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A selective method for sequential splitting of *O*- and *N*-linked glycans from *N*,*O*-glycoproteins

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ABSTRACT

O-Linked oligosaccharides from *N*,*O*-glycoproteins were selectively splitt off by treatment with alkaline sodium borohydride in the presence of cadmium salt. The side reaction of reductive cleavage of *N*-glycosylamide and peptide bonds, observed under standard conditions of splitting of *O*-linked chains (M $NaBH_4$ and 50mM NaOH, 16 h, 50°), was inhibited by addition of 5–10mM cadmium acetate and 5–10mM $EDTA \cdot Na_4$, as shown by treatment of model compounds and several glycoproteins (ovomucoid, group-specific glycoproteins H and B, fetuin, and asialofetuin). This treatment, in combination with the previously developed procedure for the release of the *N*-linked oligosaccharide chains by lithium borohydride, allows a sequential, selective cleavage of *O*-, and then *N*-linked oligosaccharides from *N*,*O*-glycoproteins by chemical methods.

INTRODUCTION

Selective release of *O*- and *N*-linked carbohydrate chains from *N*,*O*-glycoproteins is an important problem which has not been solved completely yet. The only known method[1] is based on the selective cleavage of the *N*-glycosylamide bonds by an enzyme, peptide–N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase with subsequent release of *O*-linked oligosaccharides (*O*–OS) by treatment with alkaline sodium borohydride. Chemical methods for the splitting of *O*- and *N*-linked carbohydrate chains are rather unspecific. Hydrazinolysis of *N*,*O*-glycoproteins, which is used to relase *N*-linked oligosaccharide chains (*N*–OS), results in a substantial splitting of *O*–OS (up to 40%), followed by their partial degradation[2]. Alkaline sodium borohydride treatment of *N*,*O*-glycoproteins gave *O*–OS in good yields, but is accompanied with a simultaneous release of *N*–OS in 10–30% yields [3–5] due to the reductive cleavage of the *N*-glycosylamide bond with sodium borohydride[6]. It is known[7–9] that the specificity of reduction of various functional groups with sodium borohydride in alcoholic solution may be modified by addition of inorganic salts. Thus, we studied the influence of some salts containing the cations Co^{2+}, Ni^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+}, and Cd^{2+} on the splitting off of *O*–OS from *N*,*O*-glycoproteins under standard conditions[10] (M sodium borohydride, 50mM sodium hydroxide, 16 h, 50°). It was found that addition of Cd^{2+} salts significantly increased the selectivity of the reaction[11].

We report herein a new procedure for the selective liberation of *O*–OS, and

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propose a method for the sequential splitting of *O*-, and then *N*–OS from *N*,*O*-glycoproteins, based on a combination of this method with the recently described[12] method for the release of *N*–OS by lithium borohydride treatment.

RESULTS AND DISCUSSION

The influence of Cd^{2+} salt upon reductive cleavage of *N*-glycosylamide and peptide bonds with alkaline sodium borohydride was tested on 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1-*N*-(4-L-aspartoyl)-2-deoxy-β-D-glucopyranosylamine (**1**) and glycylglycine as model compounds. The mixture obtained after treatment of **1** with aqueous M sodium borohydride–50mM sodium hydroxide for 16 h at 50° contained, in addition to the starting glycosylamino acid **1** (80%), 2-amino-4-hydroxybutyric acid (**3**, ~ 15%; the product of reductive cleavage of the *N*-glycosylamide bond), and aspartic acid (**4**, ~ 3%; the product of alkaline hydrolysis). The reduced disaccharide **5** (18%) was also present; it was formed from the oligosaccharide released by alkaline hydrolysis of glycosylamine **2**, the initial product of the reductive cleavage of the *N*-glycosylamide bond (**2** is stable under the conditions of the reaction with lithium borohydride[12]).

After treatment of glycopeptide **1** with alkaline sodium borohydride containing cadmium sulfate (from 0.1 up to 1mM), starting **1** remained mainly intact, small proportions (3%) of **4** and **5** were detected, and the product of reductive cleavage **3** was not found. Analogous results were obtained with glycylglycine which gave 2-aminoethanol in a yield of 9% (the product of reductive cleavage) only after treatment with alkaline sodium borohydride without cadmium sulfate. These results demonstrated that the presence of a cadmium salt in alkaline sodium borohydride solution leads to inhibition of the reductive cleavage of *N*-glycosylamide and peptide bonds.

