Use of Hydrazine To Release in Intact and Unreduced Form both N- and O-Linked Oligosaccharides from Glycoproteins

Thakor Patel,[‡] James Bruce,[‡] Anthony Merry,[‡] Colette Bigge,[‡] Mark Wormald,[§] Alan Jaques,[§] and Raj Parekh*,[‡]

Oxford GlycoSystems Limited, Unit 4, Hitching Court, Blacklands Way, Abingdon, Oxon OX14 1RG, England, and Oxford Glycobiology Unit, Department of Biochemistry, Oxford University, South Parks Road, Oxford OX1 3QU, England

Received June 17, 1992; Revised Manuscript Received October 9, 1992

ABSTRACT: The use of hydrazine to release unreduced N- and O-linked oligosaccharides from glycoproteins has been investigated using several "standard" glycoproteins of previously defined glycosylation. It is shown that hydrazinolysis can be used to release intact N- and O-linked oligosaccharides in an unreduced form. The release of O-linked oligosaccharides occurs with a lower temperature dependence than the release of N-linked oligosaccharides, and the kinetic parameters governing release of oligosaccharides from these standard glycoproteins have been determined. These parameters allow a definition of reaction conditions under which anhydrous hydrazinolysis can be used to selectively release O-linked oligosaccharides (60 °C, 5 h) or release both N- and O-linked oligosaccharides (95 °C, 4 h) in high yield (>85%) from all glycoproteins investigated (n = 11). Under these reaction conditions, the recovered N- and O-linked oligosaccharides are structurally intact (as judged by 600-MHz ^{1}H-NMR, laser-desorption mass spectrometry, HPAEC-PAD, gel filtration, and glycosidase digestion), with the possible exception of certain *N*- and *O*-acyl substituents of sialic acid. This use of mild hydrazinolysis therefore allows both the simultaneous and sequential chemical release from glycoproteins of O- and N-linked oligosaccharides in their intact unreduced form.

The majority of cell-surface and secreted polypeptides of eukaryotic cells are glycosylated. Structural analysis of these covalently attached oligosaccharides is proving increasingly important in understanding the structure–function relationship for many glycoproteins. As a rule, several oligosaccharides are attached to a single polypeptide chain, and each individual glycosylation site in a population of polypeptide molecules is associated with many different oligosaccharide structures (Rademacher et al., 1988). Consequently, a full analysis of polypeptide glycosylation, involving both an estimation of the relative molar content of individual oligosaccharides and detailed structural analysis on one or more oligosaccharides, is currently impractical while the oligosaccharides are still attached to the polypeptide.

Excluding the GPI-membrane anchor, oligosaccharides are principally attached to peptide through an N- or O-glycosidic bond (Montreuil, 1982). Both enzymatic and chemical methods have been used to achieve the release of N- and/or O-linked oligosaccharides from glycoproteins. Examples of enzymatic methods include the use of a variety of peptide N-glycosidases (Damm et al., 1987; Nuck et al., 1990) and peptide O-glycosidase (Umemoto et al., 1977). Examples of chemical methods include the use of hydrazine to release N-linked oligosaccharides (Bayard & Montreuil, 1974; Takasaki et al., 1982), β-elimination under mild alkaline conditions to release O-linked oligosaccharides (Carlson, 1968; Fukuda, 1989), and very strong alkali (Lee & Scocca, 1972) or lithium aluminum borohydride (Likhosherstov et al., 1990) to release both N- and O-linked oligosaccharides. The success of enzymatic methods depends on the substrate specificity of the enzyme and the choice of hydrolytic conditions. The use of alkaline conditions or lithium aluminum borohydride can cause extensive degradation of alkali-labile linkages unless performed in the presence of excess reducing agent, which therefore leads to the recovery of oligosaccharides as the alditol derivatives.

Our efforts have been directed first toward defining a general reaction yielding release of intact unreduced N- and O-linked oligosaccharides from a glycoprotein and second to comparing such a reaction to currently practiced alternative methods. In order to define such a reaction, the use of hydrazine as a cleaving agent has first been investigated in detail. The results, reported in this paper, indicate that hydrazinolysis when performed under controlled and optimized conditions can be used to release intact unreduced N- and O-linked oligosaccharides in high yield. In subsequent papers, we compare the use of hydrazinolysis with PNGase F[1] (*Flavobacterium meningosepticum*) for release of N-linked oligosaccharides and hydrazinolysis with alkaline β-elimination for the release of O-linked oligosaccharides from glycoproteins.

MATERIALS AND METHODS

General. Standard glycoproteins were all purchased from Sigma (U.K.) and were of the highest purity obtainable. All solvents and reagents were of the highest grade available. Dowex AG50X 12 (H^+), Dowex AG3X 4A (OH^-), Chelex 100 (Na^+), and Bio-Gel P4 (–400 mesh) were from Bio-Rad (U.K.). All exoglycosidases and standard oligosaccharides (both unreduced and tritiated alditols) were from Oxford GlycoSystems (U.K.).

Release and Isolation of Oligosaccharides. An aliquot of the glycoprotein to be analyzed (0.1–10 mg) was exhaustively dialyzed (microflow dialysis using a BRL 1200 MA apparatus with 5–10-KDa-cutoff dialysis membrane) against 0.1% (v/

* To whom correspondence should be addressed.
‡ Oxford GlycoSystems Limited.
§ Oxford University.

[1] Abbreviations: PNGase F, peptide N-glycosidase F; FID, flame-ionization detector; TIC, total ion current; HPAEC–PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; LD-MS, laser desorption mass spectrometry; gu, glucose unit; E^{ACT}, activation energy; A, Arrhenius constant; Ac, acetate; HDO, residual protons in ^2H_2O; NMR, nuclear magnetic resonance; PMAA, partially methylated alditol acetate.

0006-2960/93/0432-679$04.00/0

v) trifluoroacetic acid and then dried either by lyophilization (<50 mtorr, ≥24 h) or by lyophilization followed by drying in vacuo over activated charcoal at −196 °C (<10^{-6} bar) for >96 h. Samples were then suspended in freshly-distilled anhydrous hydrazine (triple-vacuum-distilled at room temperature) of water content ≤1% v/v (as determined by direct GC analysis with FID quantitation) at 5–10 mg/mL. The hydrazinolysis reaction was performed under controlled conditions of temperature and time, after which excess hydrazine was removed by evaporation at 25 °C. N-Acetylation of any primary amino groups generated during the reaction was achieved by the addition of excess acetic anhydride (0.5 M final concentration) in saturated $NaHCO_3$, first at 4 °C for 20 min and then at ambient temperature for 30 min. Sodium ions were then removed from samples by passage through a column of Dowex AG50X 12 (H^+) at room temperature, and the samples were then rotary-evaporated to dryness (27 °C) and applied to Whatman 3MM chromatography paper. Chromatography to remove peptide material was performed at 27 °C, 60% relative humidity for 18 h using as solvent 1-butanol/ethanol/water (4/1/1, v/v). The oligosaccharide pool remaining at the origin (all carbohydrates of disaccharide or larger do not move in this solvent system under these conditions) was recovered by elution with water, filtered (0.2-μm Teflon filter), rendered 1 mM in copper(II) acetate, and incubated at 27 °C for 30 min. After passage through a mixed bed column of Chelex 100 (Na^+)/Dowex AG50X 12 (H^+), the oligosaccharide pool was rotary-evaporated to dryness (27 °C) and prepared for further analysis.

