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The Effect of Alkaline Borohydride Treatment on *N*-Linked Carbohydrates of Glycoproteins

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The effects of treatments of the glycoprotein ribonuclease-B, the proteins ribonuclease-A and myoglobin, and the glyco-amino acid GlcNAcβ(1-*N*)Asn with alkali, alkaline sodium borohydride, and aqueous sodium borohydride were systematically studied as a function of the concentration of the reagents, the temperature, and the length of the treatment. High-field ^{1}H-NMR spectroscopy, chromatographic methods and amino-acid analysis were used to characterize products of the treatments of the various compounds. Our results indicate that mild alkaline borohydride treatment, as well as aqueous borohydride treatment alone, is capable of extensively degrading polypeptides and of partially releasing the *N*-linked glycans from ribonuclease-B. Initially, glycopeptides are produced, the peptide portion of which consists of several amino acids, which are further hydrolyzed to yield a mixture of glyco-asparagines and oligosaccharide-alditols in the ratio of ~4:1. Strong alkaline borohydride treatment of ribonuclease-B is capable of completely releasing the *N*-linked carbohydrates as oligosaccharide-alditols.

For many years it has been widely accepted that the *N*-glycosidic linkage between *N*-acetylglucosamine and asparagine in glycoproteins is considerably more stable under mild alkaline conditions than the *O*-glycosidic linkages between *N*-acetylgalactosamine and serine or threonine [1-3]. This differential stability has been employed to distinguish between *N*- and *O*-linked carbohydrate chains on glycoproteins (e.g., [1, 2]). However, a number of investigators have recently given evidence that *N*-linked oligosaccharide chains are at least partially cleaved from the protein under conditions previously thought to cleave only *O*-glycosidic carbohydrate-peptide linkages.

Rasilo and Renkonen [4] reported that both *N*- and *O*-linked oligosaccharides are liberated from glycoproteins under mild alkaline borohydride conditions (0.05 M NaOH, 1.0 M

Abbreviation: RNase, ribonuclease.

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$NaBH_4$, 45°C, 16 h) and that $NaBH_4$ alone, but not alkali alone, cleaved the GlcNAcβ(1-*N*)Asn linkage of GC-4 glycopeptide. Ogata and Lloyd [5] studied the effects of three conditions of alkaline $NaBH_4$ treatment on desialylated fetuin, transferrin, and glycophorin, and found that both *N*- and *O*-linked carbohydrate chains were released under all conditions tested and the approximately 60% of the *N*-linked chains were released even under the mildest conditions (0.05 M NaOH, 1.0 M $NaBH_4$, 50°C, 16 h). They concluded that mild alkaline $NaBH_4$ treatment cannot be used as a method for distinguishing *N*- and *O*-glycosides.

Neither of the two aforementioned studies [4, 5], however, dealt with the detailed structural identification of the released *N*-glycosides. Debray *et al.* [6] found, in their studies on human transferrin, lactotransferrin, and α_1-acid glycoprotein, that only small amounts (0-6%) of *N*-glycosidic chains are released as reduced glycans during mild alkaline borohydride treatment using the conditions (0.05 M NaOH, 1.0 M $NaBH_4$, 45°C, 16 h) of Iyer and Carlson [7] or the conditions (0.1 M NaOH, 0.8 M $NaBH_4$, 37°C, 68 h) of Spiro and Bhoyroo [8]. They indicated that peptide bond cleavage during mild alkaline $NaBH_4$ treatment could release glycopeptides and "make it appear as if" oligosaccharide-alditols were being produced from cleavage of the GlcNAcβ(1-*N*)Asn linkage. Hounsell *et al.* [9] also found that only a small percentage (10-20%) of *N*-linked oligosaccharides were released from fetuin during mild alkaline $NaBH_4$ treatment (using the Iyer-Carlson conditions) and that the polypeptide backbone was totally degraded.

In the present study we set out to investigate systematically the effects of numerous conditions of alkali, alkaline $NaBH_4$, and aqueous $NaBH_4$ treatments on RNase-B, a 13 kDa glycoprotein with one oligosaccharide moiety *N*-glycosidically linked to Asn-34 [10], on pure proteins, and also on GlcNAcβ(1-*N*)Asn in order to assess the effect of the length of the peptide moiety on the outcome of the events.

