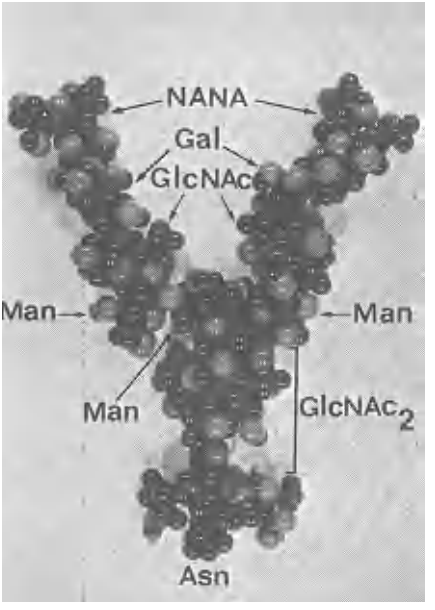


Figure 48. Molecular model of human serotransferrin glycan according to the structure given in *Figure 23.*

acid residues in variable proportions, terminate the antennae and that the glycan structure described in *Table 10* can be substituted by a sialic acid residue (lower or upper antenna) and by a fucose residue (upper or lower antenna); (c) that in other cases there may be only one sialic acid (or fucose) residue per mole of isoglycan and therefore that one of the antennae (the lower or the upper one) possesses a galactose residue in a terminal position (*Figure 29*).

(3) If, again, we suppose that several glycoproteins possess the same basic structure described in *Table 10* and that, in addition, each antenna terminates with a *N*-acetylneuraminic residue, the recognition specificity can be determined by the mode of sialic acid linkage on each galactose residue: α-(2 $\rightarrow$ 2), α-(2 $\rightarrow$ 3), α-(2 $\rightarrow$ 4) or α-(2 $\rightarrow$ 6), leading to 16 different relative positions in space of the two sialic acid residues.

(4) If glycoproteins play different biological roles and possess glycans that are absolutely identical, their recognition specificity can depend upon the number of glycan groups conjugated to the different proteins.

(5) Even if we suppose that different specific glycoproteins possess the same number of glycan groups and that the structures of these glycans are identical, the recognition specificity could result (i) from the relative positions on the protein of the carbohydrate moieties one with regard to the others, and (ii) from their relative orientations in space. These two parameters, distance and orientation, being dependent upon the structure of the protein itself, one could assume, for love of paradox, that all the recognition specificities could be held by two glycans only, having identical structures. But this is not so.

In this connection, it becomes urgent to undertake physical studies on the conformation of glycoproteins in order to localize and to orient in space the glycan group they carry.

CONCLUSIONS

The considerable progress which has been made in the last few years in the methodology concerning the structure of glycoproteins does not result from pure chance but from necessity. In fact, because the discovery of the important biological role that membrane glycoconjugates play, on the one hand, and the evidence that the carbohydrate moieties of glycoproteins are recognition signals, on the other hand, the improvement of the methods for studying the structures of glycans appeared as a necessity. At the end of this brief review, we can conclude that the problem of determination of the primary structure of glycans is virtually solved. However, it remains to miniaturize the procedures in order to extend their application to very small quantities of biological substrates. Thus, we can look forward and claim that the future belongs to Biophysics the role of which will be to determine precisely the spatial arrangement of the groups of glycans and the conformation of glycoproteins in order to understand the mechanism of their action. The most exciting and marvellous age of the history of glycoproteins starts right now. At this time, we would like to remember the pioneers whose discoveries have established the basis of our knowledge of the physicochemical properties and structure of glycoconjugates. Not all of them have seen the fruits of the trees they planted. In this connection, I would like to conclude with A. Gottschalk who wrote in 1973: 'About the outlook of glycoprotein research, we are not at the end of all progress, but at the beginning. We have but reached the shores of a great unexplored continent'.

REFERENCES

[1] D. Aminoff, C. C. Huang, C. McLean and D. A. Werner, 'Carbohydrate protein linkage specific cleavage by chemical and enzymatic methods', in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 271. Ed. CNRS: Paris (1974).

[2] T. Arima and R. G. Spiro, *J. Biol. Chem.* **247**, 1836 (1972).