Monosaccharide Composition Analysis. The absolute content of individual monosaccharides in a known aliquot of either a standard glycoprotein or an oligosaccharide pool was determined by methanolysis (0.5 N methanolic hydrochloric acid, 16 h, 75 °C) followed by GC-MS analysis of the re-N-acetylated and trimethylsilylated *O*-methyl glycosides (direct on-column injection onto a CP-Sil 8CB coated fused silica column (25 m × 0.32 mm i.d.) from Chrompack) using an HP 5890 Series II GC with 5971A MS. The temperature program used was as follows: 90 °C (held for 0.5 min) followed by a linear increase to 150 °C (at 30 °C/min) and then to 200 °C (at 3 °C/min) and then to 240 °C (at 7.5 °C/min). Identification of individual methyl glycosides was by reference to the elution times and mass spectra of standard compounds, and quantitation was by reference to an internal standard (*scyllo*-inositol) added to each sample prior to addition of methanolic hydrochloric acid (Chaplin, 1982). All samples were analyzed at least in duplicate, and mean values are quoted in all cases. The yield of each monosaccharide in the final oligosaccharide pool obtained by subjecting a standard glycoprotein to hydrazinolysis under particular conditions is stated in percentage terms relative to the expected recovery of that monosaccharide as determined from a direct monosaccharide analysis of the intact glycoprotein.

^{1}H-NMR Spectroscopy. Samples for NMR analysis were prepared by repeated dissolution in ^2H_2O followed by rotary-evaporation. Finally, samples were dissolved in 0.5 mL of ^2H_2O and transferred to an NMR tube. One-dimensional ^{1}H-NMR spectra were recorded at 500 MHz or 600 MHz on a Bruker AM500 or Bruker AM600 spectrometer, at a probe temperature of 300 K. Typically, 512 free induction decays were accumulated with a sweep width of 8000 Hz, solvent supression, and a total recycle time of 2s. All spectra were Fourier transformed directly with no prior mathematical manipulation. Initial spectral analysis was performed by comparison of resonance chemical shifts with reported standard values (peptides, Bundi and Wuthrich (1979); N-linked oligosaccharides, Vliegenthart et al. (1983); other references given in text when necessary).

Laser Desorption Mass Spectrometry (LD-MS). Oligosaccharides to be analyzed by LD-MS were rendered salt-free by passage through a Chelex 100 (Na^+)/Dowex AG50X 12(H^+) mixed-bed column, filtered (0.22-μm Teflon filter), and rotary-evaporated to dryness. The oligosaccharides were then resuspended in glass-distilled water and analyzed directly (i.e., without any chemical derivatization) using a Finnigan MAT (Hemel Hempstead, U.K.) laser desorption mass spectrometer.

Radiolabeling of Oligosaccharides. This was performed as described previously (Parekh et al., 1989).

Titration of Reducing Termini in a Pool of Oligosaccharides. To measure the relative molar content of released oligosaccharides in a given hydrazinolysate, 1.0 nmol of the unreduced glucose heptamer derived by partial acid hydrolysis of dextran was added to the sample directly after the sodium bicarbonate buffer during the re-N-acetylation step. The relative radioactivity subsequently recovered in the glucose-7 alditol and the sample-derived oligosaccharide alditols is directly proportional to the molar content of unreduced oligosaccharides contained in that sample.

Fractionation of Oligosaccharide Alditol Pools. This was performed in one or more of the following three ways. (i) Charge-based separation using anion-exchange chromatography: this was performed as described previously (Parekh et al., 1987). (ii) Gel filtration: this was performed as described previously (Parekh et al., 1989). Pools of unreduced oligosaccharides were fractionated without co-application of a dextran hydrolysate. (iii) HPAEC–PAD: unreduced oligosaccharide samples were fractionated by HPAEC–PAD (Lee et al., 1990) using a Dionex BioLC with data collection and analysis by Perkin Elmer Nelson Chromacol 2000 software. Chromatographic conditions were as follows: column, Carbopac PA1 (40 × 25 ϕ mm); flow rate, 1 mL/min; elution, 10 mL of 20 mM sodium acetate in 100 mM sodium hydroxide followed by a linear gradient (over 50 min) to 250 mM sodium acetate in 100 mM sodium hydroxide, held for 20 mins. PAD settings: E_1 = +50 mV, E_2 = +600 mV; E_3 = −600 mV, output = 1000 nA, 0.0005-in. gasket. Fractions (0.5 mL) were collected and immediately brought to neutral pH, and oligosaccharide pools were re-N-acetylated and de-salted prior to further analysis.

Identification of Reducing Terminal Monosaccharide Alditols. A radiolabeled oligosaccharide alditol or pool of alditols was incubated with a mixture of exoglycosidases (see below) so as to hydrolyze all internal O-glycosidic linkages. Quantitative liberation of the reducing terminal monosaccharide alditol was assessed by gel-filtration chromatography (vide infra). An aliquot of the radioactive fraction obtained by gel-filtration chromatography was directly applied to a Shodex sugar SP1010 column, maintained at 80 °C, equilibrated, and isocratically eluted with 20% ethanolic water (Takeuchi et al., 1987). Monosaccharide alditols were identified on the basis of elution time by reference to standard alditols. The composition of the mixture of exoglycosidases (in 0.2 M citrate-phosphate buffer, pH 5.0) used to achieve complete cleavage of O-glycosidic linkages in an oligosaccharide alditol pool was as follows: α-D-neuraminidase (*Arthrobacter ureafaciens*) at 1.0 unit/mL; β-D-galactosidase (bovine testes) at 2.0 unit/mL; α-D-mannosidase (jack bean) at 60 units/mL; β-*N*-acetyl-D-hexosaminidase (jack bean) at

Table I: Summary of the Major Glycosylation Characteristics[a] of the Standard Glycoproteins

glycan property	standard glycoprotein[a]							
	BSF	RNase B	PTG	ovalb	HRP	pogen	IgA	α_1-AGP
O-glycans	+[b]	–[b]	–	–	–	+	+	–
N-glycans	+	+	+	+	+	+	+	+
biantennary complex	+	–	+	–	–	+	+	+
triantennary complex	+	–	+	–	–	–	+	+
tetraantennary complex	(+)[c]	–	(+)	–	–	–	–	+
hybrid	–	–	–	+	–	–	–	–
oligomannose	–	+	+	+	–	–	–	–
polylactosaminoglycan	–	–	–	–	–	–	–	+
"bisect" GlcNAc	–	–	–	+	–	–	+	–
xyloseβ1→2	–	–	–	–	+	–	–	–
core fucoseα1→3	–	–	–	–	+	–	–	–
core fucoseα1→6	–	–	+	–	–	–	+	–
galactoseα1→3	–	–	+	–	–	–	–	–
outer-arm fucoseα1→3	–	–	–	–	–	–	–	+
NeuNAcα2→3	+	–	+	–	–	+	+	+
NeuNAcα2→6	+	–	+	–	–	+	+	+

[a] These glycosylation characteristics are as defined in the following references: BSF (fetal calf serum fetuin; Takasaki & Kobata, 1986); RNase B (bovine pancreatic ribonuclease B; Liang et al., 1980); PTG (porcine thyroglobulin; Anamula & Taylor, 1991); ovalb (hen egg ovalbumin; Yamashita et al., 1978); HRP (horseradish peroxidase; Ashford et al., 1987); pogen (human serum plasminogen; Hayes & Castellino, 1979); IgA (human serum immunoglobulin A; Field et al., 1989); α_1-AGP (human serum α1-acid glycoprotein; Yoshima et al., 1981). [b] A + sign designates presence and a – sign designates absence of a particular glycan property. [c] (+) indicates presence but at <5% molar incidence of a particular glycan property.

20 units/mL; β-D-mannosidase (*Helix pomatia*) at 0.8 unit/mL; α-L-fucosidase (bovine kidney) at 3 units/mL; β-D-xylosidase (almond emulsin) at 1.5 units/mL. All unitages are as previously defined (Parekh et al., 1989; Ashford et al., 1987) and represent the activity in the final mixture. Incubations were performed at 37 °C for 18 h under toluene and terminated by heating at 100 °C for 2 min.