Materials and Methods

General

Bovine pancreatic ribonuclease-A (RNase-A) (type 1A) and ribonuclease-B (RNase-B) (type XII B) were purchased from Sigma (St. Louis, MO, USA). Sperm whale myoglobin was purchased from Schwarz/Mann (Cambridge, MA, USA) GlcNAcβ(1-*N*)Asn was purchased from Sigma. NaOH and $NaBH_4$ were from Aldrich (Milwaukee, WI, USA).

Pronase Digestion of RNase-B

RNase-B (5.0 mg) was incubated with 1.5 mg Pronase (Calbiochem, La Jolla, CA, USA) in 2.5 ml of 0.2 M sodium phosphate buffer (pH 8.0) at 37°C for five days. Four additional amounts of Pronase (1.5 mg/addition) were added every 24 h of incubation time. The digest was centrifuged (3000 x *g* for 15 min) and the resulting supernatant fluid lyophilized, dissolved in 0.6 ml of 50 mM pyridine acetate buffer (pH 5.0) and subjected to Sephadex G-50 gel permeation chromatography.

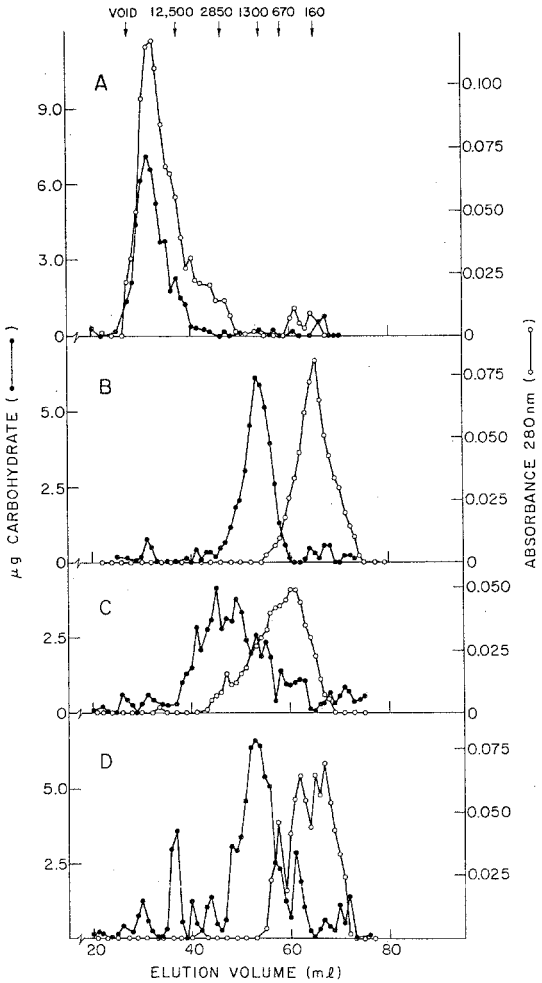

Figure 1. Elution profiles of RNase-B from Sephadex G-50 after the following treatments:
A. 0.05 M NaOH, 45°C, 8 h; B. 0.05 M NaOH, 1.0 M $NaBH_4$, 45°C, 16 h; C. 1.0 M $NaBH_4$, 100°C, 15 h.
Elution volumes for molecular weight standards are indicated by arrows at the top of the Figure. See the Materials and Methods section for details.

Gel Permeation Chromatography

The Pronase-digested RNase-B and the reaction mixtures from the alkali, alkaline $NaBH_4$ and aqueous $NaBH_4$ treated RNase-A, myoglobin and RNase-B (neutralized and brought to pH 5.0 with 1.0 M acetic acid) were analyzed by gel permeation chromatography on a Sephadex G-50 column (0.9 x 95 cm) (Pharmacia, Piscataway, NJ, USA). The column was eluted with 50 mM pyridine-acetate buffer (pH 5.0) at an approximate rate of 257 ml/h at room temperature (19-20°C) and fractions (1.0 ml) were collected and analyzed for protein and peptides by absorbance at 280 nm and for carbohydrate by the orcinol-H_2SO_4 assay [11].

Table 1. Effects of various conditions of alkali, alkaline $NaBH_4$ and aqueous $NaBH_4$ treatment on myoglobin, RNase-A and RNase-B.

Protein	Treatment conditions				Degradation of protein backbone[a]
	NaOH (M)	$NaBH_4$ (M)	Temp (°C)	Time (h)	
Myoglobin	0.05	—	45	16	none
	0.05	1.0	45	16	complete
RNase-A	0.05	—	45	16	none
	0.05	1.0	45	8	complete
RNase-B	0.05	—	3	16	none
	0.05	—	45	8	none
	0.05	—	45	16	none
	0.05	1.0	45	8	complete
	0.05	1.0	45	16	complete
	0.05	1.0	45	23	complete
	0.05	1.0	45	32	complete
	0.1	0.8	37	68	complete
	—	1.0	45	16	complete
	0.2	—	100	15	complete
	0.2	1.0	100	15	complete

[a] none (<5%); complete (≥95%).