[3] N. N. Aronson, 'Degradation of glycoproteins by lysosomal enzymes', in A. Gottschalk, *Glycoproteins*, Part B, p 1211. Elsevier: Amsterdam (1972).

[4] G. Ashwell and A. G. Morell, 'Galactose: a cryptic determinant of glycoprotein catabolism', in G. A. Jamieson and T. J. Greenwalt, *Glycoproteins of Blood Cells and Plasma*, p 173. J. B. Lippincott Co.: Philadelphia (1971).

[5] B. M. Austen and R. D. Marshall, 'The stability of linkages involving 4-*N*-(2-acetamido-2-

deoxy-β-D-glucopyranosyl)-L-asparagine to aqueous alkali and to alkaline sodium borohydride solutions', in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 219. Ed. CNRS: Paris (1974).

[6] H. V. Baelen and G. Mannaerts, *Arch. Biochem. Biophys.* **163**, 53 (1974).

[7] J. Baenziger, S. Kornfeld and S. Kochwa, *J. Biol. Chem.* **249**, 1889 (1974).

[8] B. Bayard, *Thesis Fac. Sci. Lille* (1974).

[9] B. Bayard, B. Fournet, S. Bouquelet, G. Strecker, G. Spik and J. Montreuil, *Carbohyd. Res.* **24**, 445 (1972).
B. Bayard and J. Montreuil, *Carbohyd. Res.* **24**, 427 (1972).

[10] B. Bayard and J. Montreuil, 'Specific cleavage of *N*-acetylglucosaminidic bonds', in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 209. Ed. CNRS: Paris (1974).

[11] B. Bayard, G. Strecker, B. Fournet, S. Bouquelet, A. Adam-Chosson, G. Spik and J. Montreuil, Unpublished results.

[12] B. Bayard, G. Strecker and J. Montreuil, *Biochimie*, **57**, 155 (1975).

[13] N. H. Behrens, H. Carminatti, R. J. Staneloni and L. F. Leloir, *Proc. Nat. Acad. Sci. USA*, **70**, 3390 (1973).

[14] H. B. Bosmann, 'Biosynthesis of glycoproteins in normal and oncogenic virus transformed cells', in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. II, p 909. Ed. CNRS: Paris (1974).

[15] H. B. Bosmann, R. J. Bernacki and B. B. Kirschbaum, 'Synthesis and structure of Mammalian glycoconjugates', in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. II, p 893. Ed. CNRS: Paris (1974).

[16] E. G. Brunngraber. 'Neuropathology of glycopeptides derived from brain glycoproteins', in V. Zambotti, G. Tettamanti and M. Arrigoni, *Glycolipids, Glycoproteins and Mucopolysaccharides of the Nervous System*, p 255. Plenum: New York (1972).

[17] E. G. Brunngraber, B. Berra and V. Zambotti, *FEBS Letters*, **34**, 350 (1973).

[18] D. M. Carlson, *J. Biol. Chem.*, **241**, 2984 (1966); **243**, 616 (1968); D. M. Carlson, 'Alkaline borohydride treatment of blood group substances and model compounds', in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifque sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 249. Ed. CNRS: Paris (1974).

[19] P. Charet, M. Monsigny, G. Spik and J. Montreuil, *CR Acad. Sci. Paris*, **269D**, 1019 (1969); P. Charet, G. Spik and J. Montreuil, *CR Acad. Sci. Paris*, **273D**, 422 (1971).

[20] P. Charet and J. Montreuil, *CR Acad. Sci. Paris*, **273D**, 533 (1971);
P. Charet, D. Tetaert, K. K. Han and J. Montreuil, *CR Acad. Sci. Paris*, **276D**, 1629 (1973).

[21] A. Cheron, Unpublished results.

[22] J. R. Clamp, T. Bhatti and R. E. Chambers, 'The examination of carbohydrate in glycoproteins by gas–liquid chromatography', in A. Gottschalk, *Glycoproteins*, Part A, p 300. Elsevier: Amsterdam (1972).

[23] J. R. Clamp, T. Bhatti and R. Chambers, 'The determination of carbohydrate in biological materials by gas–liquid chromatography', in D. Glick, *Methods Biochem. Anal.* **19**, 229 (1971).

