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Structural studies of glycans isolated from rat plasma hemopexin.

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Résumé.

Les glycopeptides obtenus par digestion pronasique de l'hémopexine de rat sont fractionnés sur une colonne de concanavaline A. Les deux premières fractions obtenues (I et II) ne sont pas retenues sur la colonne, seule la fraction III réagit avec la concanavaline A.

D'après la composition molaire en glucides, et en associant une analyse par perméthylation à celle par résonance magnétique nucléaire des protons, nous avons déterminé la structure primaire des glycannes de la fraction III. Celle-ci est formée d'un mélange de molécules diantennées, mono- et di-sialylées, de type N-*acétyllactosaminique.*

L'hydrazinolyse de chacune des fractions non retenues sur la colonne de concanavaline A donne un mélange d'oligosaccharides, qui sont ensuite fractionnés par HPLC. La composition molaire de chacun d'eux est donnée. Cette dernière suggère que l'hémopexine de rat contient, entre autres, une structure diantennée comportant trois acides sialiques.

Mots-clés : hémopexine / hydrate de carbone / ^{1}H-RMN / rat.

Summary.

After exhaustive pronase digestion, purification by gel filtration and affinity chromatography on concanavalin A, three glycopeptide fractions were obtained from rat hemopexin. Two fractions (I and II) were concanavalin A non-reactive and one (III) was concanavalin A reactive.

On the basis of carbohydrate composition, methylation analysis and proton nuclear magnetic resonance spectroscopy, the primary structure of the glycan in fraction III is proposed as being a mixture of mono- and di-sialo-diantennae of the N-*glycosidic,* N- *acetyllactosamine type.*

Hydrazinolysis of glycopeptides not binding to concanavalin A yielded mixtures of oligosaccharides for both fractions. These oligosaccharides were separated by HPLC ; the molar composition of each of them is given. These data suggest that rat hemopexin contains, among others, a diantennary structure bearing three sialic acid residues.

Key-words : hemopexin / carbohydrate / ^{1}H-NMR / rat.

List of Abbreviations :

- *ConA : concanavalin A.*
- *TLC : thin-layer chromatography.*
- *HPLC : high-performance liquid chromatography.*
- *NMR : nuclear magnetic resonance.*
- *GLC(—MS) : gas liquid chromatography (coupled with mass spectrometry).*

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Introduction.

Hemopexin is a plasma β-glycoprotein which binds heme with high affinity. Its biological function is to remove heme from the circulation and thus plays an important physiological role in case of severe hemolysis : if the hemoglobin level is higher than the binding capacity of haptoglobin, it dissociates into globin and heme, the latter being immediately bound by hemopexin [1]. The complex thus formed is taken up by liver parenchymal cells [2, 3].

Rat hemopexin has been isolated in our laboratory and its physical and chemical properties have been studied [4, 5]. We have shown that, upon heme binding [6, 7], there was no major modification either of the physical and chemical properties of hemopexin, or in asialo-hemopexin uptake by isolated hepatocytes.

We became interested in the rat carbohydrate moiety of the hemopexin because of the microheterogeneity found in this glycoprotein [6]. Glycoproteins often exhibit molecular heterogeneity due to structural variations in their carbohydrate chains. This is the case for rat hemopexin : by crossed-immuno-affino-electrophoresis, with free ConA in the first dimension, it has been shown that part of the molecular heterogeneity of hemopexin was due to the glycan moiety (unpublished results). The aim of the present study was to elucidate the microheterogeneity of these carbohydrate chains by structural studies.

Materials and Methods.

Materials.

Rat hemopexin was prepared as previously reported [5]. Ultrogel AcA202 was purchased from IBF (Genevilliers, France). ConA/Sepharose was obtained from Pharmacia (Uppsala, Sweden) and Bio-Gel P_2 from Bio-Rad (Richmond, California).

Acetic anhydride [^{14}C] (5-10 mCi/mmole) was purchased from New England Nuclear (Boston, Massachusetts/USA). Pronase (B grade) was obtained from Calbiochem (San Diego, California/USA). Trifluoroacetic anhydride was purchased from Fluka (Buchs, Switzerland). All other reagents used were of the highest grade available and were used without further purification.

The standards used for TLC were oligosaccharides of known structure :

Proteolytic digestion of hemopexin and isolation of glycopeptides.