The column was calibrated with the following molecular weight standards: blue dextran (M_r 2,000,000; Sigma), RNase-B (M_r 13,000), RNase-A (M_r 11,830), horse heart cytochrome C (M_r 12,500; Schwarz/Mann), mellitin (M_r 2648; Sigma), isomalto-octaose (M_r 1314; Bio-Carb, Lund, Sweden). stachyose (M_r 666; Sigma), and L-fucose (M_r 164; Sigma). A plot of log M_r versus elution volume of the molecular weight standards yielded a straight line from which the apparent molecular weights of the reaction products were determined.

Bio-Gel P-2 Chromatography

The fractions of the orcinol-positive peak (which eluted with an approximately M_r of 1500 Da) derived from Sephadex G-50 fractionation of Pronase-digested RNase-B were combined, lyophilized and the resulting residue was dissolved in 0.5 ml distilled H_2O and chromatographed on a column (0.9 x 95 cm) of Bio-Gel P-2 (Bio-Rad, Richmond, CA, USA). The column was eluted with distilled H_2O at an approximate rate of 9.6 ml/h at 19-20°C and 0.5 ml fractions were collected and analyzed for carbohydrate by the orcinol-H_2SO_4 assay [11]. The P-2 column was calibrated with the following molecular weight standards: blue dextran (M_r 2,000,000), stachyose (M_r 666), glucose (M_r 180) (all from Sigma) and isomalto-octaose (M_r 1314) (BioCarb), and the data were plotted as described above for the G-50 column.

500-MHz ^{1}H-NMR Spectroscopy

RNase-B derived samples for NMR analysis were repeatedly dissolved in ^{2}H (99.96 atm% ^{2}H, Aldrich, Milwaukee, WI, USA) at room temperature and p^{2}H 6 with intermediate lyophilization. The deuterium-exchanged samples were subjected to ^{1}H-NMR spectroscopy at 500 MHz (Bruker AM-500 instrument equipped with Aspect-3000 computer). Further experimental details have been described previously [12]. ^{1}H Chemical shifts are expressed in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). They were actually measured at 27°C relative to internal acetone (δ 2.225) with an accuracy of 0.002 ppm.

Amino-acid Analysis

Amino acid analyses of the glycopeptides yielded by some of the alkaline $NaBH_4$, treatments and Pronase digestion of RNase-B were performed according to [13].

Thin Layer Chromatography

The reaction mixtures from the alkali, alkaline $NaBH_4$ and aqueous $NaBH_4$ treated GlcNAcβ(1-*N*)Asn were analyzed by TLC on silica gel plates (250 μ, 20 x 20 cm Redi/plate; Fisher, Pittsburgh, PA, USA) with single development using *N*-propanol/acetic acid/H2O, 6/2/1 by vol, as the solvent system. The developed plates were dried and the compounds were visualized by spraying with 2% (w/v) ninhydrin (Sigma) in acetone and/or 1.6% (w/v) orcinol (Sigma) in 60% (v/v) H_2SO_4 followed by heating for 15 min at 100°C. Authentic GlcNAcβ(1-*N*)Asn and *N*-acetylglucosamine (from Sigma) and authentic L-Asn (Nutritional Biochemicals, Cleveland, OH, USA) were run as standards on the thin layer plates.

Results

RNase-B, RNAse, myoglobin, and GlcNAcβ(1-*N*)Asn were subjected to various treatment conditions of temperature (3°, 4°, 37°, 41°, 45°, 50°, 55°, and 100°C), time of incubation (8, 15, 16, 23, 24, 32, and 68 h), and concentrations of NaOH (0.05, 0.1 and 0.2 M) and $NaBH_4$ (0.8 and 1.0 M). The results of treating the first three of these compounds, obtained by monitoring the elution profiles of the reaction products on the Sephadex G-50 column (Fig. 1),are summarized in Table 1. Inspection of the Table indicates that mild alkali treatment (0.05 M NaOH, 16 h, 45°C) had no significant effect on the two proteins studied (myoglobin, RNase-A) which eluted as intact proteins in the void volume. However, when the mild alkali treatment included 1.0 M $NaBH_4$ as a reagent, both proteins were completely (≥ 95%) degraded. Similarly, mild alkali treatment had no effect on RNase-B, which eluted as an intact glycoprotein in the void volume (Fig. 1A). Mild alkaline borohydride treatment (1.0 M $NaBH_4$, 45°C, 0.05 M NaOH) of RNase-B for 8, 16 (Fig. 1B), 23 or 32 h led to complete degradation of its protein backbone and the production of a carbohydrate-containing fraction (orcinol-positive peak) with an apparent M_r of 1300 to 1500 Da. Pronase treatment of RNase-B for five days gave an elution profile comparable to the one depicted in Fig. 1B (profile not shown). Aqueous borohydride treatment (1 M $NaBH_4$, pH 10.3, 45°C, 16 h) of RNase-B led to extensive degradation of its protein backbone and the appearance of several