Desialylation by Incubation with Neuraminidase. The oligosaccharide pool (unreduced or alditols) was incubated with α-D-neuraminidase (*A. ureafaciens*) under a toluene atmosphere at 37 °C for 18 h. Substrate concentration was in the range 20–50 μM, enzyme was used at 1.0 unit/mL, and the reaction buffer was 0.1 M sodium acetate, pH 5.0. The reaction mixture was then passaged through a column of Dowex AG50X 12 (H^+), and eluant and washings were combined, rotary-evaporated to dryness, and analyzed further.

Standard Oligosaccharides. These were prepared as described previously (Parekh et al., 1989).

PNGase F Incubations. PNGase F was purified from culture supernatants of *Flavobacterium meningosepticum* by a combination of ammonium sulfate fractionation and hydrophobic-interaction chromatography and was used to de-N-glycosylate glycoproteins essentially according to previously defined procedures (Nuck et al., 1990). Glycoprotein to be de-N-glycosylated was denatured by heating at 100 °C for 2 min in reaction buffer (20 mM sodium phosphate, pH 7.5, 50 mM EDTA) containing 0.5% SDS and 5% β-mercaptoethanol. A 5-fold molar excess over SDS of *n*-octyl glucoside was then added, and the denatured glycoprotein incubated (at ~10 mg/mL) with 10 (IUB) units/mL PNGase F (where unitage is defined against [^{3}H]dansylfetuin glycopeptide) in reaction buffer for 18 h at 37 °C. Liberated oligosaccharides were recovered by passage of the incubation mixture first through a column of Dowex AG50X 12 (H^+) with elution in water. Eluant and washings were pooled and directly applied to a C-18 silica reverse-phase cartridge (Waters, Sep-Pak cartridge), and eluant and wash (10% aqueous methanol) were combined, rotary-evaporated to dryness, and prepared for further analysis.

Release of O-Linked Oligosaccharides by Reductive β-Elimination. O-Linked oligosaccharides were released from bovine serum fetuin as the tritiated alditols as described previously (Fukuda, 1989). Liberated oligosaccharide alditols were recovered by passage of the reaction mixture first through a column of Dowex AG50X 12 (H^+) with elution in water. Eluant and washings were pooled, rotary-evaporated to dryness, evaporated (5×) from methanol (acidified with acetic acid), resuspended in water, and applied to a C-18 silica reverse-phase cartridge. Eluant and wash (10% aqueous methanol) were combined, rotary-evaporated to dryness, and prepared for further analysis.

RESULTS

Temperature Dependence of the Cleavage from Peptide of the N- and O-Glycosidic Bonds by the Hydrazinolysis Reaction. Several standard glycoproteins of previously defined glycosylation were used to investigate the effect of reaction temperature on the rate of release of N- and O-linked oligosaccharides by hydrazine. The standard glycoproteins used in this study were bovine serum fetuin, human serum IgA, human serum plasminogen, porcine thyroglobulin, hen egg ovalbumin, and horseradish peroxidase. Between them, these glycoproteins carry most of the major N- and O-linked oligosaccharide structures reported on eukaryotic glycoproteins (see Table I for a summary of the glycosylation characteristics of each standard glycoprotein).

The relative rate of release of oligosaccharides from polypeptide as a function of reaction temperature was measured as follows. Equal aliquots (corresponding to 0.20 mg as determined by amino acid analysis of a glycoprotein preparation using an ABI amino acid analyzer Model 420A) of a salt-free lyophilized glycoprotein preparation were incubated in a sealed glass vessel under an argon atmosphere with anhydrous hydrazine (100 μL) at various temperatures (35 °C, 45 °C, 55 °C, 65 °C, 75 °C, 85 °C, 95 °C, 105 °C, 115 °C, 125 °C) for a fixed reaction time of 8.0 h. At the end of this period, unreacted hydrazine was removed by evaporation, and the samples were re-N-acetylated in the presence of an aliquot (1 nmol) of unreduced glucose-7 linear oligomer. After peptide removal and radiolabeling, the total oligosaccharide alditol pool was desialylated and applied to a P4 gel filtration column (see Figure 1), and the release of N- and O-linked oligosaccharides relative to the glucose-7 standard was measured as follows. For all the standard glycoproteins, the desialylated N-linked oligosaccharide al-

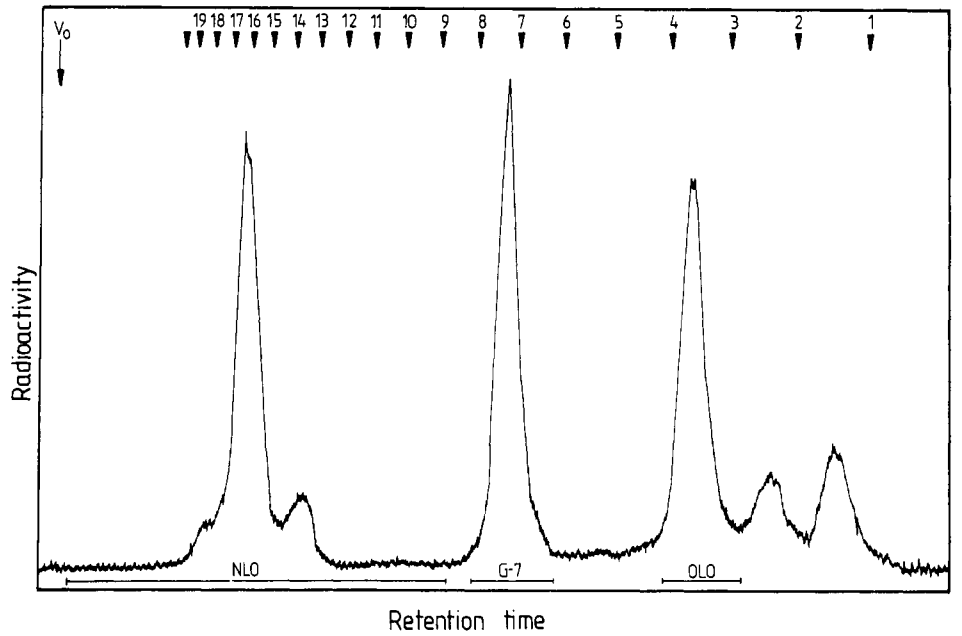


FIGURE 1: P4 gel filtration chromatogram of the desialylated oligosaccharide alditols (with the glucose-7 (control) alditol) recovered from bovine serum fetuin after controlled hydrazinolysis. The content of N-linked oligosaccharide alditols relative to the internal standard glucose-7 is defined as the total measured radioactivity in the pool NLO (containing all alditols eluting between 8.5 gu and v_0) relative to the total measured radioactivity in the pool G-7 (centred at 7.5 gu). Similarly, the content of intact O-linked oligosaccharide alditols is defined as the total measured radioactivity in the pool OLO (centered at 3.5 gu) relative to that measured in pool G-7.

ditols elute at ≥8.5 gu in P4 gel filtration chromatography, the principal O-linked oligosaccharide alditols at 3.5 gu (corresponding to galactose(β1→3)*N*-acetylgalactosaminitol), and alditols from "peeled" O-linked oligosaccharides at 1.5 gu (corresponding to galactitol). The alditol from the standard glucose-7 elutes at 7.5 gu. In all cases, therefore, the radioactivity in oligosaccharide alditols of elution volume ≥8.5 gu to that in the glucose-7 alditol (at 7.5 gu) is a measure of the net release of N-linked oligosaccharides, and the radioactivity in the oligosaccharide alditol eluting at 3.5 gu relative to that in the glucose-7 alditol is a measure of the net release of intact O-linked oligosaccharides (see Figure 1). This method of measurement was chosen for four reasons. First, any rearrangement or degradation of the N- or O-linked oligosaccharides during either the chemical release or the subsequent recovery would lead to the oligosaccharide becoming either nonreducing or altered with respect to hydrodynamic volume. Second, the principal source of heterogeneity in both the O- and N-linked oligosaccharides is due to variable sialylation. O-Linked oligosaccharide recovery is therefore quantified more accurately after desialylation (and consequent conversion of almost all such oligosaccharides to a single disaccharide), and the errors in quantifying N-linked oligosaccharide recovery are likewise reduced by reducing the number of different structures to be quantified. Third, any glycopeptide or other nonreducing oligosaccharide conjugate would not be detected by this method. Fourth, the desialylated O-linked oligosaccharides carried by these glycoproteins are completely resolved from desialylated N-linked oligosaccharides by gel filtration.