Glycopeptides were prepared from purified rat hemopexin by pronase (2 per cent w/w) digestion in 0.2 M Tris-HCl buffer (pH 7.8) containing 15 mM $CaCl_2$ and 1 per cent toluene at 37°C for 72 h, according to Zinn *et al.* [8].

The pronase digest was purified by gel filtration on an Ultrogel AcA202 column (2.6 × 49 cm). Elution was carried out with 0.1 M pyridine acetate buffer (pH 5.0). The eluate was monitored for peptides with ninhydrin and for carbohydrate by the orcinol/sulfuric acid reagent using a Technicon automated analyzer [9].

Labelling of glycopeptides.

In order to follow their elution on different columns, hemopexin glycopeptides were *N*-[^{14}C] acetylated on their peptide moiety with acetic anhydride [^{14}C] (5-10 mCi/mmole) in 0.1 M $NaHCO_3$ (1 ml) at room temperature for 20 min. The *N*-[^{14}C] acetylated glycopeptides were separated from other radioactive material by gel filtration on a column of Bio-gel P_2 (1.5 × 62 cm) equilibrated with distilled water.

Affinity chromatography on ConA-Sepharose.

The labelled hemopexin glycopeptides were fractionated on a column (1 × 24 cm) of ConA/Sepharose. The column was equilibrated with 0.05 M sodium acetate buffer (pH 6.0) containing 100 mM NaCl and 1 mM of $CaCl_2$, $MgCl_2$ and $MnCl_2$, each. The sample containing about 2000 µg (hexose content) was applied to the column and the elution was started with equilibration buffer which eluted the non-retained glycopeptides (fractions I and II).

In order to recover the retained glycopeptides (fraction III), 15 mM methyl α-D-glucopyranoside was added to the eluting buffer. The position of unretained glycopeptides was determined by the elution of an albumin solution (50 mg/ml). The different fractions eluted from the ConA/Sepharose column were desalted and separated from α-methylglucoside by gel filtration on a column of Bio-Gel P_2 (1.5 × 35 cm) equilibrated with distilled water.

Preparation and HPLC-fractionation of oligosaccharides from the ConA-unretained fractions.

Unadsorbed material eluted from the ConA/Sepharose column was subjected to hydrazinolysis, according to Bayard and Montreuil [10]. The residue was dissolved in saturated $NaHCO_3$ and the deacetylated GlcNac residues were completely re-*N*-acetylated with acetic anhydride [11].

A) NeuAcα (2 → 6) Galβ (1 → 4) GlcNAcβ (1 → 2) Manα (1 → 3)
   NeuAcα (2 → 6) Galβ (1 → 4) GlcNacβ (1 → 2) Manα (1 → 6) >Manβ (1 → 4) GlcNAc

B) NeuAcα (2 → 3) Galβ (1 → 4) GlcNAcβ (1 → 4) \
   NeuAcα (2 → 6) Galβ (1 → 4) GlcNAcβ (1 → 2) Manα (1 → 3)
   NeuAcα (2 → 6) Galβ (1 → 4) GlcNAcβ (1 → 2) Manα (1 → 6) >Manβ (1 → 4) GlcNAc

The resulting oligosaccharides were separated by HPLC on a Spectra Physics Liquid Chromatograph model SP 8,700 with a Micro Pak AX10 column (Varian Associates Orsay, France) (4 mm × 30 cm) eluted with water (10 min), 25 mM KH_2PO_4, pH 4.0 (10 min) and then a linear gradient of 25 to 150 mM KH_2PO_4, pH 4.0 (45 min). Before being analyzed, each fraction eluted from the column was desalted on a Bio-Gel P_2 column (1.5 × 38 cm) equilibrated with distilled water.

Thin-layer chromatography.

A thin-layer chromatographic system was used in order to assess the purity of the glycopeptide-derived oligosaccharides. Samples (5 and 10 µl) were applied to commercial plates (10 × 20 cm) coated with silica gel 60 (Merck, Darmstadt, FRG). The chromatograms were developed for 15 h using *n*-butanol/ethanol/acetic acid/pyridine/water (20:200:6:20:60, v/v) [12]. The plates were sprayed with orcinol/sulfuric acid reagent (200 mg orcinol in 20 per cent H_2SO_4) and heated at 105°C to visualize the oligosaccharides.

