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# G.L.C. OF *O*-METHYLOXIME AND ALDITOL ACETATE DERIVATIVES OF NEUTRAL SUGARS, HEXOSAMINES, AND SIALIC ACIDS: "ONE-POT" QUANTITATIVE DETERMINATION OF THE CARBOHYDRATE CONSTITUENTS OF GLYCOPROTEINS AND A STUDY OF THE SELECTIVITY OF ALKALINE BOROHYDRIDE REDUCTIONS*

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## ABSTRACT

A new procedure for the quantification by g.l.c. of the carbohydrate constituents of glycoproteins is proposed which involves (*a*) simultaneous action of neuraminidase and neuraminic acid aldolase, (*b*) hydrolysis with 4M trifluoroacetic acid at 125° for 1 h, and (*c*) conversion of the products into *O*-methyloxime acetates and g.l.c. The procedure has been successfully tested on fetuin, transferrin, $\alpha_1$-acid glycoprotein, and mucin. The g.l.c. conditions used also enabled the complete separation of *O*-methyloxime and alditol acetate derivatives in one run, so that the release of carbohydrate chains from glycoproteins by treatment with alkaline borohydride can be investigated conveniently. There was complete release of *O*-linked oligosaccharides from fetuin on treatment with 0.1M NaOH/0.8M $NaBH_4$ (68 h, 37°) or 0.05M KOH/M $KBH_4$ (24 h, 45°) and also release of ~75% and 35–40%, respectively, of *N*-asparagine-linked chains. Reduced oligosaccharides were formed only from *O*-linked chains; the mechanism by which *N*-linked chains were released is still not clear.

## INTRODUCTION

We have recently developed[1] a simple, rapid, and sensitive method for the quantification of neutral and amino sugars from glycoproteins and polysaccharides, involving hydrolysis of very dilute solutions in 4M trifluoroacetic acid at 125° for 1 h (conditions which ensure complete release of amino sugars and minimum loss of neutral sugars) followed by g.l.c. of the *O*-methyloxime acetates of the products. By using a Carbowax 20 M capillary column, the *syn* and *anti* oximes from neutral and amino sugar derivatives could be separated.

We sought next to include *N*-acylneuraminic (sialic) acids in the above

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methodology. Many methods have been proposed[2] for the derivatisation of *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycoloylneuraminic acid (Neu5Gl) followed by g.l.c. However, the development of a "one-pot" method for all the component sugars of glycoproteins requires a hydrolysis procedure which ensures release of the neutral and amino sugars from the backbone, without destruction of the sialic acids which are acid-sensitive[3]. Liberation of sialic acids without loss requires conditions (*e.g.*, 25mM $H_2SO_4$, 80°, 2 h) which would not release other monosaccharides. The simultaneous action of neuraminidase and neuraminic acid aldolase can liberate neuraminic acids from glycoproteins and convert these components into 2-amino-2-deoxymannose derivatives[4]. Because 2-acylamino-2-deoxymannose derivatives are resistant towards acid[3] and because 2-acetamido-2-deoxymannose (ManNAc) can be separated from other amino sugars as the *O*-methyloxime acetate[1], the treatment of glycoproteins with neuraminidase and neuraminic acid aldolase prior to acid hydrolysis in the above analytical procedure was studied, and we now report a simple "one-pot" method for the quantification of the neutral sugars, hexosamines, and sialic acids from glycoproteins.