For each glycoprotein, the maximal value of the content of N- and O-linked oligosaccharide alditols relative to the added glucose-7 alditol (as determined after extrapolation of the values obtained over the temperature range studied) is taken as representing "100%" recovery. The release relative to the 100% recovery value as a function of reaction temperature of both N- and O-linked oligosaccharides from bovine serum fetuin is shown in Figure 2A, and for N- and O-linked oligosaccharides from several of the other standard glycoproteins it is shown in parts B and C, respectively, of Figure 2.

Analysis of the Order of the Reaction Leading to Release of N-Linked Oligosaccharides and of the Reactions Leading to Release and Degradation of O-Linked Oligosaccharides. For a pseudo-first-order reaction between glycoprotein (A) and hydrazine (B), where hydrazine is in large molar excess:

$$\frac{d[A]}{dt} = -K_R^{app}[A]$$

$$\ln K_R^{app} = \ln\left[\frac{\ln (A_2/A_1)}{t_1 - t_2}\right]$$

where $K_R^{app} = \psi$ (first-order rate constant) = $K_R[B]$, where K_R = true second-order rate constant. For each standard glycoprotein (where appropriate), ln $K_{R,N}^{app}$, ln $K_{R,O}^{app}$, and ln $K_{D,O}^{app}$ can be calculated for each reaction temperature from the numerical values of the data presented graphically in Figure 2A–C, where $K_{R,N}^{app}$ and $K_{R,O}^{app}$ are defined as the apparent rate constants for release of N- and O-linked oligosaccharides, respectively, and $K_{D,O}^{app}$ is defined as the apparent rate constant for degradation of O-linked oligosaccharides. The data in Figures 2 and 3 support the assumption that the degradation of N-linked oligosaccharides is not significant at $T \leq 398$ K with $t = 8$ h and that release and degradation of O-linked oligosaccharides are independent at the extremes of temperature. Plots of log $K_{R,N}^{app}$ vs $1/T$, log $K_{R,O}^{app}$ vs $1/T$, and log $K_{D,O}^{app}$ vs $1/T$ can therefore be made for each standard glycoprotein. These plots are shown for the standard glycoprotein bovine serum fetuin in Figure 3. For each standard glycoprotein, each plot of the relevant log K^{app} vs $1/T$ is linear with all individual correlation coefficients in the range $0.916 \leq r^2 \leq 0.988$. This linearity establishes that the reactions leading to the release of N- and O-linked oligosaccharides and degradation of O-linked oligosaccharides when performed under the conditions described herein (of large molar excess of hydrazine) can be considered to be first-order. Assuming

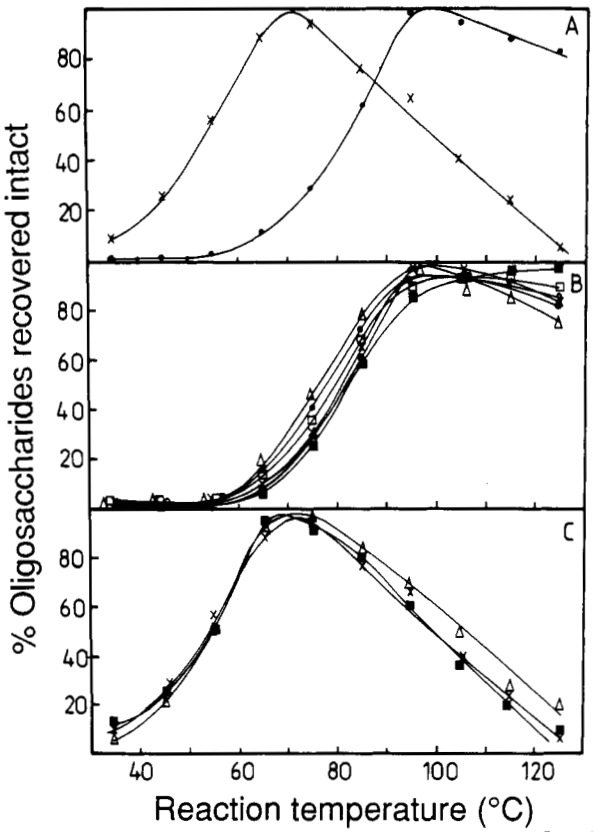


FIGURE 2: (A) Graphs of the percent recovery of intact O-linked oligosaccharides (×) and intact N-linked oligosaccharides (●) released from bovine serum fetuin versus reaction temperature of hydrazinolysis. (B) Graphs of the percent recovery of intact N-linked oligosaccharides from the following standard glycoproteins versus reaction temperature of hydrazinolysis: bovine serum fetuin (●); horseradish peroxidase (□); porcine thyroglobulin (×); ovalbumin (○); human serum IgA (△); human serum plasminogen (■). (C) Graphs of the percent recovery of intact O-linked oligosaccharides from bovine serum fetuin (×), human serum IgA (△), and human serum plasminogen (■) versus reaction temperature of hydrazinolysis.

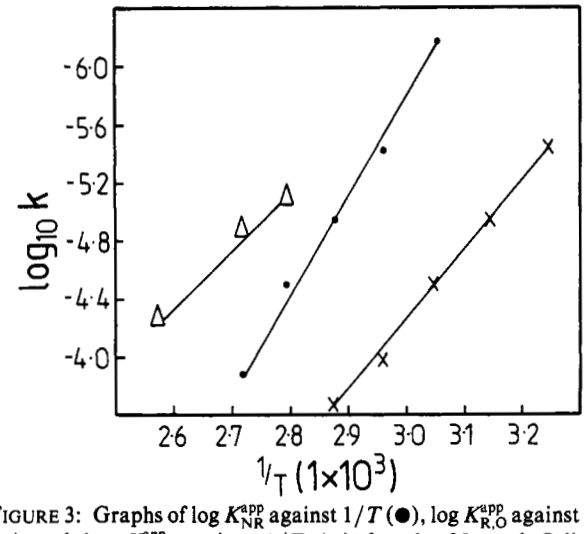


FIGURE 3: Graphs of log K_{NR}^{app} against $1/T$ (●), log $K_{R,O}^{app}$ against $1/T$ (×) and log $K_{D,O}^{app}$ against $1/T$ (△) for the N- and O-linked oligosaccharides from bovine serum fetuin. Each set of data points fits a straight line as indicated by regression analysis. For log K_{NR} against $1/T$, $r^2 = 0.992$, for log $K_{R,O}$ against $1/T$, $r^2 = 0.997$, and for log $K_{D,O}$ against $1/T$, $r^2 = 0.965$.

a constant reaction mechanism over the temperature range of study, each reaction can therefore be analyzed in terms of the Arrhenius equation, ignoring second-order and higher coefficients.

Calculation of $E_{R,N}^{ACT}$, $E_{R,O}^{ACT}$, $E_{D,O}^{ACT}$, $A_{R,N}$, $A_{R,O}$, and $A_{D,O}$ at [Hydrazine] = 32 M. The Arrhenius equation states that $k = Ae^{-E^{ACT}/RT}$, where k = rate of reaction, A = Arrhenius constant, E^{ACT} = activation energy, R = gas constant, and T = reaction temperature.

In accordance with the Arrhenius equation, $E_{R,N}^{ACT}$ and $A_{R,N}^{app}$ are determined from the plots of log $K_{R,N}^{app}$ vs $1/T$ for 318 K $\leq T \leq$ 398 K, $E_{R,O}$ and $A_{R,O}$ are determined from the plots of log $K_{R,O}^{app}$ vs $1/T$ for 308 K $\leq T \leq$ 353 K, and the $E_{D,O}^{ACT}$ and $A_{D,O}$ are determined from the plots of log $K_{D,O}^{app}$ vs $1/T$ for 353 K $\leq T \leq$ 398 K. Values for the Arrhenius constant A are calculated after extrapolation of the corresponding plot to $1/T = 0$, and E^{ACT} values are calculated from the slope of each plot. The values so determined are summarized in Table II.