Analytical Methods.

Radioactivity was determined in a Packard Tricarb liquid scintillation counter.

Carbohydrate analysis of glycopeptides or oligosaccharides was carried out by classical colorimetric methods [13] and by gas-liquid chromatography after methanolysis with 0.5 M HCl/methanol for 24 h at 80°C and trifluoroacetylation according to the method of Zanetta *et al.* [14]. Analyses were carried out on a Varian Aerograph 2,700 (glass column packed with 5 per cent OV 210 ; temp. 90-210°C ; 2°C/min).

Methylation analysis was carried out as follows: glycopeptides were methylated, according to Hakomori [15], and methanolyzed in 0.5M HCl/methanol. The partially methylated methylglycosides were acetylated [16] (pyridine-acetic anhydride, 1:1, v/v ; 0.2 ml) and the products were analyzed by GLC-MS (Riber, model 10-10, Rueil-Malmaison, France) using a capillary column (0.35 mm × 60 m) coated with OV-101 (temperature programme, 100-220°C, at 4°C/min).

TABLE I.

Molar carbohydrate composition (a) of rat hemopexin and of glycopeptides resulting from pronase digestion of rat hemopexin.

Fraction	Gal	Man	GlcNAc	NeuAC
Hemopexin	2.0	3	4.0	2.4
Pronase glycopeptides	2.1	3	4.0	2.4
ConA Fraction I	2.4	3	4.5	2.9
ConA Fraction II	2.0	3	3.9	3.1
ConA Fraction III	2.0	3	3.8	2.0

(a) Calculated on the basis of three mannose residues per glycan.

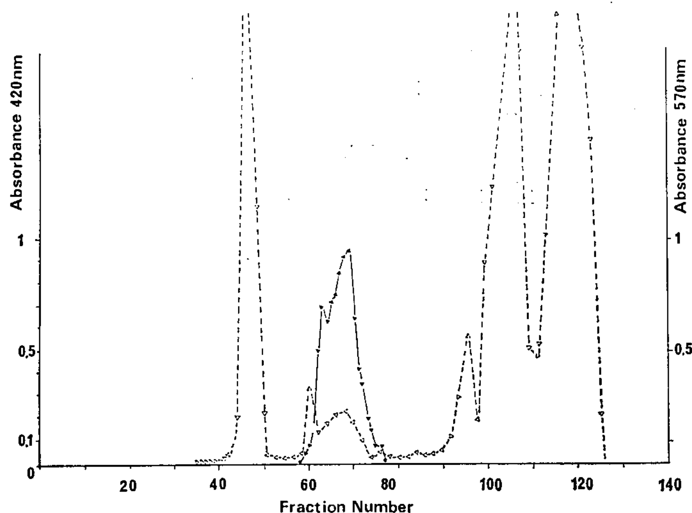

FIG. 1. — *Elution profile of pronase hydrolyzate of hemopexin on an AcA202 column (2.6 × 49 cm), equilibrated in 0.1 M pyridine acetate buffer (pH 5.0).* The column was eluted with the equilibration buffer at a rate of 17 ml/h, 2 ml fractions were collected, and analyzed for carbohydrate (▲——▲) and for peptides (△——△), as described in Experimental Procedures. Fractions 58 to 77 were pooled to obtain the glycopeptide material.

For NMR analysis, the neutralized glycopeptide was treated five times with ^2H_2O with intermediate lyophilisation, finally using 99.96 per cent ^2H_2O (Aldrich, Milwaukee, Wisconsin, USA). A Bruker WM-500 spectrometer operating at 500 MHz in the Fourier-transform mode was used. The probe temperature was 27°C. Resolution enhancement of the spectrum was achieved by Lorentzian to Gaussian transformation from quadrature phase detection [17]. The chemical shifts (δ) are expressed in p.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS) but were actually measured by reference to internal acetone (δ 2.225) with an accuracy of 0.001 p.p.m.

TABLE II.

Recovery of hexose in fractions obtained by pronase digestion of 100 mg of hemopexin, and by ConA/Sepharose chromatography of hemopexin glycopeptides.