Until recently, it was accepted that the *N*-glycosylic linkages between GlcNAc and asparagine were stable towards mild alkali, whereas *O*-glycosylic linkages between serine and threonine and GalNAc were very labile under similar conditions[5]. However, Ogata and Lloyd showed that both *N*- and *O*-linked chains were released, even under relatively mild conditions[6]. The release of *O*-glycosylically linked oligosaccharides from glycoproteins involves alkali-catalysed *β*-elimination[5], but the mechanism for the liberation of asparagine-*N*-linked moieties is still not understood. When borohydride is present in the reaction mixtures, the liberated oligosaccharides are converted into alditols, and we have therefore investigated the g.l.c. of mixtures of acetylated alditols and *O*-methyloximes which would be obtained after hydrolysis of the reduced oligosaccharides and derivatisation of the products. A procedure similar to that investigated here (enzymic release of sialic acids from glycoproteins, mild treatment with alkaline borohydride, deionisation, acid hydrolysis, *O*-methyloximation, acetylation, and g.l.c.) has been proposed for the determination of the d.p. of glycosylaminoglycans[7].

## RESULTS AND DISCUSSION

Sialic acids (Neu5Ac and Neu5Gl) may be acetylated[8] at positions 4, 7, 8, and 9, and it is clear that the procedure proposed above will lead to a reduction of the great variety of neuraminic acid derivatives, because of the acid-lability of acetic esters and the choice of acetylated *O*-methyloxime derivatives for g.l.c. analysis. Since ManNAc is well resolved from other amino sugars as the *O*-methyloxime acetate[1], we first investigated the resolution of standard mixtures of sugar derivatives containing 2-deoxy-2-glycoloylaminomannose (ManNGl), arising from the enzymic cleavage of Neu5Gl by neuraminic acid aldolase. Using the g.l.c. conditions previously described[1] (see Experimental), two well-separated peaks for the

TABLE I

CARBOHYDRATE CONSTITUENTS (%) OF FOUR GLYCOPROTEINS

| *Glycoprotein* | | *Fuc* | *Man* | *Gal* | *GlcNAc* | *GalNAc* | *Neu5Ac* |
|---|---|---|---|---|---|---|---|
| Fetuin | Found | — | 2.9 | 4.2 | 5.9 | 1.0 | 5.8 |
| | Reported | — | *3.0*[a] | *4.6*[a] | *5.6*[a] | *0.7*[a] | *5.3*[b]–*6.3*[c] |
| $\alpha_1$-Acid glycoprotein | Found | 0.8 | 5.6 | 7.2 | 12.3 | — | 9.3 |
| | Reported[d] | *0.7* | *5.5* | *7.6* | *13.2* | — | *10.9* |
| Transferrin | Found | — | 1.2 | 1.5 | 2.3 | 0.7 | 1.6 |
| | Reported[e] | — | *2.4* | | *2.0* | — | *1.4* |
| Mucin | Found | 0.4 | 0.6 | 1.1 | 4.3 | 10.0 | 5.0 |
| | Reported | *0.5*[f] | *0.5*[f] | *1.0*[f] | *4.0*[f] | *9.7*[f] | *1.8*[b]–*5.0*[c] |

[a]From ref. 9. [b]From ref. 4. [c]Estimated by the supplier (Sigma). [d]From ref. 10. [e]From ref. 11. [f]From ref. 1.

*O*-methyloxime acetate of ManNGl were obtained (*T* 1.90 and 1.97 relative to that of acetylated *myo*-inositol) in the ratio 17:83, the total run-time being <40 min. The simultaneous action of neuraminidase and neuraminic acid aldolase on glycoproteins having been demonstrated[4], its combination with subsequent hydrolysis with 4M trifluoroacetic acid and conversion of the products into *O*-methyloxime acetates was applied to foetal calf serum fetuin, human transferrin, human $\alpha_1$-acid glycoprotein, and bovine submaxillary mucin. 3-*O*-Methyl-D-glucose (3-MeGlc) and *myo*-inositol are equally suitable internal standards for the determination of neutral and amino sugars[1]. We have now shown that 3-acetamido-3-deoxy-D-glucose (Glc3NAc) can be used as a specific internal standard for hexosamines. The results in Table I show that, using 3-MeGlc and Glc3NAc as internal standards for neutral and amino sugars, respectively, excellent agreement was found in the percentages of neutral sugars, hexosamines, and sialic acids, when comparing our data with those obtained by other procedures. The data in Table I indicate that the use of two different internal standards for neutral and amino sugars gave values slightly closer to those previously reported[9] for the fetuin than when a single standard was used[1]. Also, Neu5Gl was not detected in our commercial sample of bovine submaxillary mucin, in contrast to the findings of other workers[4].