Determination of Standard Hydrazinolysis Conditions for the Release from Peptide either of O-Linked Oligosaccharides Only or of both N- and O-Linked Oligosaccharides. From the preceding analysis the release of N- and O-linked oligosaccharides is considered to occur according to the following models:

$$\text{release of N-linked oligosaccharides: } P \rightarrow O_N \rightarrow D_N$$

$$\text{release of O-linked oligosaccharides: } P \rightarrow O_O \rightarrow D_O$$

where P = glycoprotein, O_N = released N-linked oligosaccharides, O_O = released, intact O-linked oligosaccharides, and D = degradation product(s) of released oligosaccharides.

From these models, the following equations can be defined for the release of intact N- and O-linked oligosaccharides from a given glycoprotein under defined reaction conditions (T, t) with large molar excess of hydrazine over glycoprotein:

$$m_N = 1 - \exp[-(A_{R,N}e^{-E_{R,N}^{ACT}/RT})t]$$

when $T \leq 398$ K and $t \leq 2.88 \times 10^4$ s and

$$m_o = \frac{\alpha}{\beta - \alpha}(e^{-\alpha t} - e^{-\beta t})$$

where

$$\alpha = A_{R,O}e^{-E_{R,O}^{ACT}/RT}$$

and

$$\beta = A_{D,O}e^{-E_{D,O}^{ACT}/RT}$$

where m_n = mole fraction of intact N-linked oligosaccharides released and m_o = mole fraction of intact O-linked oligosaccharides released.

The experimentally determined values of $E_{R,N}^{ACT}$, $E_{R,O}^{ACT}$, $E_{D,O}^{ACT}$, $A_{R,N}$, $A_{R,O}$, and $A_{D,O}$ for each standard glycoprotein are used to derive the plots of m_n vs T vs t and of m_o vs T vs t. These plots are extremely similar for all the standard glycoproteins, and the relevant contours of each surface are shown for bovine serum fetuin in parts A and B of Figure 4, respectively. Superimposing the contours in Figure 4A,B allows a definition of conditions under which O-linked oligosaccharides can be selectively released from protein (arbitrarily defined as $m_o \geq 0.90$ and $m_n \leq 0.1$) and conditions under which both N- and O-linked oligosaccharides can be released in equal yield (arbitrarily defined as $m_o, m_n \geq 0.85$). The standard reaction conditions chosen for selective release of O-linked oligosaccharides are 60 °C for 5 h, and for release of both N- and O-linked oligosaccharides are 95 °C for 4 h.

Table II: Activation Energies and Arrhenius Constants (at 32 M hydrazine concentration) for the Release of N- and O-Linked Oligosaccharides and the Degradation of O-Linked Oligosaccharides from Standard Glycoproteins

glycoprotein	$E_{R,N}^{ACT}$[a]	$E_{R,O}^{ACT}$	$E_{D,O}^{ACT}$	$A_{R,N}$[b]	$A_{R,O}$	$A_{D,O}$
BSF	122	96.4	81.2	2.8×10^{13}	1.6×10^{11}	3.6×10^{6}
PTG	114			2.5×10^{13}		
ovalb	118			1.7×10^{13}		
HRP	128			3.9×10^{14}		
pogen	nd[c]	97.4	82.1	nd	2.2×10^{11}	5×10^{6}
IgA	126	96.3	82.5	3.6×10^{14}	2.15×10^{11}	4.5×10^{6}

[a] $E_{R,N}^{ACT}$, $E_{R,O}^{ACT}$, and $E_{D,O}^{ACT}$ are as defined in the text and are stated to 3 sf in units of kilojoules per mole. [b] $A_{R,N}$, $A_{R,O}$, and $A_{D,O}$ are as defined in the text and are stated to 2 sf in units of seconds^{-1}. The Arrhenius constant for each reaction is calculated from the intercept at $1/T = 0$ of the plot of $\log K^{app}$ against $1/T$. Since $K^{app} = K_{true}$[hydrazine] $= K_{true} \times 32$ M, the calculated Arrhenius constant is 32× the true Arrhenius constant if the reaction is also assumed to be first-order with respect to hydrazine. [c] nd = not determined due to experimental inaccuracies.

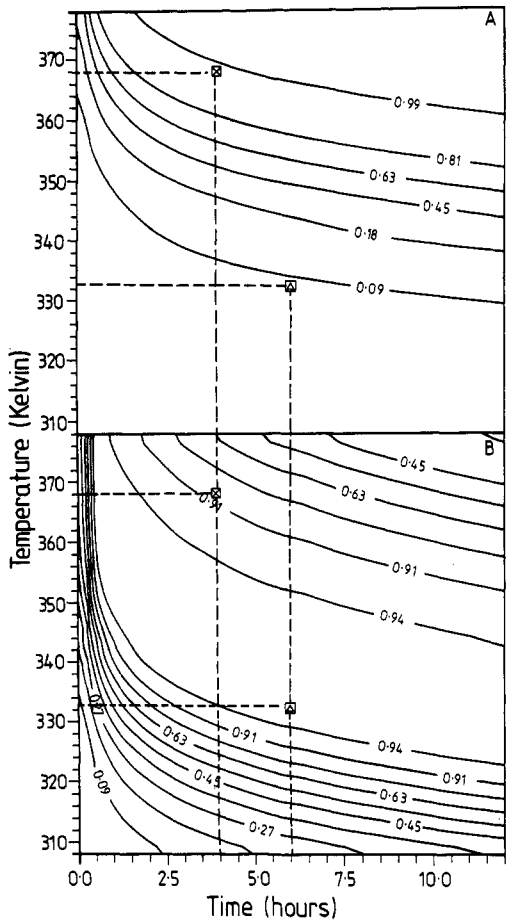


FIGURE 4: Graphical simulations of m_n (A) and m_o (B) for bovine serum fetuin as a function of temperature and time based on the experimentally determined values of $E_{R,N}^{ACT}$, $E_{R,O}^{ACT}$, $E_{D,O}^{ACT}$, $A_{R,N}$, $A_{R,O}$, and $A_{D,O}$ (see text for details). For m_n, all (T, t) on or above a given line are consistent with the designated value of m_n. For m_o, all (T, t) on or between the given lines are consistent with the designated value of m_o. The boxed × indicates standard N+O reaction conditions, and the boxed triangles indicate the standard O-only reaction conditions.

The general validity of these standard reaction conditions was next investigated in detail.

Yield of Recovered Oligosaccharides under Standard Reaction Conditions. This was determined in two different ways: First, by comparing the quantity of individual monosaccharides in an aliquot of a glycoprotein directly and in the oligosaccharide pool recovered from an equal aliquot of that glycoprotein after release using the standard reaction conditions (the average percentage yield for each monosaccharide for each glycoprotein studied is summarized in Table III); second, by titrating the reducing termini in the recovered pool of oligosaccharides as a function of reaction time (at 60 °C or 95 °C) by reduction with alkaline sodium borotritide in the presence of 1.0 nmol of the glucose-7 linear oligomer as internal standard. These time-course data are presented relative to the "100%" recovery value (see earlier) for bovine serum fetuin, hen egg ovalbumin, human serum IgA, and porcine thyroglobulin in Figure 5.