[24] H. Clauser, G. Herman, B. Rossignol and S. Harbon, 'Biosynthesis at the cellular and subcellular level', in A. Gottschalk, *Glycoproteins*, Part B, p 1151. Elsevier: Amsterdam (1972).

[25] A. L. De Vries, *Science*, **172**, 1152 (1971);
W. T. Shier, Y. Lin and A. L. De Vries, *Biochim. Biophys. Acta*, **263**, 406 (1972);
Y. Lin, J. G. Duman and A. L. De Vries, *Biochem. Biophys. Res. Commun.*, **46**, 87 (1972).

[26] J. T. Dingle and H. B. Fell, *Lysosomes in Biology and Pathology*, Vol. II. North-Holland: Amsterdam (1969).

[27] B. A. Dmitriev, Yu. A. Knirel and N. K. Kotchetkov, *Carbohyd. Res.* **29**, 451 (1973); **30**, 45 (1973).

[28] P. Durand, 'Progress and problems on fucosidosis and mucolipidosis', in V. Zambotti, G. Tettamanti and M. Arrigoni, *Glycolipids, Glycoproteins and Mucopolysaccharides of the Nervous System*, p 293. Plenum: New York (1972).

[29] G. S. Dutton, 'Applications of gas–liquid chromatography to carbohydrates', *Advanc. Carbohyd. Chem.* **28**, 12 (1973).

[30] H. Eylar, 'On the biological role of glycoproteins', *J. Theoret. Biol.* **10**, 89 (1965).
[31] J. Fielding and B. E. Speyer, *Biochim. Biophys. Acta*, **363**, 387 (1974).
[32] B. Fournet, *Thesis Fac. Sci. Lille* (1973).
[33] B. Fournet, Y. Leroy and J. Montreuil, 'General procedure of identification of methylated monosaccharides present in permethylated glycans', in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 111. Ed. CNRS: Paris (1974).
[34] B. Fournet, Y. Leroy and J. Montreuil, Unpublished results.
[35] B. Fournet, Y. Leroy, J. Montreuil and H. Mayer, *J. Chromatogr.* **92**, 185 (1974).
[36] B. Fournet and J. Montreuil, *J. Chromatogr.* **75**, 29 (1973).
[37] B. Fournet, G. Takerkart, J. Brohon and J. Montreuil, *Bull. Soc. Chim. Biol.* **50**, 1351 (1968).
[38] M. Fujinaga and Y. Matsushima, *Bull. Chem. Soc. Japan*, **37**, 468 (1964).
[39] M. Fukuda and F. Egami, *Biochem. J.* **123**, 415 (1971).
[40] A. Gottschalk, 'Biosynthesis of glycoproteins and its relationship to heterogeneity', *Nature, London*, **222**, 452 (1969).
[41] A. Gottschalk, *Glycoproteins. Their Composition, Structure and Function.* Elsevier: Amsterdam (1972).
[42] N. Handa and R. Montgomery, *Carbohyd. Res.* **11**, 467 (1969).
[43] V. B. Hatcher and R. W. Jeanloz, in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 329. Ed. CNRS: Paris (1974).
[44] J. Haverkamp and J. F. G. Vliegenthart, in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 131. Ed. CNRS: Paris (1974).