There are two minor limitations to the above "one-pot" determinations. First, as recommended by Varki and Diaz[8], if di-*O*-acetylated sialic acid species are suspected, the use of a mixture of neuraminidases from *S. sanguis* and *A. ureafaciens* should be investigated, instead of the commercial *C. perfringens* enzyme, in order to ensure quantitative release of such species from glycoproteins. Secondly, since 4-*O*-acetylated neuraminic acid derivatives are resistant to known neuraminidases[12], they cannot be determined by the present procedure.

Prior to investigating the sequential release of carbohydrates under reductive and non-reductive conditions, g.l.c. of mixtures of *O*-methyloxime acetates from neutral and amino sugars together with alditol acetates was investigated. Fig. 1

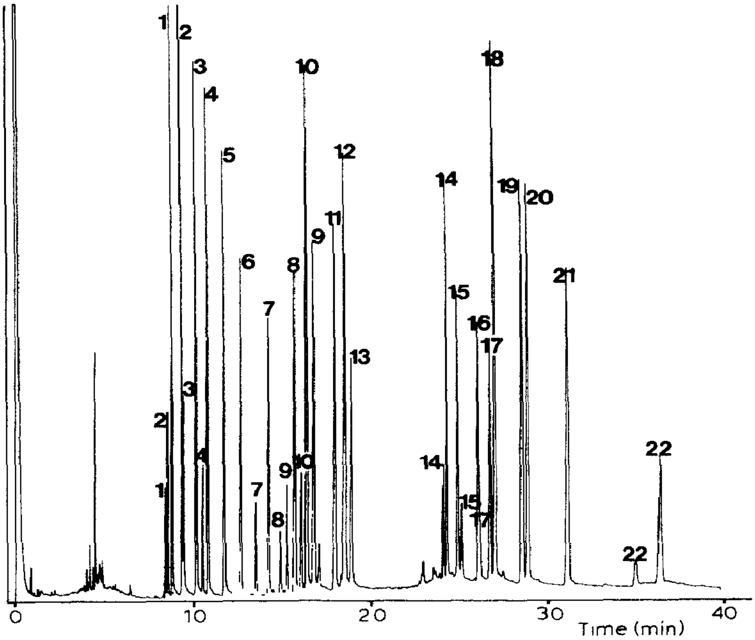

Fig. 1. Gas chromatogram of *O*-methyloxime and alditol acetates from neutral and amino sugars separated on a fused-silica Carbowax 20 M capillary column; co-injection of 2 × 0.7 µL containing an average of 0.05 µg of each sugar; initial temperature, 80°; final temperature, 230°. Peaks: *1* rhamnose, *2* fucose, *3* arabinose, *4* xylose, *5* arabinitol, *6* xylitol, *7* 3-MeGlc, *8* mannose, *9* galactose, *10* glucose, *11* mannitol, *12* *myo*-inositol, *13* galactitol, *14* Glc3NAc, *15* GlcNAc, *16* GalNAc, *17* ManNAc, *18* GlcNAcol, *19* GalNAcol, *20* ManNAcol, *21* Glc6NAcol, *22* ManNGl.

shows a gas chromatogram obtained in <40 min for the *O*-methyloxime acetates (*syn* and *anti* isomers) obtained from eight neutral sugars and five hexosamines, together with alditol acetates obtained from four neutral sugars and four hexosamines. The components of the mixture were fully resolved. During the preparation of this manuscript, the resolution of mixtures of neutral and amino sugars (as aldononitrile or *O*-methyloxime acetates) together with sugar alcohols (as alditol or cyclitol acetates) was reported[13].