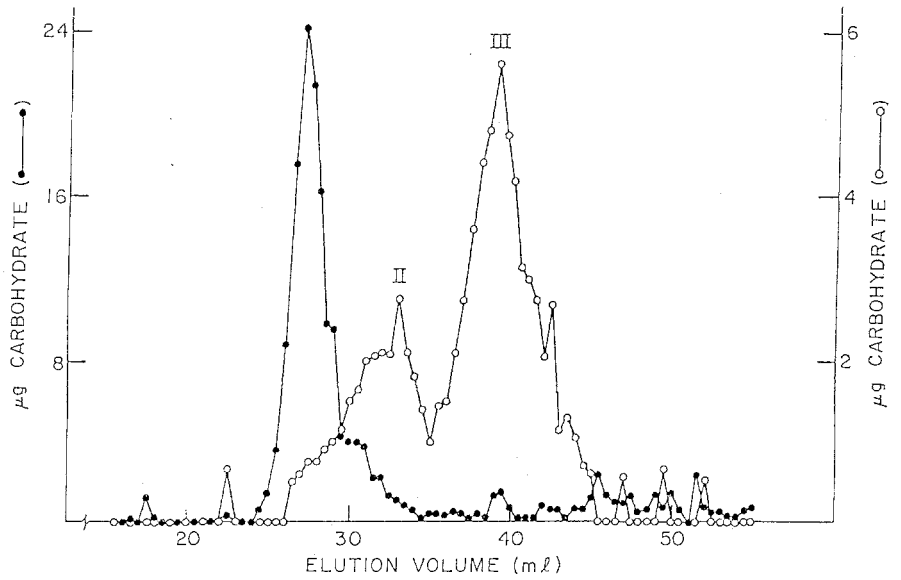

Figure 2. Elution profiles from Bio-Gel P-2 of RNase-B after exhaustive Pronase digestion (●) and after mild alkaline borohydride treatment (0.05 M NaOH, 1.0 M $NaBH_4$, 45°C, 16 h) (○). See the Materials and Methods section for details..

orcinol-positive peaks centered around an apparent M_r of 2900 Da (Fig. 1C). Strong alkali treatment (0.2 M NaOH, 15 h, 100°C) and strong alkaline borohydride treatment (1.0 M $NaBH_4$, 100°C, 0.2 M NaOH, 15 h) (Fig. 1D) of RNase-B both led to extensive degradation of the protein backbone and the appearance of several orcinol-positive peaks. The major peak (apparent M_r of 1300 to 1500 Da) from the strong alkaline $NaBH_4$ treatment was comparable to that seen for mild alkaline $NaBH_4$ treatment (Fig. 1B).

RNase-B (50 mg) was treated mildly with alkaline borohydride (0.05 M NaOH, 1 M $NaBH_4$, 45°C, 16 h), and the reaction product subjected to Sephadex G-50 chromatography as described above (compare Fig. 1B). The fractions of the orcinol-positive peak recovered from Sephadex G-50 (which eluted with an apparent M_r of 1500 Da) were combined, lyophilized, and desalted over Bio-Gel P-2. Fig 2 indicates that two orcinol-positive peaks (II and III) eluted from the column. Peak I represents Pronase-treated RNase-B after Sephadex G-50 chromatography; its position, included here for reference, indicates that this material elutes earlier from Bio-Gel P-2 than the alkaline borohydride released products.

Bio-Gel P-2 fractions I, II and III were each subjected to ^{1}H-NMR spectroscopy at 500 MHz. The ^{1}H chemical shifts of the structural-reporter groups of the monosaccharides present in the proposed structures are summarized in Table 2. As far as the oligomannoside portion of

Table 2. ^{1}H Chemical shifts of pertinent structural-reporter groups of monosaccharides present in compounds derived from RNase-B by mild alkaline borohydride treatment, compared to compounds of related structure.