Fraction	Hexose (µg)	Yield
Hemopexin	6,600	—
Pronase glycopeptides	5,900	89.4
ConA Fraction I	300	5
ConA Fraction II	1,020	17.2
ConA Fraction III	2,550	43.2

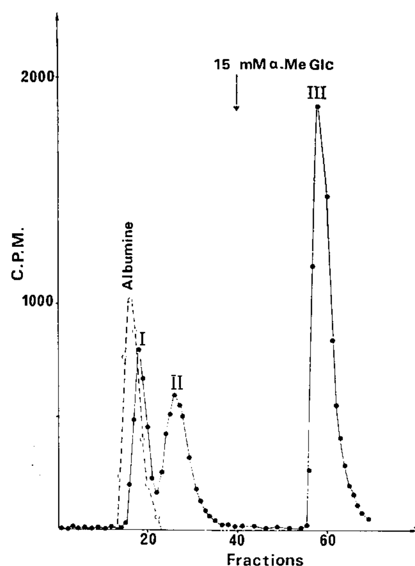

FIG. 2. — *Fractionation of hemopexin glycopeptides on a ConA/Sepharose column (1 × 24 cm) equilibrated with 0.05 M sodium acetate buffer (pH 6.0), containing 100 mM NaCl and 1 mM of $CaCl_2$, $MgCl_2$, $MnCl_2$ each.* Elution was started with equilibration buffer and fractions of 1 ml were collected and analyzed for radioactivity. When the radioactivity of the eluate had returned to the base-line elution was carried out with the starting buffer containing 15 mM α-methylglucoside; fractions of 1 ml were collected and analyzed for radioactivity.

Results.

The carbohydrate composition of rat hemopexin has been previously reported [4]. The total carbohydrate content was calculated to be 18.3 per cent. Only Man, Gal, GlcNAc and NeuAc were present. The molar ratios of these sugars in the intact glycoprotein are given in table I. No GalNAc was detected, indicating that the glycoprotein has only asparagine-linked sugar chains.

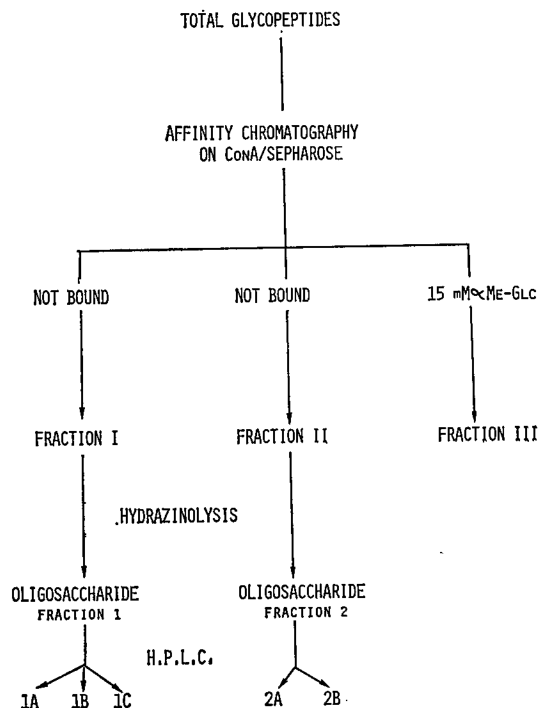

FIG. 3. — *Scheme for the fractionation of rat hemopexin glycopeptides.*

Pronase digestion.

In order to determine the structure of the oligosaccharide units of rat hemopexin, this glycoprotein was first digested extensively with pronase. Gel filtration on Ultrogel AcA202 resulted in good separation of the glycopeptides from the bulk of peptides and amino acids (fig. 1). Carbohydrate-containing material eluted in a single peak; the corresponding fractions were pooled and evaporated to dryness under reduced pressure in a rotary evaporator. About 90 per cent of the hexose content of the glycoprotein was recovered (table II) in this glycopeptide fraction; the sugar ratio was the same as in the undegraded material (table I).

Fractionation of glycopeptides.

N-acetylated [^{14}C]-labelled glycopeptides were fractionated by affinity chromatography on ConA/Sepharose into three fractions (fig. 2). Fractions I and II were eluted with the starting buffer. Fraction III, quantitatively the most important one, was bound by the lectin and eluted with 15 mM methyl α-D-glucopyranoside in the same buffer (fig. 3). Upon rechromatography, fractions I and II were both eluted as single peaks in the same position as in the first run, indicating that the ConA binding capacity of the column was not exceeded.