Integrity of Recovered Oligosaccharides. (i) ^{1}H-NMR and LD-MS Analysis. In order to obtain sufficient carbohydrate sample for NMR analysis, the N- and O-linked oligosaccharides were recovered from 10 mg each of bovine serum fetuin, bovine pancreatic RNase B, and horseradish peroxidase using the reaction conditions of 95 °C for 4 h. In the case of bovine serum fetuin, the NMR analysis was performed first on the total pool of recovered oligosaccharides, and the spectrum obtained is shown in Figure 6A. This pool of oligosaccharides was then desialylated by incubation with neuraminidase (*A. ureafaciens*) and fractionated by P4 gel filtration chromatography (Figure 6B). Individual fractions were pooled as indicated and separately subjected to NMR analysis, and the spectra are shown for fractions A and B in parts C and D of Figure 6, respectively. From this analysis, it is concluded that fraction A consists principally of a single oligosaccharide of primary sequence as shown in Figure 6C and fraction V contains peptide material and free sialic acid but no other carbohydrate or glycopeptide material (not shown). Fraction B gives a relatively complex spectrum (Figure 6D) which is consistent with the presence of complex conformers of the disaccharide galactose(β1→3)*N*-acetylgalactosamine (as the major component) and possibly some breakdown products of this disaccharide (see Discussion). An aliquot of fraction A was also directly analyzed by LD-MS. The spectrum (not shown) indicates that fraction A consists of a single molecular species with respect to mass consistent with the sequence assignment derived from the NMR analysis (see Table IV).

In the case of RNase B, NMR analysis was first performed on the glycosylated form (RNase B) and the nonglycosylated form (RNase A), and the spectra are shown in parts A and B of Figure 7, respectively. The difference spectrum is shown in Figure 7C. The NMR spectrum of the total pool of unreduced oligosaccharides recovered after hydrazinolysis of the glycosylated form of RNase B is shown in Figure 7D,E. This pool of oligosaccharides was then directly fractionated by P4 gel filtration chromatography (Figure 7F), and individual fractions were pooled as indicated. Each fraction was then separately subjected to NMR analysis, and the spectra for fractions C and D are shown in parts G and H of Figure 7, respectively. From this analysis, it is concluded that each fraction consists of oligosaccharides of primary sequence as shown in Figure 7G,H and Table IV. (Note: The fraction shown in Figure 7G (but not 7H) also contains the monosaccharide xylose, resonances labeled X. This species

Table III: Average Percentage Monosaccharide Yields[a] Obtained from Standard Glycoproteins under Standard Reaction Conditions for Release of O-Linked Oligosaccharides and for Release of N- and O-Linked Oligosaccharides

standard glycoprotein	reaction condition[c]	monosaccharide[b]						
		NeuNAc	Gal	Man	Fuc	Xyl	GalNAc	GlcNAc
BSF	O-only[d]	29 ± 3.2	21 ± 1.8	9.2 ± 3.1				
	O-only[e]	87 ± 6.7	85 ± 7.4		-[f]	-	79 ± 8.4	11 ± 4.1
	N+O	87 ± 7.7	86 ± 5.4	89 ± 5.1	-	-	82 ± 9.2	88 ± 8.8
IgA	O-only[d]	59 ± 4.4	46 ± 2.1	9.6 ± 1.8				
	O-only	81 ± 7.9	83 ± 4.3		11.1 ± 2.1	-	76 ± 8.2	9.3 ± 2.6
	N+O	81 ± 6.2	89 ± 5.1	86 ± 6.2	80 ± 6.3	-	83 ± 8.3	81 ± 9.3
pogen	O-only[d]	49 ± 2.6	53 ± 2.1	7.3 ± 1.2				
	O-only	79 ± 8.4	80 ± 4.9		-	-	71 ± 6.5	9.2 ± 3.1
	N+O	76 ± 6.7	79 ± 5.5	83 ± 6.1	-	-	81 ± 7.9	79 ± 8.4
RNase B	N+O	-	-	88 ± 5.8	-	-	-	86 ± 9.1
PTG	N+O	88 ± 7.8	86 ± 5.2	89 ± 5.4	79 ± 4.1	-	-	86 ± 8.3
ovalb	N+O	-	-	80 ± 6.2	-	-	-	83 ± 7.9
HRP	N+O	-	-	82 ± 6.1	83 ± 5.6	85 ± 4.9	-	89 ± 9.1
α-$_1$AGP	N+O	83 ± 8.1	81 ± 6.2	87 ± 7.0	81 ± 5.7	-	-	86 ± 8.6

[a] Average percentage yields for each monosaccharide were determined from the amount of a given monosaccharide in the recovered oligosaccharide pool derived from an amount (~0.1 mg) of a glycoprotein compared to that directly measured in an equal amount of glycoprotein. Yields are quoted to 2 sf as the average ±1 sd (n = 7) of serial analyses on different aliquots of each standard glycoprotein under each reaction condition studied. [b] Abbreviations are as follows: NeuNAc = N-acetylneuraminic acid; Gal = galactose; Man = mannose; Fuc = fucose; Xyl = xylose; GalNAc = N-acetylgalactosamine; GlcNAc = N-acetylglucosamine. [c] O-only refers to hydrazinolysis reaction conditions of 60 °C for 5 h; N+O refers to hydrazinolysis conditions of 95 °C for 4 h. [d] Values of recovery in the O-only mode not normalized to the expected content of O-linked oligosaccharides. [e] Values of recovery in the O-only mode normalized for the content of O-linked oligosaccharides expected from the measured content of GalNAc. [f] Not measured.

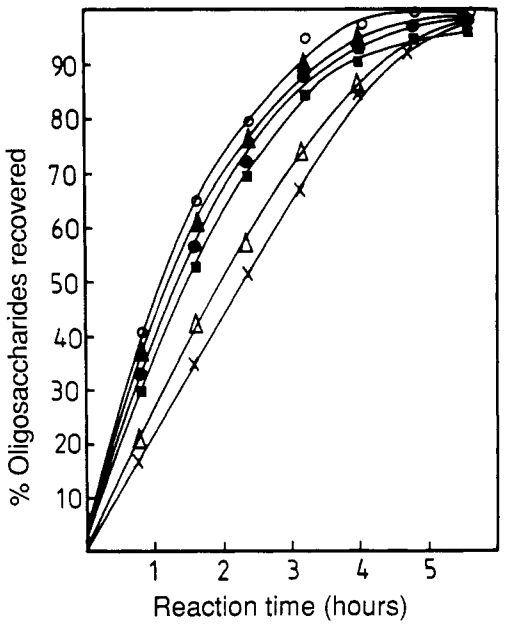


FIGURE 5: Graphs of the percent recovery of N- and O-linked oligosaccharides as a function of reaction time (at 95 °C for N-linked oligosaccharides and 60 °C for O-linked oligosaccharides) from various standard glycoproteins: (×) O-linked oligosaccharides from bovine serum fetuin; (△) O-linked oligosaccharides from human serum IgA; (○) N-linked oligosaccharides from ovalbumin; (▲) N-linked oligosaccharides from human serum IgA; (●) N-linked oligosaccharides from bovine serum fetuin; (■) N-linked oligosaccharides from porcine thyroglobulin.

was not present in the whole oligosaccharide pool and is therefore assumed to have been introduced as a contaminant during fractionation.) An aliquot of each fraction (Figure 7F) was also directly analyzed by LD-MS. The corresponding mass spectra (not shown) also indicate that each fraction is essentially homogeneous with respect to mass and, further, that this mass is consistent with the sequence assignments derived from the NMR analysis (see Table IV).