[45] E. C. Heath, *Annu. Rev. Biochem.* **40**, 29 (1971).
[46] H. G. Hers and F. Van Hoff, *Lysosomes and Storage Diseases*, Academic Press: New York (1973).
[47] M. Höök, U. Lindahl and P-H. Iverius, *Biochem. J.* **137**, 33 (1974).
[48] D. Horton and K. D. Philips, *Carbohyd. Res.* **30**, 367 (1973).
[49] C. C. Huang, H. E. Moyer and R. Montgomery, *Carbohyd. Res.* **13**, 127 (1970).
[50] S. Isemura and K. Schmid, *Biochem. J.* **124**, 531 (1971).
[51] E. Jakubczak and J. Montreuil, *CR Acad. Sci. Paris*, **271D**, 537 (1970).
[52] G. A. Jamieson, *Biochem. Biophys. Res. Commun.* **17**, 775 (1964);
G. A. Jamieson, *J. Biol. Chem.* **240**, 2914 (1965).
[53] G. A. Jamieson and T. J. Greenwalt, *Glycoproteins of Blood Cells and Plasma*, J. B. Lippincott Co.: Philadelphia (1971).
[54] J. H. Jandl and J. H. Katz, *J. Clin. Invest.* **42**, 314 (1963).
[55] F. A. Jenner and R. J. Pollitt, *Biochem. J.* **103**, 48 P (1967);
R. J. Pollitt, F. A. Jenner and H. Merskey, *Lancet*, **ii**, 253 (1968);
J. Palo and K. Mattsson, *J. Mental Deficiency Res.* **14**, 168 (1970);
S. Autio, *J. Mental Deficiency Res.*, Monograph n° 1 (1972).
[56] M. Jutisz and P. De La Llosa, 'Glycoproteins as hormones and as enzymes', in A. Gottschalk, *Glycoproteins*, Part B, p 1019. Elsevier: Amsterdam (1972).
[57] E. D. Kaverzneva and T. De-Fan, *Biokhimiya*, **26**, 702 (1961); Plenum's English edition p 675.
[58] J. F. Kennedy, 'Chemical aspects of glycoproteins, proteoglycans and carbohydrate–protein complexes of human tissues, The Meldola Medal Lecture'. *Chem. Soc. Rev.* **2**, 355 (1973).
[59] K. Kochetkov and O. S. Chizhov, 'Mass spectrometry of carbohydrates', in R. Whistler and J. N. BeMiller, *Methods Carbohyd. Chem.*, Vol. VI, p 540. Academic Press; New York (1972).
[60] N. Koide and T. Muramatsu, *J. Biol. Chem.* **249**, 4897 (1974).
[61] R. Kornfeld, J. Keller, J. Baenziger and S. Kornfeld, *J. Biol. Chem.* **246**, 3259 (1971).
[62] R. Kuhn, H. H. Baer and A. Gauhe, *Chem. Ber.* **89**, 2519 (1936).
[63] L. F. Leloir, 'Two decades of research on the biosynthesis of saccharides', *Science*, **172**, 1299 (1971).
[64] W. J. Lennarz, *Annu. Rev. Biochem.* **39**, 359 (1970).
[65] W. J. Lennarz, in S. J. Wakil, *Lipid Metabolism*, p 155. Academic Press: New York (1970).
[66] W. J. Lennarz and M. G. Scher, 'Metabolism and function of polyisoprenol sugar intermediates in membrane-associated reactions', *Biochim. Biophys. Acta*, **265**, 417 (1972).
[67] J. G. Leroy and R. Demars, *Science*, **157**, 804 (1967).