About 70 per cent of the carbohydrate (hexose content) applied to the column was recovered (table II). No radioactivity was found either with 100 mM or 300 mM α-methylglucoside in the starting buffer, indicating the absence of neutral mannose-rich glycopeptides in hemopexin. Each of these three fractions was pooled, evaporated and then desalted and separated from α-methylglucoside on a column of Bio-Gel P_2. Their carbohydrate composition is reported in table I.

Methylation analysis.

In order to obtain more detailed information about the structure of the hemopexin glycopeptide fraction III, substitution of the sugar residues of this glycopeptide was studied by methylation analysis. The methyl-glycosides obtained by methanolysis of the fully methylated glycopeptide III were analysed by GLC. The results thus obtained suggest that the glycan moiety of glycopeptide III possesses a diantennary structure of the *N*-acetyllactosamine type. Methylation products were identified as methyl-glycosides of 2,3,4-tri-*O*-methylgalactose, 2,3,4,6-tetra-*O*-methyl-galactose, 3,4,6-tri-*O*-methylmannose, 2,4-di-*O*-methylmannose, 3,6-di-*O*-methyl-*N*-(methyl) acetylglucosamine and 4,7,8,9-tetra-*O*-methyl-*N*-(methyl) acetylneuraminic acid in the approximative ratios : 2:0.3:2:1:4:2 (fig. 4).

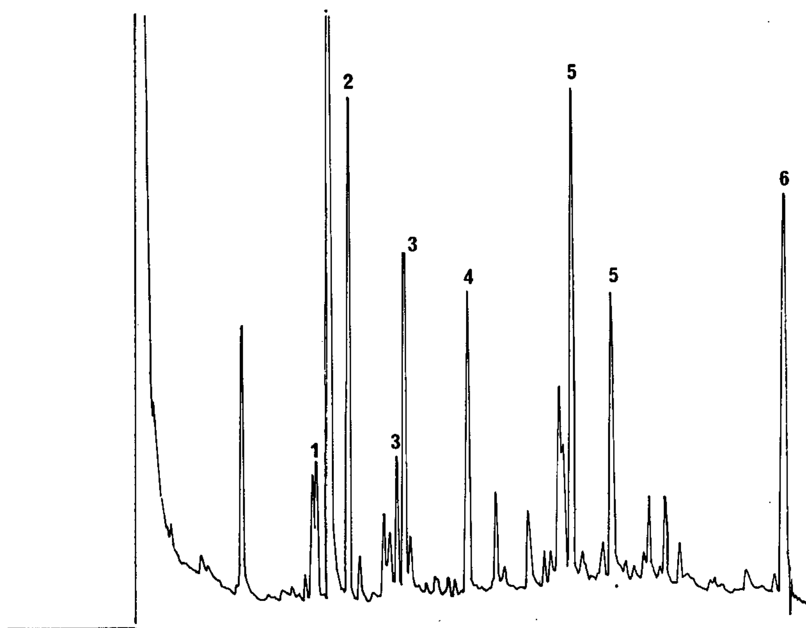

FIG. 4. — *GLC analysis of partially methylated monosaccharide derivatives present in the methanolysate of the permethylated glycopeptide fraction III :*

1 : 2,3,4,6-tetra-*O*-Me-Gal.
2 : 3,4,6-tri-*O*-Me-Man ; + 3 : **2,3,4-tri-*O*-Me-Gal** ;
4 : 2,4-di-*O*-Me-Man ; + 5 : **3,6-di-*O*-Me-GlcN (Me) Ac** ;
6 : 4,7,8,9-tetra-*O*-Me-NeuAc.

Unnumbered peaks corerspond to non-carbohydrate material.

Capillary column (0.35 mm × 60 m) coated with OV-101. Temperature 100-220°C at 4°C/min.

500-MHz ¹H-NMR Spectroscopy.

The 500-MHz ¹H-NMR spectrum of the hemopexin glycopeptide fraction III revealed the characteristic features of a partially sialylated diantennary, *N*-glycosidic carbohydrate chain of the *N*-acetyllactosamine type. The structure has a very close resemblance to that of the glycopeptide « compound 2 » from human serotransferrin [18]. The presence of the mannotriosido-*N*, ↕ *N'*-diacetylchitobiose core unit is readily inferred from the structural-reporter group signals of the constituting residues, namely : for GlcNAc-1, linked to Asn, δH-1 5.043, δNAc 2.006 ; for GlcNAc-2, δH-1 4.616, δNAc 2.078 ; and for Man-3, δH-1 ~ 4.78 [18]. The diantennary type of branching is evidenced by the set of Man H-2 chemical shifts : for Man-3, δH-2 4.251 ; for Man-4, δH-2 4.190, and for Man-4', δH-2 4.112 [18].