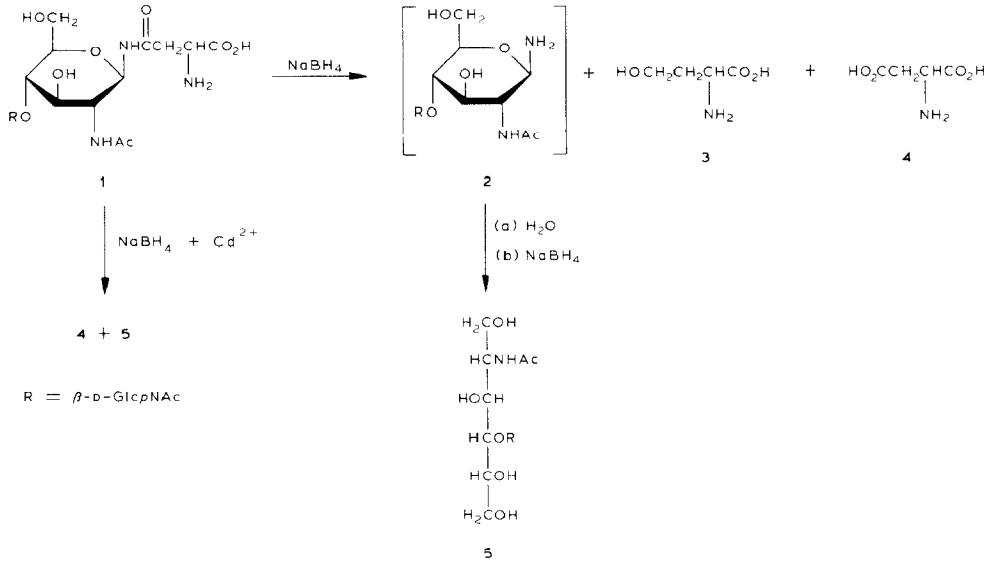


TABLE I

Yields of olygosaccharides after alkaline borohydride treatment of glycoproteins.

	Reagents							*Yields (%) of*	
Glycoprotein	*$LiBH_4$* (M)	*LiOH* (*m*M)	*$NaBH_4$* (M)	*NaOH* (*m*M)	*Cd^{2+}*	*Time* (*h*)	Temp. (°)	O–*OS*	N–*OS*
Fetuin or asialofetuin			1	50		16	50	50–55	15–20
			1	57	+	16	50	67–73	3–4
			2	27		16	50	48–53	23–25
			2	27	+	16	60	85–90	7–8
			2	27	+	10	60	75–80	3.5–4
	1.4	25				5	45	32	41
	2	25				5	45	10–15	60–63
Glycopeptide fraction[a]	2	25				5	45	[b]	50–55
Ovomucoid			1	50		16	50		15–20
			1	57	+	16	50		3–4
			2	27	+	16	60		4.3
			2	27	+	10	60		3.5
GSG H or GSG B			1	50		16	50	61–66	
			1	57	+	16	50	70–75	
			2	27		16	60	65	
			2	27	+	16	60	85–90	
			2	27	+	10	60	78–83	
			2	27	+	5	60	65	

[a] From fetuin or asialofetuin after $NaBH_4$–Cd^{2+} treatment. [b] Traces.

Subsequently, we investigated the influence of Cd^{2+} salt in alkaline sodium borohydride on the splitting of *O*–OS and *N*–OS, and the cleavage of the peptide chain of glycoproteins by use of fetuin and asialofetuin (containing both *O*- and *N*-linked chains), ovomucoid (containing *N*-linked chains), and group-specific glycoproteins (GSG) H and B (containing *O*-linked chains). Inhibition of the reductive cleavage of the amide bonds could be achieved by the use of a 10-fold higher concentration of Cd^{2+} salt. The addition of tetrasodium ethylenediaminetetraacetate (EDTA · Na_4) was found to be necessary to prevent the precipitation of cadmium hydroxide from the alkaline solution. The chelating agent showed no influence on the splitting of *O*- and *N*–OS with alkaline sodium borohydride in the absence of Cd^{2+} salt.