Reporter group	Residue[a]	Chemical shift[b] in [c]						
		RNase-B P-2 I -D/+D	RNase-B P-2 II -D/+D	RNase-B P-2 III -D/+D	Man-GlcNAc$_2$	Glc$_3$-Man$_9$-GlcNAc$_2$	Man$_5$-GlcNAc-GlcNAc-ol	Man$_5$-GlcNAc$_2$-Asn
H-1	GlcNAc-Asn	5.086	5.05	5.05	—	—	—	5.071
	GlcNAc-1α	—	5.184	—	5.189	5.182	—	—
	GlcNAc-1β	—	n.d.	—	4.696	n.d.	—	—
	GlcNAc-2	4.606	(4.60/4.62)	4.620	4.605	4.6[f]	4.627	4.606
	Man-3	4.77	4.77	4.77	4.764	4.769	n.d.	4.781
	Man-4[d]	5.096	5.093	5.092	—	—	5.098	5.099
	Man-4	5.345/5.338	5.342/5.335	5.346/5.341	—	5.334	—	—
	Man-C	5.057/5.304	5.049/5.299	5.052/5.304	—	5.302	—	—
	Man-D$_1$	— /5.044	— /5.038	— /5.041	—	5.046[g]	—	—
	Man-4'	4.872	4.865	4.868	—	4.873	4.868	4.872
	Man-A	5.096/5.403	5.087/5.396	5.088/5.398	—	5.398	5.089	5.093
	Man-D$_2$	— /5.057	— /5.049	— /5.052	—	5.065	—	—
	Man-B	4.908/5.144	4.903/5.138	4.907/5.143	—	5.137	4.907	4.908
	Man-D$_3$	— /5.044	— /5.038	— /5.041	—	5.046	—	—
H-2	GlcNAc-ol	—	4.23	—	4.060	—	4.232	—
	Man-3	4.251/4.233[e]	4.248/4.229[e]	4.250/4.232[e]	—	n.d.	4.253	4.251
	Man-4	4.109	4.10	4.10	—	n.d.	4.076	4.077
	Man-C	4.070/4.109	4.063/4.10	4.069/4.10	—	n.d.	—	—
	Man-D$_1$	— /4.070	— /4.063	— /4.069	—	n.d.	—	—
	Man-4'	4.146	4.141	4.141	—	n.d.	4.145	4.144
	Man-A	4.070/4.109	4.063/4.104	4.069/4.10	—	n.d.	4.066	4.066
	Man-D$_2$	— /4.070	— /4.063	— /4.069	—	n.d.	—	—
	Man-B	4.023	n.d./n.d.	n.d./n.d.	—	n.d.	3.985	3.985
	Man-D$_3$	— /4.070	— /4.063	— /4.069	—	n.d.	—	—
NAc	GlcNAc-Asn	2.008	2.010	2.007	—	—	—	2.012
	GlcNAc-ol	—	2.055	—	—	—	2.055	—
	GlcNAc-1(α,β)	—	2.038	—	2.038	2.039	—	—
	GlcNAc-2	2.062	2.063	2.059	2.064	2.068	2.063	2.060

[a] For coding of residues, see Fig. 3 (compare [12]).
[b] Chemical shifts were acquired at 500 MHz, in neutral ^2H_2O solutions at 27°C.
[c] The subdivision of the RNase-B fractions I, II and III chemical shift values shows, in the left columns, their respective values for the pertinent reporter groups in branches not terminated by a Man-D residue and, in the right columns, the values when the branches are terminated by a Man-D. Centering δ-values indicates that these chemical shifts are not sensitive to heterogeneity with regard to the Man-D residues. Data for the reference compounds were taken from refs. [12], [14], [15], and from van Halbeek (unpublished results).
[d] This δ value refers to terminal Man-4, i.e., without Man-C (and -D$_1$).
[e] In this case, without/with Man-C, respectively.
[f] Spectrum was recorded at 37°C; the H-1 signal of GlcNAc-2 was obscured by the HO^{2}H peak, and could therefore not be located more precisely.
[g] Extended by Glcα(1-2)Glcα(1-3)Glcα(1-3).
n.d., value could not be determined.