In the case of horseradish peroxidase, the total pool of recovered oligosaccharides was directly fractionated by P4 gel-filtration chromatography (Figure 8A), and fraction A was pooled as indicated. This fraction was then separately subjected to NMR analysis and the spectrum obtained is shown in Figure 8B. From a comparison of the NMR parameters with reported values (Ashford et al., 1987), fraction A is deduced to contain a single oligosaccharide of primary sequence as depicted in Figure 8B. An aliquot of fraction A was also directly analyzed by LD-MS. The corresponding mass spectrum indicates that fraction A consists of a single molecular species with respect to mass and, further, that this mass is consistent with the sequence assignments derived from the NMR analysis (see Table IV). To further probe the structural integrity of recovered O-linked oligosaccharides, the following analysis was performed. The oligosaccharides were released from 10 mg of bovine serum fetuin using each of the standard conditions. The total pool of recovered oligosaccharides was directly fractionated by HPAEC–PAD, and the chromatograms are shown in Figure 9A,B. Fractions were pooled as indicated, desalted by passage through a column of Dowex AG50X 12 (H^+) followed by rotary-evaporation, filtered through a 0.22-μm Teflon filter, and subjected to NMR analysis. Both fractions O-A-1 and N-A-1 gave essentially identical NMR spectra, corresponding to unreduced N-acetylneuraminic acid. This free sialic acid is thought to be largely present in the starting glycoprotein preparation for three reasons. First, its relative content is the same under both standard reaction conditions (i.e., more vigorous hydrazinolysis reaction conditions do not lead to the recovery of more free sialic acid). Second, standard sialyated oligosaccharides are not desialylated by these hydrazinolysis reaction conditions (see later). Third, a similar recovery of free sialic acid is obtained when oligosaccharide pools are released using PNGase F from bovine serum fetuin and other sialylated serum glycoproteins (not shown). Fractions O-A-2 and N-A-2 also gave very similar NMR spectra and very similar LD-MS spectra (not shown, but see Table IV). The NMR spectrum is therefore shown for N-A-2 in Figure 9C. From this spectrum, fractions O-A-2 and N-A-2 are deduced to contain a single oligosaccharide of primary sequence as depicted in Figure 9C. This conclusion is supported by the LD-MS analysis on O-A-2 and N-A-2 which further indicates that each consists of a single molecular species with respect to mass and that this mass is consistent with the primary sequence deduced from the NMR analysis.

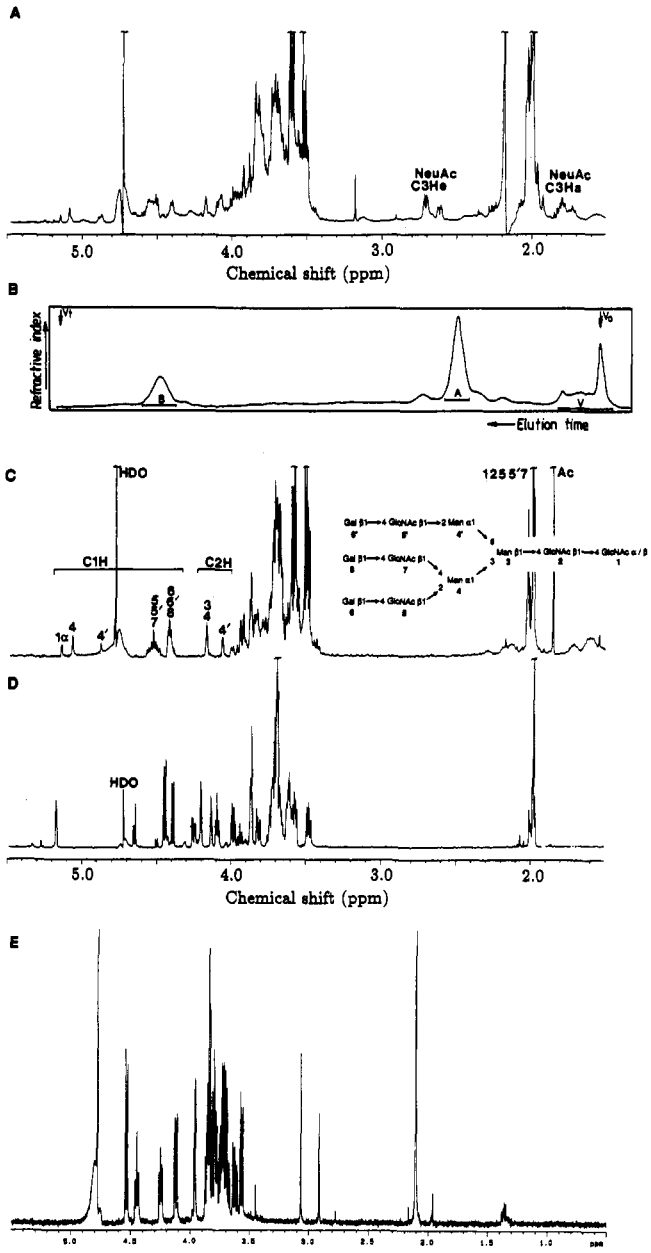
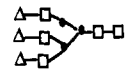
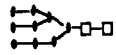
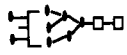


FIGURE 6: One-dimensional 600-MHz ^{1}H-NMR spectrum of the pool of unreduced oligosaccharides recovered from fetuin after hydrazinolysis under conditions to release both N- and O-linked oligosaccharides (A). This pool of unreduced oligosaccharides was treated with neuraminidase (see text) and fractionated by gel-filtration chromatography (B). Fractions were pooled as indicated and individually subjected to NMR analysis. The spectra for fractions A and B are shown in panels C and D, respectively, and for fraction B after reduction with sodium borohydride in Figure 6E. Resonances are labeled according to the residue numbering scheme shown on the inset structure.

(ii) Comparison of N-Linked Oligosaccharide Pools Released from Glycoproteins by Hydrazinolysis and PNGase F. N-Linked oligosaccharides were released from porcine thyroglobulin, hen egg ovalbumin, and human α_1-acid glycoprotein either by hydrazinolysis (under reaction conditions of 95 °C for 4 h) or by exhaustive incubation with PNGase F. An aliquot of the oligosaccharide pools so obtained was radiolabeled, desialylated by incubation with neuraminidase, and fractionated by gel-filtration chromatography. Chromatograms are shown in Figure 10.

(iii) Comparison of O-Linked Oligosaccharide Alditol Pools Obtained from Bovine Serum Fetuin by Hydrazinolysis and Reductive β-Elimination. Three major O-linked oligosaccharides are reported to occur on bovine serum fetuin, namely mono- and disialylated Gal-GalNAc and disialylated Gal-GlcNAc (Gal-) GalNAc. The oligosaccharides released and recovered from fetuin using hydrazinolysis under O-only conditions were radiolabeled. Each pool of radiolabeled alditols was then separated first by anion-exchange chromatography into neutral, mono-acidic, and di-acidic fractions. Each fraction was individually pooled, its total content of radioactivity was determined, and then each acidic fraction was desialylated by incubation with neuraminidase, and the desialylated products were fractionated by P4 gel filtration chromatography. In this way, the relative molar content in each alditol pool of neutral, mono-sialylated, and di-sialylated Gal-GalNAc and Gal-GlcNAc(Gal-)GalNAc could be measured, and these data are summarized in Table V.

Table IV: Comparison of Expected and Observed Molecular Mass for Individual Oligosaccharide Fractions Analyzed by LD-MS

fraction	structure assigned by ^{1}H-NMR[a]	expected mass[b]	observed mass[c]
BSF-Fraction A		2007	2007
RNaseB-Fraction A		1884	1884
RNase B-Fraction B		1722	1722
RNase B-Fraction C		1397	1397
RNase B-Fraction D		1235	1235
HRP-Fraction A		1205	1205
BSF-Fraction O-A-2		966	967
BSF-Fraction N-A-2		966	967

[a] The expected molecular mass is calculated assuming that unreduced monosaccharides have the following relative molecular weights: fucose = 164.16; xylose = 166.13; galactose = 180.16; mannose = 180.16; *N*-acetylglucosamine = 221.21; *N*-acetylgalactosamine = 221.21; *N*-acetylneuraminic acid = 309.27. [b] Symbolic abbreviations for monosaccharides are as follows: ✡ = fucose; ○ = xylose; △ = galactose; ● = mannose; □ = *N*-acetylglucosamine; ■ = *N*-acetylgalactosamine; ▲ = *N*-acetylneuraminic acid. [c] Oligosaccharides are detected as the [oligosaccharide Na]$^+$ ions, and the observed mass is quoted after correction for the attached Na$^+$. Both expected and observed mass are stated to the nearest integral value.