[68] Y-T. Li and Y. C. Lee, *J. Biol. Chem.* **247**, 3677 (1972).
[69] Y-T. Li and S. C. Li, in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 339. Ed. CNRS: Paris (1974).
[70] S-C. Li and Y-T. Li, Unpublished results.
[71] B. Lindberg, 'Methylation analysis of polysaccharides', in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 28 B, p 178. Academic Press: New York (1972).
[72] P. Louisot, 'Biosynthesis of brain glycoproteins', in V. Zambotti, G. Tettamanti and M. Arrigoni, *Glycolipids, Glycoproteins and Mucopolysaccharides of the Nervous System.* Plenum: New York (1972).
[73] W. A. Lukowsky and R. H. Painter, *Canad. J. Biochem.* **50**, 909 (1972).
[74] A. Lundblad, S. Svensson and P. A. Ockerman, *Abstracts 7th Internat. Symp. Carbohyd. Chem., Bratislava*, 221 (1974).
[75] R. D. Marshall, 'Glycoproteins', *Annu. Rev. Biochem.* **41**, 673 (1972).
[76] R. D. Marshall and A. Neuberger, 'Aspects of the structure and metabolism of glycoproteins', *Advanc. Carbohyd. Chem.* **25**, 407 (1970).
[77] R. D. Marshall and A. Neuberger, 'Structural analysis of the carbohydrate groups of glycoproteins', in A. Gottschalk, *Glycoproteins*, Part A, p 322. Elsevier: Amsterdam (1972).
[78] Y. Matsushima and N. Fujii, *Bull. Chem. Soc. Japan*, **30**, 48 (1957).
[79] F. Miller, *Immunochemistry*, **9**, 217 (1972).
[80] J. Molnar, 'A proposed pathway of plasma glycoprotein synthesis in the liver', in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. II, p 921. Ed. CNRS: Paris (1974).
[81] R. Montgomery, 'Glycoproteins', in W. Pigman and D. Horton, *The Carbohydrates, Chemistry and Biochemistry*, Vol. 2B, p 515. Academic Press: New York (1970).
[82] J. Montreuil, 'Procédés chimiques et enzymatiques d'exploration de la structure des isoglycannes', *Pure Appl. Chem.* **27**, 549 (1971).
[83] J. Montreuil, *Actes* du Colloque International du Centre National de la Recherche Scientifique n° 221: Méthodologie concernant la structure et le métabolisme des glycoconjugués, Villeneuve d'Ascq (France), 20–27 juin 1973, Ed. CNRS: Paris (1974).
[84] J. Montreuil and S. Mullet, *CR Acad. Sci. Paris*, **250**, 1376 (1960);
J. Montreuil, J. Tonnelat and S. Mullet, *Biochim. Biophys. Acta*, **45**, 413 (1960).
[85] J. Montreuil, G. Spik and A. Chosson, *CR Acad. Sci. Paris*, **255**, 3493 (1962).
[86] A. G. Morell, R. A. Irvine, I. Sternlieb, I. H. Scheinberg and G. Ashwell, *J. Biol. Chem.* **243**, 155 (1968).
[87] E. H. Morgan and E. Baker, *Biochim. Biophys. Acta*, **363**, 240 (1974).
[88] E. H. Morgan and C. B. Laurell, *Brit. J. Haematol.* **9**, 471 (1963).
[89] T. Muramatsu, *J. Biol. Chem.* **246**, 5535 (1971).
[90] T. Muramatsu and F. Egami, *J. Biochem. (Tokyo)*, **62**, 700 (1967);
F. Egami, in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 289. Ed. CNRS: Paris (1974).
[91] G. L. Nelsestuen and J. W. Suttie, *J. Biol. Chem.* **247**, 6096 (1972).
[92] A. Neuberger, A. Gottschalk, R. D. Marshall and R. G. Spiro, 'Carbohydrate peptide linkages in glycoproteins and methods for their elucidation', in A. Gottschalk, *Glycoproteins*, Part A, p 450. Elsevier: Amsterdam (1972).
[93] A. Neuberger and R. D. Marshall, 'Aspects of the structure of glycoproteins', in H. W. Schultze, R. F. Cain and R. W. Wrotstad, *Symposium on Foods—Carbohydrates and Their Roles*, p 115. Oregon State University; Avi Publishing Co.: Westport, Conn. (1969).
[94] M. Nishigaki, T. Muramatsu and A. Kobata, *Biochem. Biophys. Res. Commun.*, **59**, 638 (1974).
[95] P. J. O'Brien and E. F. Neufeld, 'Glycosyltransferases', in A. Gottschalk, *Glycoproteins*, Part B, p 1170. Elsevier: Amsterdam (1972).
[96] P. A. Öckerman, N. E. Norden and L. Szabo, 'Problems in the chemical diagnosis of glycoprotein storage disease', in V. Zambotti, G. Tettamanti and M. Arrigoni, *Glycolipids, Glycoproteins and Mucopolysaccharides of the Nervous System*, p 281. Plenum: New York (1972).
[97] M. J. Osborn, *Annu. Rev. Biochem.* **38**, 501 (1969).