Excellent recoveries are obtained[1] when dilute solutions of standard mixtures of sugars in 4M trifluoroacetic acid have been kept for 1 h at 125°. In extending the method, a standard mixture of sugars was treated with 0.1M NaOH/0.8M NaBH$_4$ for 68 h at 37°, the resulting alditols were isolated and added to a new standard mixture of sugars, and the combined mixture was treated with 4M trifluoroacetic acid at 125° for 1 h. The recoveries of carbohydrates are shown in Table II. Losses of alditol and hexosaminitol during the alkaline treatment are unlikely since alditols are well known to be stable under such conditions. Therefore, it appears that the observed recoveries of alditols (70–85%) reflect losses during treatment with acid, and the concomitant losses of neutral sugars have to be compared with the excellent recoveries previously observed for the same carbohydrates treated with acid in the

TABLE II

CARBOHYDRATE RECOVERIES RELATIVE TO *myo*-INOSITOL AFTER TREATMENT WITH 4M TRIFLUOROACETIC ACID AT 125° FOR 1 h

| *Peak*[a] | *Carbohydrate* | *Recoveries (%)* | |
|---|---|---|---|
| | | *Without alditol*[b] | *In the presence of alditols*[c] |
| *1* | Rha | 82 | 27 |
| *2* | Fuc | 89 | 30 |
| *3* | Ara | 86 | 48 |
| *4* | Xyl | 72 | 54 |
| *5* | Araol | — | 80 |
| *6* | Xylol | 88 | 85 |
| *7* | 3-MeGlc | 97 | 73 |
| *8* | Man | 86 | 60 |
| *9* | Gal | 81 | 68 |
| *10* | Glc | 97 | 86 |
| *11* | Manol | — | 72 |
| *12* | *myo*-Inositol | 100 | 100 |
| *13* | Galol | — | 74 |
| *14* | Glc3NAc | — | 105 |
| *15* | GlcNAc | 100 | 102 |
| *16* | GalNAc | 88 | 104 |
| *17* | ManNAc | 87 | 92 |
| *18* | GlcNAcol | — | 75 |
| *19* | GalNAcol | — | 76 |
| *20* | ManNAcol | — | 76 |
| *21* | Glc6NAcol | — | 70 |
| *22* | ManNGl | — | not tested |

[a]Peak numbers as in Fig. 1. [b]Results from ref. 1. [c]See text and Experimental.

absence of alditols (see Table II). It appears that the alditol derivatives participate in reversion-type reactions when treated with acid in the presence of reducing sugars, leading mainly to important losses of 6-deoxyhexoses and pentoses, whereas the recoveries of other carbohydrates are still acceptable.

Recent studies of the carbohydrate chains of fetuin have led[14,15] to the proposed structures depicted in Fig. 2. The occurrence of both *O*- and *N*-carbohydrate/peptide linkages in this glycoprotein made it an ideal model compound for the present study. Therefore, fetuin was subjected in sequence to (*a*) simultaneous action of neuraminidase and neuraminic acid aldolase in order to obtain asialofetuin and ManNAc, (*b*) treatment with 0.1M NaOH/0.8M $NaBH_4$ or 0.05M KOH/M $KBH_4$ in order to liberate and reduce the alkali-labile carbohydrate chains from the backbone, (*c*) deionisation on Dowex ($H^+$) resin in order to retain only the neutral moieties, (*d*) hydrolysis with 4M trifluoroacetic acid at 125° for 1 h, and (*e*) *O*-methyloximation, acetylation, and g.l.c. Fig. 3 shows a typical, resulting chromatogram. Using 6-acetamido-6-deoxyglucose (Glc6NAc), 3 MeGlc, and Glc3NAc as internal standards for hexosaminitols, neutral sugars, and amino