The treatment (16 h, 50°) of fetuin, asialofetuin, and ovomucoid with a solution of M sodium borohydride in 57 mM sodium hydroxide containing 6mM cadmium acetate and 6mM EDTA · Na_4 led to liberation of *O*–OS (70%) from fetuin or asialofetuin, and only a minor release of *N*–OS (3–4%) as compared to 15–20% in the absence of Cd^{2+} salt (see Table I). That the splitting of *N*–OS was due to alkaline hydrolysis of the *N*-glycosylamide bond was confirmed by control experiments in which ovomucoid and fetuin were treated with sodium hydroxide alone.

No reductive cleavage of the peptide chain was observed upon alkaline treatment

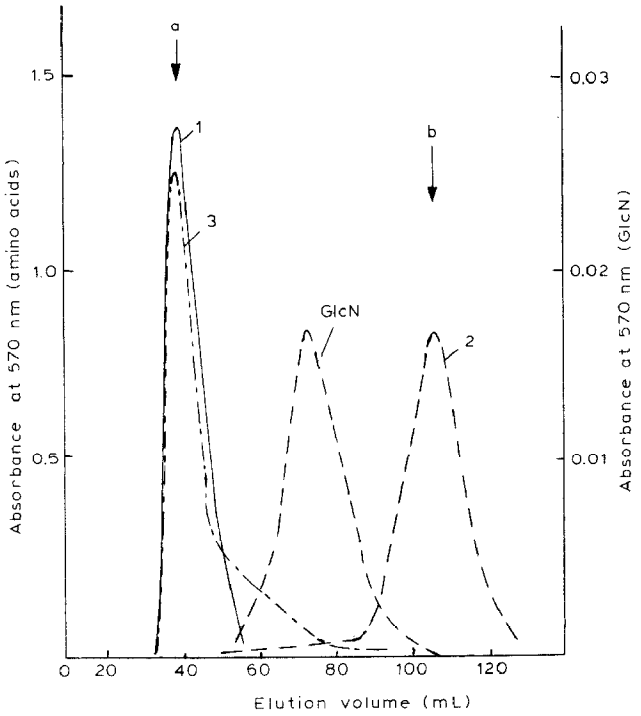


Fig. 1. Gel chromatography on a Sephadex G-75 column (1.55 × 62 cm) in 4mM NH_4OH of: (1) fetuin; (2) fetuin treated with M $NaBH_4$ and 50 mM NaOH for 16 h at 50°; and (3) fetuin treated with M $NaBH_4$ at 57mM NaOH and 6mM cadmium acetate–6mM EDTA·Na_4 solution for 16 h 50°. Fractions were analyzed after hydrolysis (4M HCl, 16 h, 100°) with an amino acid analyzer (buffer *C*). Absorbance at 570 nm after ninhydrin reaction gave a sum of neutral amino acids for (1), (2), and (3); and 2-amino-2-deoxy-D-glucose (GlcN) for (2). The arrows show the elution volume of reference compounds: (a) Dextran Blue; (b) triglycylglycine.

with sodium borohydride–Cd^2 of glycoproteins as well. This conclusion could be drawn from gel chromatography data. Thus, gel filtration on Sephadex G-75 resulted in almost identical elution curves for fetuin before and after treatment with alkaline sodium borohydride–Cd^{2+}, whereas treatment without Cd^{2+} produced a considerable depolymerization with complete absence of high-molecular-weight products (Fig. 1). The depolymerization was accompanied by a decrease in content of several amino acids (Table II). For example, the proportions of aspartic and glutamic acids, and glycine in GSG H diminished by 37–46% after treatment with akaline sodium borohydride, whereas no change was observed upon treatment with alkaline sodium borohydride–Cd^{2+}. Increase in alanine content and formation of 2-aminobutyric acid in both cases indicated the partial reduction by sodium borohydride of 2-aminopropenoic and 2-amino-2-butenoic acid residues that were formed upon β-elimination of *O*–OS from serine and threonine residues.