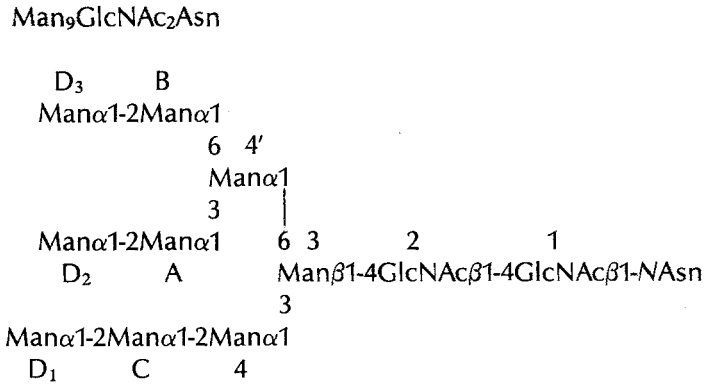

Figure 3. System used for the coding of monosaccharide residues in oligomannoside-type chains.

the structures is concerned, they are comparable to those reported for RNase-B by Liang *et al.* [16]. The NMR spectra of all three fractions showed the typical features of oligomannoside-type carbohydrates, varying in the number of mannose residues from 5 to 8 (compare [12, 17-19]). Table 2 has been arranged precisely as the analogous tables in [17] and [19], in order to facilitate comparison.

Focusing on the nature of the potential reducing end, RNase-B fractions I and III appeared to consist of glycopeptides and glyco-asparagines, respectively, that is, the oligomannosides are attached *via* GlcNAcβ(1-4)GlcNAc in β-linkage to asparagine. This is evident from the typical resonance positions of the two *N*-acetyl methyl singlets in the spectral region around δ 2 ppm: the signal at δ 2.008 (I), c.q. 2.007 (III) is assigned to the asparagine-linked *N*-acetylglucosamine (denoted GlcNAc-1, see Fig. 3), the one at δ 2.062 (I), c.q. 2.059 (III) to GlcNAc-2 [12, 17, 19]. The peptide moiety of the components of fraction I was found to be homogeneous both at the N- and the C-terminal side of asparagine (as evidenced by the singularity of the signal for the *N*-acetyl group of GlcNAc-1). However, the peptide portion of the Pronase digest glycopeptides (I) was ~7-8 amino-acids long, as suggested by the elution of fraction I on the Bio-Gel P-2 column (apparent molecular mass ~2000 Da; see Fig. 2); the latter was confirmed qualitatively by the abundance of amino-acid proton signals in the ^{1}H-NMR spectrum of fraction I, and by amino-acid analysis (results not shown). The alkaline borohydride product (fraction III) consisted essentially of glyco-asparagines, that is, only Asn-34 was left of the peptide portion of RNase-B.

In RNase-B fraction II, ~80% of the oligomannoside chains end in reduced chitobiose [GlcNAcβ(1-4)GlcNAc-ol] units. NMR evidence for this structural element stems from the occurrence of the GlcNAc-ol H-2 and *N*-acetyl signals at δ 4.23 and 2.055, respectively (compare Man_5GlcNAc-GlcNAc-ol from human placenta α-glucosidase; Table 2 [15, 19]. The remaining 20% of fraction II is made up of asparagine-linked carbohydrates (glycopeptides) similar to those in fraction I, but with a shorter peptide portion. The relative amounts

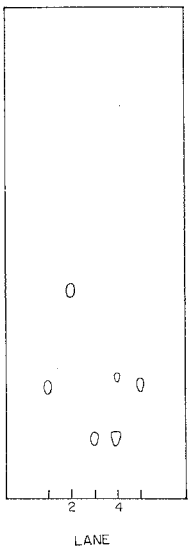

Figure 4. Diagramatic representation of results TLC of authentic standards and hydrolysate after 16 h alkaline $NaBH_4$ treatments of GlcNAcβ(1-*N*)Asn.
1. asparagine (2 mg); 2. *N*-acetylglucosamine (10 mg); 3. GlcNAcβ(1-*N*)Asn (2 mg); 4. GlcNAcβ(1-*N*)Asn (10 mg) treated with 0.05 M NaOH, 1.0 M $NaBH_4$ at 45°C; 5. GlcNAcβ(1-*N*)Asn (10 mg) treated with 0.2 M NaOH, 1.0 M $NaBH_4$ at 100°C. See the Materials and Methods section for details.

of reduced oligosaccharides and glycopeptides in fraction II (molar ratio 4:1) were deduced from the intensity ratios of the *N*-acetyl signals; their estimated accuracy is 5%.