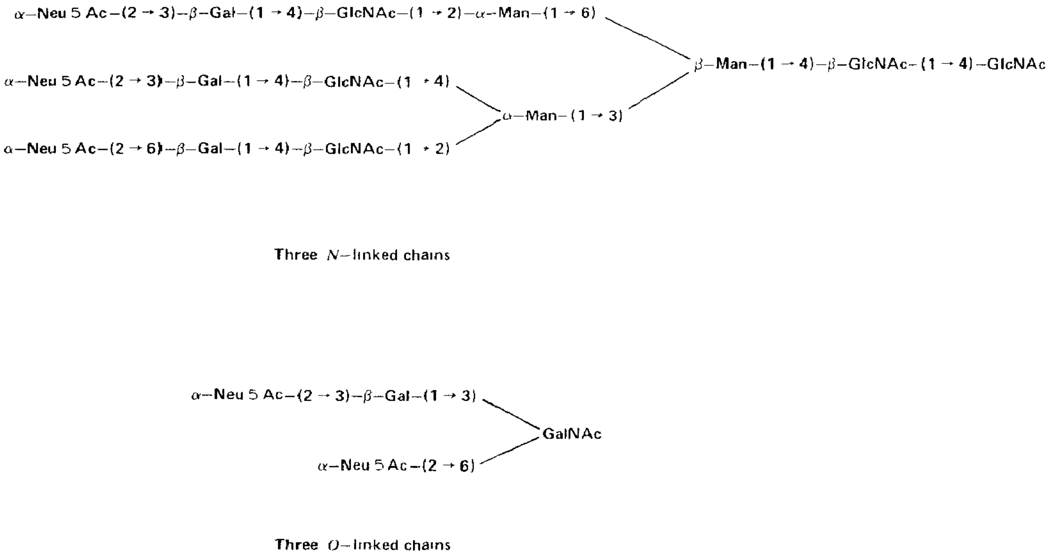

Fig. 2. Proposed structures for the carbohydrate components of fetuin (from refs. 14 and 15).

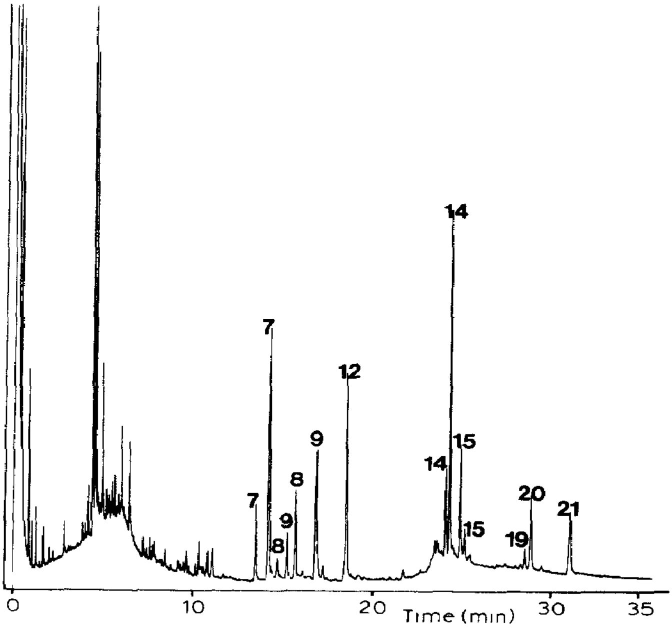

Fig. 3. Gas chromatogram of alditol and *O*-methyloxime acetates obtained after derivatisation of carbohydrates released from fetuin by alkali–borohydride (see Experimental): key as in Fig 1.