The addition of Cd^{2+} salt to alkaline sodium borohydride not only increased the selectivity of *O*–OS splitting, but also resulted in higher yields of oligosaccharide alditols (Table I). This increase probably resulted from the inhibition of the cleavage of

TABLE II

Amino acid composition of GSG H after treatment with various reagents [a]

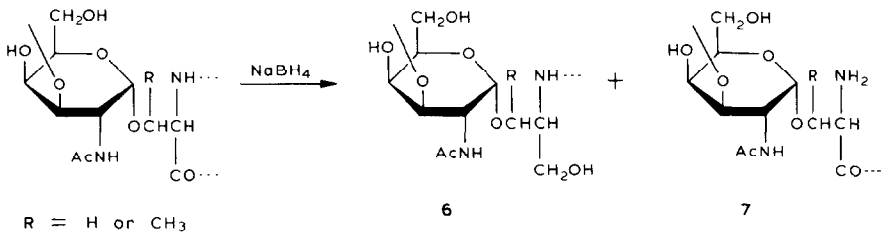
Amino acid	*Conditions*		
	A	*B*	*C*
Aspartic acid	62.5	100	100
Threonine	12.9	25	24.5
Serine	10.7	19.5	22.5
Glutamic acid	63	97.5	100
Proline	76	92	88
Glycine	54	97.5	95
Alanine	136	204	201
2-Aminobutyric[b] acid	41	14	19

[a](A) M $NaBH_4$, 50mM NaOH 16h, 50°; (B) M $NaBH_4$, 57mM NaOH, 6mM EDTA·Na_4, 6mM cadmium acetate, 16h, 50°; and (C) 2M $NaBH_4$, 27mM NaOH, 6mM EDTA·Na_4, 6mM cadmium acetate, 10h, 60° (Percentage with respect to initial content). [b]From threonine in initial GSG H.

the peptide chain. In fact, 2-amino-1,3-propanediol and 2-amino-1,3-butanediol, the products of reductive cleavage of peptide bonds at serine and threonine residues, were identified among the amino alcohols formed by alkaline sodium borohydride treatment of fetuin. It may be expected that the reductive cleavage of the bonds of the 2-amino-3-hydroxy acid residues bearing carbohydrate chains gives compounds **6** or **7**, which are either stable against or rather resistant to β-elimination of *O*–OS.

For determination of the influence of reductive cleavage of the peptide chain on the yield of *O*–OS, fetuin was treated with lithium borohydride, which produced a reductive cleavage of peptide bonds much more extensive than did sodium borohydride[12]. It was found that the increase in concentration of lithium borohydride (from 1.4 to 2M), and therefore of the velocity of the reductive cleavage led to a decrease in the yield of *O*–OS (from 32 to 12%; Table I). The *O*-linked oligosaccharides remaining unsplit (88% after 2M lithium borohydride and 45% after M sodium borohydride treatment) were found to be stable under mild alkaline conditions, and remained practically intact after repeated treatment with alkaline sodium borohydride or sodium borohydride–Cd^{2+}.

The inhibition by Cd^{2+} salt of the reductive cleavage of the peptide chain by alkaline sodium borohydride treatment opened the route to a more efficient procedure for the liberation of *O*-OS from glycoproteins. When fetuin or GSG were treated with a 2M solution of sodium borohydride in 27mM sodium hydroxide containing 6mM EDTA·Na_4 and 6mM cadmium acetate for 16h at 60°, *O*-OS were released in yields of



R = H or CH_3

6 7

85–90% (Table I). Under these conditions, however the alkaline hydrolysis of the *N*-glycosylamide bond was more significant and *N*-linked chains (7–8%) were split off from fetuin. For this reason, it seems advisable to use these conditions for release of *O*-OS from *O*-glycoproteins. To split of *O*-OS from *N,O*-glycoproteins in rather high yields (70–80%) and sufficiently selectively, it is recommended to use alkaline M or 2M sodium borohydride–Cd^{2+} (16 h, 50°; or 10h, 60°, respectively). It should be noted that under all conditions applied, including those described earlier[10], *N*-deacetylation of 2-acetamido-2-deoxy-D-hexose residues was not more than 2–3% for GSG H or B, and was not detected for fetuin and asialofetuin.