[98] V. Patel and A. L. Tappel, 'Glycosidases in glycoprotein catabolism', in G. A. Jamieson and T. J. Greenwalt, *Glycoproteins of Blood Cells and Plasma*, p 133. J. B. Lippincott Co.: Philadelphia (1971).

[99] J. H. Pazur and N. N. Aronson Jr, 'Glycoenzymes: enzymes of glycoprotein structure', *Advanc. Carbohyd. Chem.* **27**, 301 (1973).

[100] W. Pigman, J. Moschera, F. Downs and T. Wakabayashi, 'Mechanisms and conditions for β-elimination of oligosaccharides from glycoproteins', in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 231. Ed. CNRS: Paris (1974).

[101] R. Piras and H. G. Pontis, 'Biochemistry of the glycosidic linkage. An integrated view', *PAABS Symposium Volume 2*, Academic Press: New York (1972).

[102] R. J. Pollitt and F. A. Jenner, *Clin. Chim. Acta*, **25**, 413 (1969);
J. Palo, P. Riekkinen, A. U. Arstila and S. Autio, *Neurology*, **21**, 1198 (1971);
J. Palo, P. Riekkinen, A. U. Arstila and S. Autio, *Acta Neuropathol. (Berlin)*, **20**, 217 (1972).

[103] W. E. Pricer Jr and G. Ashwell, *J. Biol. Chem.* **246**, 4825 (1971).

[104] L. Robert and J. Polonovski, 'Colloque sur les Glycoprotéines', in P. Boulanger, M.-F. Jayle and J. Roche, *Exp. Ann. Biochim. Méd.*, Vol. 30. Masson: Paris (1970).

[105] S. Roseman, 'The synthesis of complex carbohydrates by multiglycosyltransferase systems and their potential function in intercellular adhesion'. *Chem. Phys. Lipids*, **5**, 270 (1970).

[106] H. Schachter, 'The biosynthesis of animal glycoproteins', in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique, Villeneuve d'Ascq, 20–27 juin 1973, Vol. II, p 937. Ed. CNRS: Paris (1974).

[107] R. Schauer, 'Chemistry and biology of the acylneuraminic acids', *Angew. Chem. Internat. Ed.* **12**, 127 (1973).

[108] K. Schmid, 'Recent advances in the study of glycoproteins', *Chimia*, **26**, 405 (1972).

[109] G. Schwarzmann, V. Reinhold and R. W. Jeanloz, 'Identification of methylated sugar derivatives by gas–liquid chromatography and mass spectrometry, in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 85. Ed. CNRS: Paris (1974).

[110] G. Spik, *Thesis Fac. Sci. Lille* (1968).

[111] G. Spik, 'Formes conjuguées et assimilation du lactofer. Etude particulière des lactotransferrines', *Ann. Nutr. Aliment.* **25**, 81 (1971).

[112] G. Spik, B. Bayard, B. Fournet, G. Strecker, S. Bouquelet and J. Montreuil, *FEBS Letters*, **50**, 296 (1975).

[113] G. Spik and B. Fournet, Personal communication.

[114] G. Spik, B. Fournet, B. Bayard, R. Vandersyppe, G. Strecker, S. Bouquelet and J. Montreuil, *Arch. Internat. Physiol. Biochim.* **82**, 29 (1974).

[115] G. Spik, M. Monsigny and J. Montreuil, *CR Acad. Sci. Paris*, **260**, 4282 (1965); **261**, 1137 (1965);
G. Spik and J. Montreuil, *Internat. Symp. Chromatogr. Electroph., Bruxelles*, 1966, p 386. Presses Acad. Europ. éd.: Bruxelles (1968);
G. Spik and J. Montreuil, *Bull. Soc. Chim. Biol.* **51**, 1271 (1969).

[116] G. Spik, R. Vandersyppe, B. Fournet, B. Bayard, P. Charet, S. Bouquelet, G. Strecker and J. Montreuil, in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 483. Ed. CNRS: Paris (1974).

[117] G. Spik, R. Vandersyppe, D. Tetaert, K. K. Han and J. Montreuil, *FEBS Letters*, **38**, 213 (1974).

[118] R. G. Spiro, 'Glycoproteins'. *Annu. Rev. Biochem.* **39**, 599 (1970).

[119] R. G. Spiro, 'Glycoproteins'. *Advanc. Prot. Chem.* **27**, 349 (1973).

[120] K. Stellner, H. Sato and S.-I. Hakomori, *Arch. Biochem. Biophys.* **155**, 464 (1973);
K. Stellner, H. Sato and S.-I. Hakomori, in J. Montreuil, *Actes* du Colloque International du CNRS sur les Glycoconjugués, Villeneuve d'Ascq (France), 20–27 juin 1973, Vol. I, p 95. Ed. CNRS: Paris (1974).

[121] G. Strecker, Th. Hondi-Assah, J.-P. Farriaux and J. Montreuil, Unpublished results.

[122] K. Sugahara, T. Okumura and I. Yamashina, *FEBS Letters*, **20**, 44 (1972).