TABLE III

MONOSACCHARIDE CONSTITUENTS (%) OF CARBOHYDRATE CHAINS RELEASED FROM FETUIN BY TREATMENT WITH ALKALI-BOROHYDRIDE AFTER ENZYMIC CLEAVAGE OF NEURAMINIC ACIDS

| | ← *On* N-*linked chains* → | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | ← *On* O-*linked chains* → | | | |
| *Conditions* | *Man*<br>*as methoxime* | *GlcNAc* | | *Gal*<br>*as methoxime* | *GalNAc* | | *Neu5Ac*<br>*as alditol* |
| | | *as methoxime* | *as alditol* | | *as methoxime* | *as alditol* | |
| 0.1M NaOH<br>0.8M NaBH$_4$<br>68 h, 37° | 2.2 (76%)[a] | 4.5 (76%) | — | 3.3 (79%) | — | 1.0 (100%) | 5.7 |
| 0.05M KOH<br>M KBH$_4$<br>24 h, 45° | 1.0 (34%) | 2.3 (39%) | — | 2.2 (52%) | — | 1.0 (100%) | 5.7 |

[a] Values in brackets indicate the percentage of release, relative to the total amount of the constituent found in fetuin (see Table I).

sugars, respectively, the carbohydrate percentages summarised in Table III were obtained. These results indicate the complete release of GalNAc after both the above alkaline treatments and confirm the quantitative liberation of *O*-linked chains by these procedures. The detection of GalNAcol, but not GalNAc, confirms that GalNAc occurs only linked to peptide in fetuin, the GalNAcol arising during borohydride reduction of the oligosaccharides liberated by the alkali-catalysed $\beta$-elimination. The determinations of Man and GlcNAc (components which occur only on *N*-linked oligosaccharides) indicate that ~75% of these chains are released as neutral moieties during the treatment with 0.1M NaOH/0.8M $NaBH_4$ but only 35–40% during the treatment with 0.5M KOH/M $KBH_4$. These results accord well with those of Ogata and Lloyd[6]. However, the fact that GlcNAc is always detected as the *O*-methyloxime derivative, but not as hexosaminitol, raises the question of the nature of the *N*-asparagine-linked chains released by the action of mild alkali. Our results do not accord with the postulated[6] release of reduced oligosaccharides from *N*-linked chains, but suggest that these chains are liberated by mild alkalis as neutral moieties in which the GlcNAc reducing-end is protected against borohydride. The subsequent acid hydrolysis releases GlcNAc which is converted into the *O*-methyloxime.

Thus, mild treatment of glycoproteins with alkaline borohydride liberates both *O*- and *N*-linked carbohydrate chains[6], but the mechanism for the release of asparagine-linked chains is still an open question.

## EXPERIMENTAL

G.l.c. was performed using a Carlo-Erba instrument (Control Module EL 490, LT Programmer Model 430, Fractovap Series 4160) equipped with a cold on-column injector and a flame-ionisation detector. Chromatograms and peak areas were measured by the use of a Spectra-Physics SP 4270 integrator. The Carbowax 20 M NP fused-silica capillary column (0.3 mm, 24 m) was prepared as described previously[1], and hydrogen at 0.7 bar was used as carrier gas. The temperature programme was 80°→180° at 20°/min, →210° at 2°/min, and →230° at 10°/min, an isothermal period of 1 min preceding each stage.

*Derivatisation procedures.* — The *O*-methyloximation reagent was prepared, as previously described[1,16], by dissolving *O*-methylhydroxylamine hydrochloride (120 mg) in dry methanol (1.0 mL) and pyridine (1.78 mL) followed by the addition of 1-dimethylamino-2-propanol (0.22 mL). Mixtures (<1.2 mg total) of neutral sugars, amino sugars, and *myo*-inositol, obtained by freeze-drying, were dissolved in the *O*-methyloximation reagent (0.2 mL). Neu5Gl (0.3 mg) was added to each mixture after conversion into ManNGl by neuraminic acid aldolase [0.3 U, 0.01M phosphate buffer (pH 7.2), 1 h at 37°]. The solutions were heated at 70–80° for 30 min in Teflon-capped reacti-tubes (Sovirel), cooled to room temperature, and concentrated with a stream of $N_2$, and the resulting oximes were acetylated with pyridine–acetic anhydride (0.5 mL, 1:3) at 70–80° for 30 min. The solutions were concentrated under reduced pressure, solutions of each residue in dichloromethane (1 mL) were washed with M hydrochloric acid (1 mL) and distilled water (3 × 1 mL), and filtered through anhydrous $Na_2SO_4$, and samples (0.5–1.5 $\mu$L) were used for g.l.c.