Selectivity of splitting of *O*-OS with alkaline sodium borohydride–Cd^{2+} and stability of the remaining *O*-OS in glycoprotein towards the action of alkaline lithium borohydride suggested a procedure of stepwise release of *O*-, and then *N*-OS from *N,O*-glycoproteins. This procedure involves the treatment of *N,O*-glycoprotein with M or 2M sodium borohydride for 16 h at 50° or 10 h at 60°, respectively, followed by addition of acetic acid to pH 7–7.5, and separation of the partially deglycosylated glycoprotein and oligosaccharides as alditols by gel chromatography in dilute ammonium hydroxide (pH ~ 9.5). It should be noted that gel chromatography in acidic media or cation-exchange chromatography are not satisfactory owing to the irreversible adsorption of a significant part of the deglycosylated glycoprotein. To split off *N*-OS from the partially deglycosylated glycoprotein, this was treated with alkaline lithium borohydride in 70% aqueous *tert*-butyl alcohol, as described previously[12], and *N*-OS were isolated in a yield of 50–55%.

This procedure, developed for asialofetuin, may be applied also for highly sialylated fetuin. In this case, separation of *N*-OS and *N*-glycopeptides on Dowex 50W-X2 (H^+) cation-exchange resin was achieved at 5° with 5% acetic acid as an eluent. It may be concluded that alkaline sodium borohydride–Cd^{2+} is the first selective reagent for splitting of nondegraded, *O*-linked oligosaccharides from *O*- and *N,O*-glycoproteins. Use of this reagent in combination with the lithium borohydride method[12] makes possible the sequential, chemical release of both *O*- and *N*-linked oligosaccharide chains from *N,O*-glycoproteins.

EXPERIMENTAL

General methods. — Fetuin (Sigma), ovomucoid[13], GSG H (ref. 14), and GSG B (ref. 15) were used as model glycoproteins. Asialofetuin was obtained by incubation (1.5 h, 80°) of fetuin in diluted formic acid (pH 2.1). The following reagent mixtures were employed: (*A*) 50mM NaOH and M $NaBH_4$; (*B*) 57mM NaOH, M $NaBH_4$, 6mM cadmium acetate and 6mM EDTA·Na_4; and (*C*) 27mM NaOH, 2M $NaBH_4$, 6mM cadmium acetate, and 6mM EDTA·Na_4. Reactions were performed in quartz test-tubes (1 × 15 cm or 0.7 × 10 cm) or in Pyrex glass test-tubes (1.8 × 20 cm) covered with Parafilm. Butanol (5% v/v) was added to the reaction mixtures containing $NaBH_4$ to prevent foaming. Yields of oligosaccharides were calculated from the quantitative determination of 2-amino-2-deoxy-D-galactose and -D-galactitol for *O*-OS or 2-amino-2-deoxy-D-glucose and -D-glucitol for *N*-OS.

Analytical methods. — Amino acids, 2-amino-2-deoxyhexoses, and amino alcohols were determined, after hydrolysis with 4M HCl (16 h, 100°), by use of a post-column ninhydrin reaction after separation with the amino acid analyzer Microtechna T-339, equipped with an Ostion LG ANB column (0.38 × 17 cm). The following buffers were used: (*A*) 0.2M sodium citrate–HCl (pH 3.25) at 63° (*B*) 0.35M sodium citrate–HCl (pH 5.28) at 63°, (*C*) borate[16] (pH 7.24) at 89°, and (*D*) 0.7M sodium citrate–HCl (pH 5.28) at 63°; at a flow rate of 16.8 mL $.h^{-1}$. 2-Amino-4-hydroxybutyric acid was determined without hydrolysis on an amino acid analyzer Biotronik LC 4010, equipped with an Aminex A-6 column (0.9 × 23 cm) eluted with buffer *A*.

Preparation of a solution of cadmium acetate–EDTA·Na_4. — A mixture of solutions of 0.1M EDTA·Na_2 (4.5 mL) and cadmium acetate (23 mg·mL^{-1}, 4.5 mL) was titrated with 0.5M NaOH (~1.7 mL) to pH 7–7.5.

Treatment of 2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1-N-(4-L-aspartoyl)-2-deoxy-β-D-glucopyranosylamine[17] (**1**). — *(a)*. To a solution of **1** (0.33 mg) in water (0.35 mL), previously titrated with 0.1M NaOH to pH 7.5–8, were added water (0.1 mL), 0.5M NaOH (0.05 mL), and $NaBH_4$ (19 mg). After being incubated for 16 h at 50°, the mixture was cooled, diluted with water (2.5 mL), acidified with conc. HCl to pH ~1.5, and diluted with water to 7 mL. Amino acid analysis of an aliquot (1 mL) indicated a content of 2-amino-4-hydroxybutyric acid (**3**) and free aspartic acid (**4**) of 15 and 3%, respectively. The remaining solution was coevaporated with methanol (3 × 3 mL). The residue was hydrolyzed with 4M HCl to give **4** (80%, buffer *A*), 2-amino-2-deoxy-D-glucitol (9%, buffer *C*), and 2-amino-2-deoxy-D-glucose (90%, buffer *C*).