The results of various conditions of alkali, alkaline $NaBH_4$ and aqueous $NaBH_4$ treatments on GlcNAcβ(1-*N*)Asn are summarized in Fig. 4 and Table 3. These results were determined by thin layer chromatographic monitoring of the reaction mixture for disappearance of the starting compound and/or appearance of sugar or amino-acid constituents of the starting compound. Mild alkali treatment (0.05 M NaOH, 16 h, 45°C) did not hydrolyze (< 5%) GlcNAcβ(1-*N*)Asn but treatment with mild alkaline borohydride (0.05 M NaOH, 1.0 M $NaBH_4$, 16 h, 45°C) led to partial (5-10%) hydrolysis. Strong alkali treatment (0.2 M NaOH, 16 h, 100°C) led to only partial hydrolysis (50-75%) of GlcNAcβ(1-*N*)Asn but strong alkaline borohydride treatment (0.2 M NaOH, 1.0 M $NaBH_4$, 16 h, 100°C) led to complete hydrolysis.

Discussion

There has been ample speculation in the literature as to the extent of *N*-glycan release during procedures commonly used to liberate *O*-glycans from glycoproteins. It seemed appropriate to carry out a study to characterize the effects of these procedures, with emphasis on the structural identification of the released products.

Table 3. Effects of various conditions of alkali, alkaline $NaBH_4$ and aqueous $NaBH_4$ treatments for 16 h on GlcNAcβ(1→*N*)Asn.

Treatment conditions			Extent of hydrolysis[a]
NaOH (M)	$NaBH_4$ (M)	Temp (°C)	
0.05	—	45	none (< 5%)
0.05	1.0	45	partial (5-10%)
—	1.0	45	partial (5-10%)
0.2	—	100	partial (50-75%)
0.2	1.0	100	complete (≥ 95%)
—	1.0	100	complete (≥ 95%)

[a] monitored on TLC by disappearance of GlcNAcβ(1→*N*)Asn and/or appearance of asparagine.

As found by Rasilo and Renkonen [4], mild alkali treatment had no significant effect on any of the molecules studied. The inclusion of 1.0 M $NaBH_4$ alone (45°C for 8-16 h; pH 10.3) led to degradation of all the protein and glycoprotein backbones and at least partial release of the *N*-linked carbohydrate from RNase-B. GlcNAcβ(1-*N*)Asn was not hydrolyzed significantly (<10%) under conditions (0.5 M NaOH, 1.0 M $NaBH_4$, 45°C, 16 h) which led to the production of a 4:1 mixture of glyco-asparagines and oligosaccharide-alditols from RNase-B. This suggests that a certain size of peptide is required to make the *N*-linkage susceptible to alkaline $NaBH_4$ or aqueous $NaBH_4$ treatments. Strong alkali treatment (0.2 M NaOH, 100°C, 15-16 h) completely degraded the protein backbone of RNase-B but the inclusion of 1.0 M $NaBH_4$ was necessary for complete hydrolysis of GlcNAcβ(1-*N*)Asn.

Pronase digestion of RNase-B produced glycopeptides which co-eluted on Sephadex G-50 with the orcinol-positive peak released by alkaline $NaBH_4$ treatment. However, G-50 chromatography probably cannot resolve the small differences in molecular weights between the glycopeptides, glyco-asparagines and oligosaccharide-alditols. Bio-Gel P-2 chromatography indicated that the glycopeptides produced from Pronase digestion of RNase-B eluted before the two carbohydrate peaks derived from alkaline $NaBH_4$ treatment of RNase-B. This suggested that the products of alkaline $NaBH_4$ treatment of RNase-B included both oligosaccharide-alditols and glyco-asparagines. This suggestion was confirmed unambiguously by 500 MHz ^{1}H-NMR spectroscopy. It is an interesting observation that the apparent M_r of Bio-Gel P-2 fraction III (which contains only glyco-asparagines) is less than the apparent M_r of Bio-Gel P-2 fraction II (which contains predominantly oligosaccharide-alditols). This finding may reflect a difference in the solution conformation of the oligomannoside chain between the oligosaccharide-alditols and the glyco-asparagines [12]. Our results, as well as those of several recent studies by others [4-6, 9], are consistent with the idea that mild alkaline $NaBH_4$ treatment (or aqueous $NaBH_4$ treatment alone) is

capable of releasing the *N*-linked glycans, in the form of oligosaccharide-alditols. Initially, glycopeptides are produced, which are then further hydrolyzed in two competing reactions to either oligosaccharide-alditols or glyco-asparagines. If the peptide backbone is degraded to the level of a single amino acid, the glyco-asparagine is no longer susceptible to the β-elimination reaction and it becomes the final product.