Both *N*-acetyllactosamine branches are terminated, by NeuAc in α(2 → 6) linkage (for NeuAc, δH-3ax 1.717 ; δH-3eq 2.669) to Gal. For each structural-reporter group sensitive to attachment of NeuAc in α(2 → 6) linkage to Gal, a pair of signals is observed ; they indicate that NeuAc is not present in both branches of all molecules in the sample. This is illustrated by Man-4, H-1 (δ5.130 and 5.116), Man-4' H-1 (δ 4.942 and 4.925), GlcNAc-5 and -5' H-1 (δ 4.598 and 4.579), Gal-6 and -6' H-1 (δ 4.441 and 4.468), and by the *N*-acetyl signals of GlcNAc-5 (δ 2.067 and 2.049) and Glc-NAc-5' (δ 2.065 and 2.045) ; for each of these reporter groups, the first value for the chemical shift corresponds to the sialylated branch, the second to the asialo one. Based on the intensity ratios within the pairs of signals, the upper branch (4-5-6) is ~ 90 per cent, the lower branch (4'-5'-6') ~ 75 per cent sialylated. It is difficult to establish whether this heterogeneity of the degree of sialylation of the branches already existed in the native hemopexin, or whether it has been introduced artificially during sample purification.

Based on the carbohydrate composition, the results of 500-MHz ¹H-NMR spectroscopy, and those of methylation analysis, the following structure is proposed for the glycans present in hemopexin fraction III :

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[NeuAcα(2→6)]0-1 Galβ(1→ 4)GlcNAcβ(1→ 2)Manα(1→ 3)
                  6          5              4        \
                                                      Manβ(1→ 4)GlcNAcβ(1→ 4)GlcNAcβ(1→N)Asn
                                                     /  3              2               1
[NeuAcα(2→6)]0-1 Galβ(1→ 4)GlcNAcβ(1→ 2)Manα(1→ 6)
                  6'         5'             4'
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Hydrazinolysis of glycopeptides I and II and fractionation of the resulting oligosaccharides.

Attempts to methylate glycopeptide fractions I and II were unsuccessful, and in order to elucidate their structure oligosaccharides were prepared from these fractions by hydrazinolysis. These oligosaccharides were re-*N*-acetylated and analyzed by TLC which revealed a heterogeneity in both oligosaccharide fractions 1 and 2 (fig. 5). Each of these fractions was chromatographed separately by HPLC. Oligosaccharide fraction 1 was resolved into three fractions namely 1_A, 1_B and 1_C, and oligosaccharide fraction 2 gave two fractions, namely 2_A and 2_B (fig. 3). The molar ratio of

FIG. 5. — *Thin-layer chromatography of re-*N*-acetylated oligosaccharides obtained by hydrazinolysis of* : (2) Glycopeptide fraction I (3) Glycopeptide fraction II and (4) Glycopeptide fraction III. Standards used were : (1) oligosaccharide A (5) oligosaccharide B.

sugars in oligosaccharide 1A could not be determined because of the small amount of this fraction.

In oligosaccharide 1B the ratio of GlcNAc to Gal was 5:3, and that of NeuAc to Gal was 3:3 (see table III). This result is compatible with a triantennary *N*-glycosidic structure.

The most striking feature of rat hemopexin is the chemical composition of oligosaccharides 1C and 2B. The ratio of GlcNAc to Gal, 4:2, is compatible with a diantennary *N*-glycosidic structure, but these two fractions contained 3 residues of NeuAc for only 2 residues of Gal (see table III).

TABLEAU III.

Molar carbohydrate composition (a) of oligosaccharides obtained from glycopeptide fractions I and II by hydrazinolysis.