(b). An analogous procedure was used for the treatment of **1** with alkaline $NaBH_4$ containing 0.1–1mM $CdSO_4$. Free aspartic acid content was determined as 3% and **3** was not found. After hydrolysis, **4** (~100%), 2-amino-2-deoxy-D-glucitol (~3%), and 2-amino-2-deoxy-D-glucose (~100%) were determined.

(c). An analogous treatment of **1** with 50 mM MaOH led to **4** (3%).

Treatment of glycylglycine. — Glycylglycine (0.6 mg) was treated as described before. After treatment with alkaline $NaBH_4$, 2-aminoethanol (buffer *D*, 9%) and glycine (buffer *A*, 13% without hydrolysis) were determined. After treatment with 50mM NaOH, or alkaline $NaBH_4$ and $CdSO_4$, glycine was determined without hydrolysis in 6% or 4% yield, respectively; 2-aminoethanol was not found.

Treatment of fetuin and asialofetuin. — (a). Analytical experiments. A solution of fetuin or asialofetuin (1.5 mg) in reagent *A*, *B*, or *C* (0.5 mL) was incubated for 16 h at 50° (for *A* and *B*), or 10 and 16 h at 60° (for *C*). The mixture was cooled, diluted with water (3.5 mL), and acidified with acetic acid to pH 6. The solution was stirred (30 min) with Dowex 50W–X2 (H^+, 3 mL) cation-exchange resin, and the resin was filtered off and washed with water. The filtrate was concentrated to dryness with addition of methanol (3 × 5 ml), and the residue was hydrolyzed and analyzed for 2-amino-2-deoxy-D-glucose, -D-galactose, -D-glucitol, and -D-galactitol. The resin was washed with M NH_4OH (20 mL), the filtrate was concentrated, the residue was hydrolyzed, and 2-amino-2-deoxy-D-glucose and -D-galactose were determined. The yields of *O*–OS and

N–OS (Table I) were calculated from the content of 2-acetamido-2-deoxy-D-glucose and -D-galactose in the starting fetuin or asialofetuin. Loss of 2-acetamido-2-deoxy-D-glucose owing to irreversible adsorption of glycopeptides on Dowex resin was as much as 25%.

(b). Isolation of O–OS and glycopeptide fractions from fetuin after treatment with reagent B. An aqueous solution (2 mL) of fetuin (7.8 mg, containing 1.52 μmol of 2-amino-2-deoxy-D-glucose and 0.25 μmol of 2 amino-2-deoxy-D-galactose) was treated with cadmium acetate–EDTA · Na_4 solution (0.43 mL), water (0.23 mL), 0.5M NaOH (0.34 mL), $NaBH_4$ (114 mg), and butanol (0.15 mL) for 16 h at 50°. The mixture was cooled, diluted with water to 15 mL, acidified with acetic to pH 7, and concentrated to 3 mL. The solution was fractionated on a Sephadex G-50 column (1.8 × 85 cm) in 4mM NH_4OH (pH ~ 9.5) to give three fractions. Fraction I ($K_{av} < 0.15$) contained 1.16 μmol (76%) of 2-amino-2-deoxy-D-glucose, 52,6 nmol (21%) of 2-amino-2-deoxy-D-galactose, and a considerable proportion of amino acids. Fraction II (K_{av} 0.15–0.3) contained 0.29 μmol (19%) of 2 amino-2-deoxy-D-glucose, 9.3 nmol (0.61%) of 2-amino-2-deoxy-D-glucitol, 12.5 nmol (5.1%) of 2-amino-2-deoxy-D-galactose, and amino acids. It was identified as a mixture of glycopeptides and of a small proportion of *N*–OS (3.1%). Both fractions were combined and concentrated to dryness, and the resulting glycopeptide fraction was used for liberation of *N*–OS. Fraction III (K_{av} 0.3–0.85) contained 0.183 μmol (73%) of 2-amino-2-deoxy-D-galactitol, 35.2 nmol (2.3%) of 2-amino-2-deoxy-D-glucose, and a small proportion of amino acids, and it was identified as oligosaccharide alditols with admixture of glycopeptides. The main portion of this fraction (0.16 μmol of amino alditols) was concentrated with addition of acetic acid in methanol (3 × 7 mL), the residue was dissolved in 5% acetic acid (1 mL), and the solution was applied to a column (1.15 × 12 cm) of Dowex 50W-X2 (H+) cation-exchange resin. The oligosaccharide alditols were eluted with 5% acetic (25 mL) at 5°, the column was washed with water (30 mL), and glycopeptides were eluted with M NH_4OH (60 mL). The oligosaccharide fraction contained 0.155 μmol of 2-amino-2-deoxy-D-galactitol and 17 nmol of 2-amino-2-deoxy-D-glucose.