In conclusion, under the mild conditions of alkaline borohydride reductive cleavage commonly used for the release of *O*-linked mucin-type carbohydrates in the form of oligosaccharide-alditols, *N*-linked glycans are being "released" predominantly (≥75-80%) as glyco-asparagines, and to a smaller extent as oligosaccharide-alditols.

Strong alkaline borohydride treatment of RNase-B is capable of completely releasing the *N*-linked carbohydrates as oligosaccharide-alditols. Ogata *et al.* [20] have released the *N*-type carbohydrate chains from Pronase-digested rat haptoglobin by strong alkaline borohydride treatment (1 M NaOH, 4 M $NaBH_4$, 80°C, 24 h), followed by *N*-reacetylation, claiming quantitative conversion of *N*-linked carbohydrates into their oligosaccharide-alditols under these harsh conditions. Reductive elimination of *N*-linked carbohydrates of glycoproteins by strong alkaline borohydride, resulting predominantly or exclusively in oligosaccharide-alditols, would be preferable for liberating such chains over other chemical methods such as hydrazinolysis followed by *N*-reacetylation and reduction [21]. In this latter method quite a few side reactions are known to occur and the final product is not a homogeneous preparation ending in the *N,N'*-diacetylchitobiitol residue [22]. The advantage *N*-glycosides have over *O*-glycosides in the β-elimination process is that they usually lack a substituent at the C-3 position on the asparagine-linked *N*-acetylglucosamine residue, and are therefore unlikely to undergo peeling reactions [23].

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References

1 Spiro RG (1972) Methods Enzymol 28:32-34.
2 Sharon N, Lis H (1982) in Proteins, vol 5, eds. Neurath H, Hill R, Academic Press, New York, p 1-144.
3 Zinn AB, Marshall JS, Carlson DM (1978) J Biol Chem 253:6761-67.
4 Rasilo M-J, Renkonen O (1981) FEBS Lett 135:38-42.
5 Ogata S-I, Lloyd KO (1982) Anal Biochem 119:351-59.
6 Debray H, Strecker G, Montreuil J (1984) Biochem Soc Trans 12:611-12.
7 Iyer RN, Carlson DM (1971) Arch Biochem Biophys 142:101-5.
8 Spiro RG, Bhoyroo VD (1974) J Biol Chem 249:5704-17.

9 Hounsell EF, Pickering NJ, Stoll MS, Lawson AM, Feizi T (1984) Biochem Soc Trans 12:607-10.
10 Bernard BA, Newton SA, Olden K (1983) J Biol Chem 258:12198-202.
11 Hewitt LF (1937) Biochem J 31:360-66.
12 Vliegenthart JFG, Dorland L, van Halbeek H (1983) Adv Carbohydr Chem Biochem 41:209-374.
13 Sinha S, Watorek W, Karr S, Giles J, Bode W, Travis J (1987) Proc Natl Acad Sci USA 84:2228-32.
14 Ronin C, van Halbeek H, Mutsaers JHGM, Vliegenthart JFG (1987) Glycoconjugate J 4:247-54.
15 Mutsaers JHGM, van Halbeek H, Vliegenthart JFG, Tager JM, Reuser AJJ, Kroos M, Galjaard H (1987) Biochim Biophys Acta 911:244-51.
16 Liang C-J, Yamashita K, Kobata A (1980) J Biochem (Tokyo) 88:51-58.
17 Marti T, Kosen PA, Honory K, Franzblau C, Schmid K, van Halbeek H, Gerwig GJ, Vliegenthart JFG (1984) Biochim Biophys Acta 799:305-12.
18 Mutsaers JHGM, van Halbeek H, Kamerling JP, Vliegenthart JFG (1985) Eur J Biochem 147:569-74.
19 Overdijk B, Beem EP, van Steijn GJ, Trippelvitz LAW, Lisman JJW, Paz-Parente J, Cardon P, Leroy Y, Fournet B, van Halbeek H, Mutsaers JHGM, Vliegenthart JFG (1985) Biochem J 232:637-41.
20 Ogata S, Misumi Y, Miki K, Ikehara Y (1986) Eur J Biochem 161:315-20.
21 Takasaki S, Mizuochi T, Kobata A (1982) Methods Enzymol 83:263-68.
22 Michalski JC, Peter-Katalinic J, Egge H, Paz-Parente J, Montreuil J, Strecker G (1984) Carbohydr Res 134:177-89.
23 Lloyd KO, Kabat EA, Licerio E (1968) Biochemistry 7:2976-90.