The alditol acetate derivatives were obtained by treating solutions of standard mixtures (<1 mg) of neutral sugars, amino sugars, and *myo*-inositol in $H_2O$ (1 mL) with sodium borohydride (15 mg) for 1 h at room temperature. The pH was adjusted to 2–3 with acetic acid, each solution was concentrated to dryness, and a solution of the residue in methanol–acetic acid (4 mL, 200:1) was concentrated to dryness. This last step was repeated three times and the resulting alditols were acetylated as described above for the oximes.

*Analytical method for glycoproteins.* — Neuraminic acids were liberated from samples (1 mg) of glycoproteins (in the presence of internal standards) and converted into mannosamine derivatives by the simultaneous action of neuraminidase (0.1 U) and neuraminic acid aldolase (0.3 U) in 0.05M phosphate buffer (0.8 mL, pH 7.0) for 1 h at 37°. After heating the mixtures for 1 min at 100° to inactivate the enzymes, the solutions were freeze-dried and the residues were treated with 4M trifluoroacetic acid (1 mL) at 125° under $N_2$ for 1 h. The acid was then evaporated and the residues were converted into *O*-methyloxime acetates as described above. Mixtures of sugars (reflecting closely the proportions found in each glycoprotein, including ManNAc calculated from the percentage of Neu5Ac) were also subjected to the above procedure. The response factors were then used in the analysis of the biological materials. A blank (containing both neuraminidase and neuraminic acid aldolase) was equally subjected to the complete procedure.

*Recoveries after treatment of carbohydrates with alkali and acid.* — The recoveries of monosaccharides and alditols after stepwise treatment with alkali–borohydride and trifluoroacetic acid were determined as follows. Freeze-dried standard mixtures (<1 mg) of sugars together with *myo*-inositol and sodium borohydride (30 mg) were dissolved in 0.1M NaOH (1 mL), the solutions were kept at 37° for 68 h and then deionised with Dowex 50W ($H^+$) resin, new freeze-dried standard mixtures (<1 mg) of sugars were added, and the solutions were concentrated to dryness. A solution of each residue in methanol–acetic acid (4 mL, 200:1) was concentrated to dryness. This last step was repeated three times. Each residue was then dissolved in 4M trifluoroacetic acid (2 mL), and the solution was flushed with $N_2$ and then kept (in reacti-tubes) at 125° for 1 h. Each solution was cooled to room temperature and concentrated under reduced pressure, and the residue was *O*-methyloximated and acetylated as described above.

*Treatment of fetuin with alkali–borohydride.* — Neu5Ac was liberated from samples (1 mg) of fetuin and converted into ManNAc (in the presence of *myo*-inositol and Glc6NAc as internal standards) as described above. After denaturation of the enzymes, borohydride and alkali were added to the crude digests in order to obtain 0.1M NaOH/0.8M $NaBH_4$ or 0.05M KOH/M $KBH_4$. The mixtures were kept for 68 h at 37° and 24 h at 45°, respectively, and then neutralised and deionised with

Dowex 50W ($H^+$) resin, and new internal standards (3-MeGlc and Glc3NAc) were added before concentration to dryness. Hydrolysis with trifluoroacetic acid, *O*-methyloximation, and acetylation were then carried out as described above. A separate hexosamine mixture was also treated with borohydride, and a second mixture reflecting closely the proportions of carbohydrate found in fetuin was added before the treatment with trifluoroacetic acid. The resulting response factors were then used in the analysis of the carbohydrates liberated from fetuin.

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