(c). Lithium borohydride treatment of a glycopeptide fraction from fetuin or asialofetuin. The glycopeptide fraction containing 1.2 μmol of 2-amino-2-deoxy-D-glucose was treated with alkaline $LiBH_4$ for 5 h at 45° in 70% *tert*-butyl alcohol (1.5 mL), and the mixture of the *N*-OS and glycopeptides was isolated by gel chromatography on a Sephadex G-15 column, as reported previously[12]. Further fractionation of the mixture prepared from asialofetuin was performed as described[12] and the *N*–OS were obtained in a yield of 55%.

In the case of fetuin, the mixture of *N*–OS and glycopeptides was dissolved in 5% acetic acid (0.1 mL), and the solution was applied to a column (0.3 × 10 cm) of Dowex 50W-X2 (H^+) cation-exchange resin in 5% acetic acid at 5°. The *N*–OS were eluted with 5% acetic acid. The first fraction (0.43 mL, 60% of the column volume) contained 0.48 μmol of 2-amino-2-deoxy-D-glucose and 18 nmol of 2-amino-2-deoxy-D-glucitol, and it was identified as a mixture of *N*–OS (34%) and *N*–OS alditols (7.5%). The second fraction (0.43 mL) contained 0.33 μmol (28%) of 2-amino-2-deoxy-D-glucose and some

amino acids, and it was identified as a mixture (~1:1) of *N*–OS and *N*-glycopeptides.

Treatment of GSG H and B. — (a). Analytical experiments. Solutions of GSG (1.5 mg) in reagent *A*, *B*, or *C* (0.5 mL) were incubated for 16 h at 50° (for *A* and *B*), or for 5 h, 10 h, and 16 h at 60° (for *C*). After cooling, water (1.5 mL) was added to each solution, which was acidified with acetic acid. The solution was concentrated with addition of methanol (3 × 3 mL), and the residue was hydrolyzed and subjected to analysis for amino acids (Table II), 2-amino-2-deoxy-D-galactose, -D-glucose, and -D-galactitol. The yields of oligosaccharides are shown in Table I.

(b). Isolation of oligosaccharides after treatment of GSG H with reagent C. An aqueous solution (1.4 mL) of GSG H (6 mg containing 2.5 μmol of 2-amino-2-deoxy-D-galactose) was mixed with water (0.2 mL), cadmium acetate–EDTA·Na_4 solution (0.29 mL), 0.5M NaOH (0.11 mL), $NaBH_4$ (152 mg), and butanol (0.1 mL). The mixture was incubated for 16 h at 60°, cooled, and diluted with water (6 mL). The solution was acidified with acetic acid to pH 7–7.5 and diluted with water to 10 mL.