Fraction	Gal	Man	GlcNAc	NeuAc
Total Oligosaccharide Fraction 1	2.6	3	3.8	2.4
Oligosaccharide 1A (b)	—	—	—	—
Oligosaccharide 1B	2.7	3	4.5	2.9
Oligosaccharide 1C	2.2	3	4.2	3.2
Total Oligosaccharide Fraction 2	1.8	3	4.1	2.9
Oligosaccharide 2A	2	3	3.9	1.8
Oligosaccharide 2B	1.6	3	3.9	2.9

(a) Calculated on the basis of three mannose residues per glycan.
(b) The amount of oligosaccharide 1A was too low to enable sugar analysis.

Discussion and Conclusion.

This study is the first on the elucidation of the carbohydrate structure of rat hemopexin. Glycopeptides resulting from pronase digestion of hemopexin present a structural heterogeneity like the undegraded glycoprotein : three fractions were separated by ConA/Sepharose affinity chromatography, which was a good method for rapidly separating the different oligosaccharide chains of rat hemopexin. The two unadsorbed peaks, ConA fractions I and II, represent about 33 per cent of the total carbohydrate chain with 1/3 of the material being in fraction I and 2/3 in fraction II. ConA fraction III represents 67 per cent of the total carbohydrate chains.

On the basis of the carbohydrate composition, methylation analysis and 500-MHz ^{1}H-NMR spectroscopy the primary structure of the carbohydrate of fraction III has been determined. It is a diantennary structure of the *N*-acetyllactosamine type consisting of a core pentasaccharide namely mannotriosido *N,N'*-diacetylchitobiose with two peripheral NeuAcα (2 $\rightarrow$ 6)-Galβ (1 $\rightarrow$ 4)-GlcNAc branches attached by β (1 $\rightarrow$ 2) linkages to the α-Man residues of the core. Microheterogeneity exists with respect to the presence of the terminal NeuAc residues.

This kind of diantennary structure of the *N*-acetyllactosamine type is widely distributed in glycoproteins and has been found in human [19] and rabbit [20] serotransferrins, human [21] and rat plasma α_1-acid glycoprotein [22], thyroxin binding globulin [23], and rat α-lactalbumin [24].

Glycopeptide fractions I and II were heterogeneous, which prompted us to separate the oligosaccharides from these fractions. HPLC enables an efficient separation of the different oligosaccharides. There are at least two types of oligosaccharides in both fractions I and II. One of these (oligosaccharide 1B) probably has a triantennary structure, *i.e.*, three peripheral NeuAc-Gal-GlcNAc branches are linked to the core pentasaccharide. The other oligosaccharide type (oligosaccharides 1C and 2B) has an unusual structure since three NeuAc residues were found to be associated with only two Gal residues. Sialic acid residues usually occupy a terminal nonreducing position in the carbohydrate chains of glycoproteins. However, a sialic acid residue may also be in a penultimate position, when substituted at C-8 by another sialic acid residue. The presence of disialosyl groups has been described in brain glycoproteins and gangliosides by Finne *et al.* [25], and in egg glyco-

protein of rainbow trout [26]. On the other hand, the location of a sialic acid residue on one of the N-acetylglucosamines of the peripheral oligosaccharide branches has been found with bovine prothrombine [27] and bovine blood-coagulation factor X [28]. However, it should be noted that in these structures a Galβ (1 → 3) GlcNAc unit is found instead of the Galβ (1 → 4) GlcNAc existing in the outer chains of most glycoproteins with asparagine-linked sugar chains. Apparently a Galβ (1 → 3) GlcNAc group allows for an increased sialic acid content. In conclusion, the third NeuAc residue in hemopexin oligosaccharides 1C and 2B may be located either at the extremity of one of the peripheral branches thus creating a disialosyl group, or it may be bound to a GlcNAc residue of one of these peripheral branches. The difference in behaviour of fractions 1C and 2B on the conA column might be explained by the supplementary NeuAc residue being attached to different branches of the oligosaccharide. On the other hand, since fractions 2A and 2B have the same mobility on the conA column, fraction 2A may in fact be composed of molecules of fraction 2B which have been desialylated during treatment posterior to the affinity chromatography (compare with glycopeptide III).

Further methylation and ^{1}H-NMR studies, at present being carried out, should enable a more exact determination of the structure of these hemopexin sugar chains. This is of interest since the heterogeneity of the rat hemopexin oligosaccharide structures may be biologically important with respect to the recognition of the heme-hemopexin complex by hepatocytes [7].

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