[123] K. Sugahara, T. Okumura and I. Yamashina, *Biochim. Biophys. Acta*, **268**, 488 (1972).

[124] T. Sukeno, A. L. Tarentino, T. H. Plummer Jr and F. Maley, *Biochem. Biophys. Res. Commun.* **45**, 219 (1971).

[125] T. Sukeno, A. L. Tarentino, T. H. Plummer Jr and F. Maley, *Biochemistry*, **11**, 1493 (1972).
[126] M. Suttajit, L. E. Reichert Jr and R. J. Winzler, *J. Biol. Chem.* **246**, 3504 (1971).
[127] M. Tanaka, M. Kohno and I. Yamashina, *J. Biochem. (Tokyo)*, **73**, 1285 (1973).
[128] A. L. Tarentino and F. Maley, *J. Biol. Chem.* **249**, 811 (1974).
[129] A. Tarentino, T. H. Plummer Jr and F. Maley, *J. Biol. Chem.*, **245**, 4150 (1970).
[130] A. L. Tarentino, T. H. Plummer Jr and F. Maley, *J. Biol. Chem.* **247**, 2629 (1972).
[131] A. L. Tarentino, T. H. Plummer Jr and F. Maley, *J. Biol. Chem.* **248**, 5547 (1973).
[132] A. L. Tarentino, T. H. Plummer Jr and F. Maley, *J. Biol. Chem.* **249**, 818 (1974).
[133] S. Toyoshima, M. Fukuda and T. Osawa, *Biochem. Biophys. Res. Commun.* **51**, 945 (1973).
[134] J. R. Vandenheede, A. I. Ahmed and R. E. Feeney, *J. Biol. Chem.* **247**, 7885 (1972); A. I. Ahmed, D. T. Osuga and R. E. Feeney, *J. Biol. Chem.* **248**, 8524 (1973).
[135] E. Werries and E. Buddecke, *Europ. J. Biochem.* **37**, 535 (1973); E. Buddecke and E. Werries, in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 299. Ed. CNRS: Paris (1974).
[136] R. L. Whistler and D. F. Durso, *J. Amer. Chem. Soc.* **72**, 677 (1950).
[137] P. J. Winterburn and C. F. Phelps, 'The significance of glycosylated proteins', *Nature, London*, **236**, 147 (1972).
[138] R. J. Winzler, 'The chemistry of glycoproteins', in C. H. Li, *Hormonal Proteins and Peptides*. Academic Press: New York (1973).
[139] L. S. Wolfe, R. G. Senior and N. M. K. Ng Ying Kin, *Fed. Proc.* **32**, 484 (1973). L. S. Wolfe, R. G. Senior and N. M. K. Ng Ying Kin, *J. Biol. Chem.* **249**, 1828 (1974).
[140] M. L. Wolfrom and B. D. Juliano, *J. Amer. Chem. Soc.* **82**, 2588 (1960).
[141] A. Wright and S. Kanegasaki, *Physiol. Rev.* **51**, 748 (1971).
[142] H. Yamaguchi, T. Ikenaka and Y. Matsushima, *J. Biochem.* **70**, 587 (1971).
[143] I. Yamashina, '4'-L-aspartylglucosylamine amido hydrolase: isolation, purification, mode of action and distribution', in A. Gottschalk, *Glycoproteins*, Part B, p 1187. Elsevier: Amsterdam (1972).
[144] Z. Yosizawa and T. Sato, *Biochim. Biophys. Acta*, **52**, 591 (1961); Z. Yosizawa and T. Sato, *J. Biochem.* **51**, 233 (1962).
[145] V. Zambotti, G. Tettamanti and M. Arrigoni, *Glycolipids, Glycoproteins and Mucopolysaccharides of the Nervous System*, Plenum: New York (1972).
[146] J. P. Zanetta, W. C. Breckenridge and G. Vincendon, *J. Chromatogr.* **69**, 291 (1972).
[147] J. P. Zanetta and C. Vincendon, in J. Montreuil, *Actes* du Colloque International n° 221 du Centre National de la Recherche Scientifique sur les Glycoconjugués, Villeneuve d'Ascq, 20–27 juin 1973, Vol. I, p 47. Ed. CNRS: Paris (1974).