Half of the solution was acidified with acetic acid to pH 5–6 and concentrated with addition of methanol (3 × 5 mL). The residue was dissolved in 5% acetic, and the solution was applied to a column (1.15 × 14.5 cm) of Dowex 50W-X2 (H^+) cation-exchange resin. The oligosaccharide fraction was eluted with 5% acetic acid (30 mL), the column was washed with water (45 mL) to neutrality, and the glycopeptide fraction was eluted with M NH_4OH (75 mL). The oligosaccharide fraction contained 1.04 μmol (83%) of 2-amino-2-deoxy-D-galactitol and 13 nmol (1%) of 2-amino-2-deoxy-D-galactose (the latter originated from the admixture of the glycopeptides). The glycopeptide fraction contained 50 nmol (4%) of 2-amino-2-deoxy-D-galactose and 29 nmol (2.3%) of 2-amino-2-deoxy-D-galactitol (from the admixture of the oligosaccharides due to *N*-deacetylation of 2-amino-2-deoxyhexoses). Loss of 2-amino-2-deoxy-D-galactose owing to irreversible adsorption of glycopeptides on Dowex resin was as much as 10%. The second half of the solution was concentrated to 2 mL and the solution was applied to a column (1.8 × 95 cm) of Sephadex G-15 which was eluted with 4mM NH_4OH (pH ~ 9.5) to give two fractions. Fraction I ($K_{av} < 0.27$) contained amino acids (~85%) and 2-amino-2-deoxy-D-galactose (0.104 μmol, 8.3%). Fraction II (K_{av} 0.27–1) was concentrated with addition of acetic acid in methanol (3 × 7 mL), the residue was dissolved in 5% acetic acid (5 mL), and the solution was applied on to a column (1.15 × 14.5 cm) of Dowex 50W-X2 (H^+) cation-exchanges resin. The oligosaccharide fraction was eluted with 5% acetic acid (30 mL), the column was washed with water (45 mL), and the glycopeptide fraction was eluted with M NH_4OH (75 mL). The oligosaccharide fraction contained 1.09 μmol (87.5%) of 2-amino-2-deoxy-D-galactitol, and the glycopeptide fraction 34 nmol (2.7%) of 2-amino-2-deoxy-D-galactitol and 21 nmol (1.7%) of 2-amino-2-deoxy-D-galactose.

Treatment of ovumucoid — (a). With alkaline sodium borohydride and Cd^{2+} (reagent B). A solution (0.6 mL) of ovomucoid (3 mg containing 1.9 μmol of 2-amino-2-deoxy-D-glucose) was mixed with water (0.14 mL), cadmium acetate–EDTA·Na_4 solution (0.14 mL), 0.5M NaOH (0.12 mL), $NaBH_4$ (38 mg), and butanol (0.05 mL). The mixture was incubated for 16 h at 50°, and then cooled. Water (1 mL) was added, and the solution was acidifield with acetic acid to pH 7 and it was applied onto a column

(1.8 × 95 cm) of Sephadex G-50 in 4mM NH_4OH and two fractions were obtained. Fraction I (K_{av} < 0.45) contained most of the amino acids and 1.71 μmol (90%) of 2-amino-2-deoxy-D-glucose, and was identified as a mixture of high-molecular-weight glycopeptides. Fraction II (K_{av} 0.45–0.75) contained 0.18 μmol (9.5%) of 2-amino-2-deoxy-D-glucose and 9.5 nmol (0.5%) of 2-amino-2-deoxy-D-glucitol, and was found to be a mixture of glycopeptides (~6%) and *N*-OS alditols (~3%).

(b). With alkaline sodium borohydride (reagent A*).* The reaction was carried out in an analogous manner. After gel chromatography on Sephadex G-50, two fractions were obtained. Fraction I (K_{av} < 0.45) contained a small proportion of amino acids and 28 nmol (1.5%) of 2-amino-2-deoxy-D-glucose, and it was identified as a mixture of glycopeptides. Fraction II (K_{av} 0.45–0.75) contained amino acids, 1.52 μmol (80.3%) of 2-amino-2-deoxy-D-glucose, and 51.2 nmol (2.7%) of 2-amino-2-deoxy-D-glucitol, and it was found to be a mixture of glycopeptides (~67%) and *N*–OS alditols (~17%).

*(c). With 0.05*M *sodium hydroxide.* The treatment was carried out in an analogous manner. After gel chromatography on Sephadex G-50, Fraction II (K_{av} 0.45–0.75) was concentrated to dryness, the residue was dissolved in 50mM NaOH (0.1 mL), and $NaBH_4$ (3 mg) was added. The mixture was incubated for 3 h at 40° and then diluted with water to 0.5 mL. Acetic acid (0.15 mL) was added and boric acid removed by coevaporation with methanol (3 × 2 mL). Analysis of the residue with an amino acids analyzer revealed the presence of amino acids, 2-amino-2-deoxy-D-glucose (0.266 μmol, 14%), and 2-amino-2-deoxy-D-glucitol (13.3 nmol, 0.7%). Thus, Fraction II is a mixture of glycopeptides (~10%) and *N*-OS (~3.5%).

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