(iv) Analysis of the Reducing Terminal Monosaccharide in Oligosaccharide Pools Released from Glycoproteins by Hydrazinolysis. The total oligosaccharide pools recovered from each standard glycoprotein using one or both of the standard hydrazinolysis reaction conditions were radiolabeled by reduction with alkaline sodium borotritide and incubated with a mixture of exoglycosidases, and the radiolabeled alditol-(s) was(were) recovered by P4 gel-filtration chromatography. The alditols so recovered were identified by reverse-phase HPLC (Takeuchi et al., 1987). The data for this analysis are summarized in Table VI.

Stability of Acidic Substituents. Radiolabeled alditols were analyzed by high-voltage paper electrophoresis before and after being subjected to hydrazinolysis (95 °C for 4 h) in the

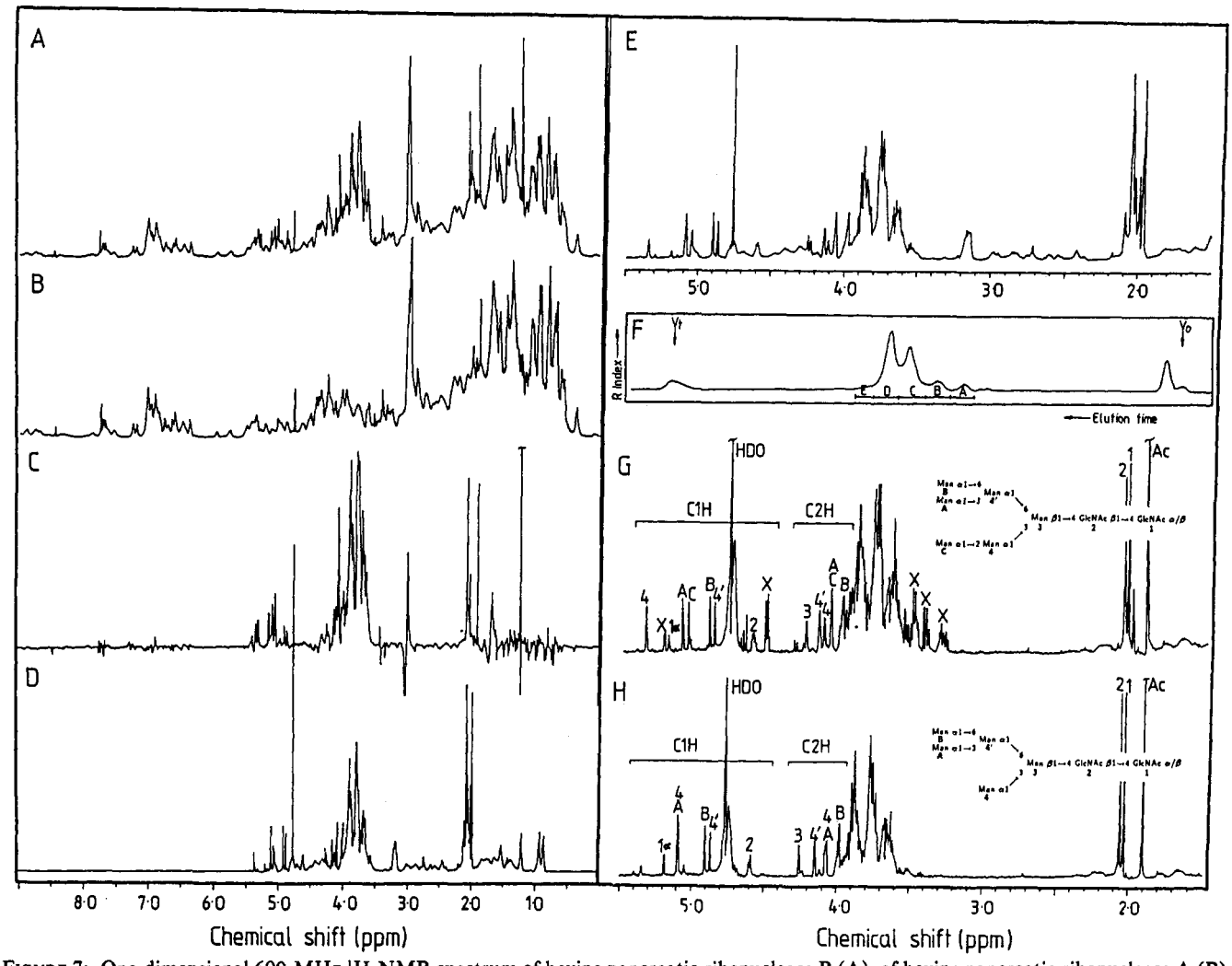


FIGURE 7: One-dimensional 600-MHz ^{1}H-NMR spectrum of bovine pancreatic ribonuclease B (A), of bovine pancreatic ribonuclease A (B), the difference spectrum between the two forms of ribonuclease (C), and of the pool of unreduced oligosaccharides (D and E) recovered from ribonuclease B after hydrazinolysis under conditions to release both N- and O-linked oligosaccharides. This pool of unreduced oligosaccharides was then fractionated by gel-filtration chromatography (F). Fractions were pooled as indicated and individually subjected to NMR analysis. Only spectra for fractions C and D are shown in panels G and H, respectively.

presence of 1 mg of bovine serum albumin. The following alditols were analyzed: *N*-acetyl-D-glucosaminitol 3-sulfate, *N*-acetyl-D-glucosaminitol 6-sulfate, D-galactitol 6-sulfate, *N*-acetyl-D-glucosaminitol 6-phosphate, D-mannitol 6-phosphate, NeuNAc(α2→3)Gal(β1→3)GalNAc$_{OL}$, and NeuNAc-(α2→6)Gal(β1→4)GlcNAc$_{OL}$. The molar percent loss of acidic substituents from each alditol under the standard hydrazinolysis reaction conditions is stated in Table VII.

DISCUSSION

Release and recovery of N- and O-linked oligosaccharides in an unreduced form is often necessary to facilitate both structural and functional analyses of protein-associated oligosaccharides. The use of alkaline sodium borohydride (Nilsson et al., 1990), whether in the presence of cadmium salt or not (Likhosherstov et al., 1990), and of lithium borohydride (Likhosherstov et al., 1988) all lead to the recovery of the reduced (alditol) form of oligosaccharides. Some groups have successfully used hydrazinolysis to release apparently structurally intact N-linked oligosaccharides from glycoproteins (Fukuda et al., 1976; Takasaki & Kobata, 1982; Parekh et al., 1989), while others have concluded that the use of hydrazine inevitably leads to the partial degradation of, in particular, the reducing terminus of N-linked oligosaccharides (Michalski et al., 1984; Van Kuik et al., 1987; Bendiak & Cumming, 1985). A systematic study of the hydrazinolysis reaction for the release of N-linked oligosaccharides has not been reported, and no standard conditions have come to prevail. A few reports addressing the recovery of O-linked oligosaccharides during hydrazinolysis indicated profound degradation of these oligosaccharides (Korrel et al., 1985; Montreuil et al., 1986). Nevertheless, the reported recovery of *some* intact O-linked oligosaccharides prompted us to investigate further the use of hydrazine to release both N- and O-linked oligosaccharides from glycoproteins.

Given that the thermodynamics of oligosaccharide release are such that equilibrium lies overwhelmingly in its favor, our strategy in evaluating the hydrazinolysis reaction was as follows: First, to establish the kinetic parameters governing release (and any degradation) of oligosaccharides from a broad range of glycoproteins, second, to use these parameters to define generally applicable standard reaction conditions, and third, to assess the molar yield and structural integrity of oligosaccharides recovered under these standard reaction conditions by a variety of physical, chemical, chromatographic, and enzymatic methods. The range of "standard" glycoproteins employed in this study together with their previously reported glycosylation characteristics is summarized in Table I. Between these various glycoproteins, all major N- and O-linked oligosaccharide structures and determinants reported

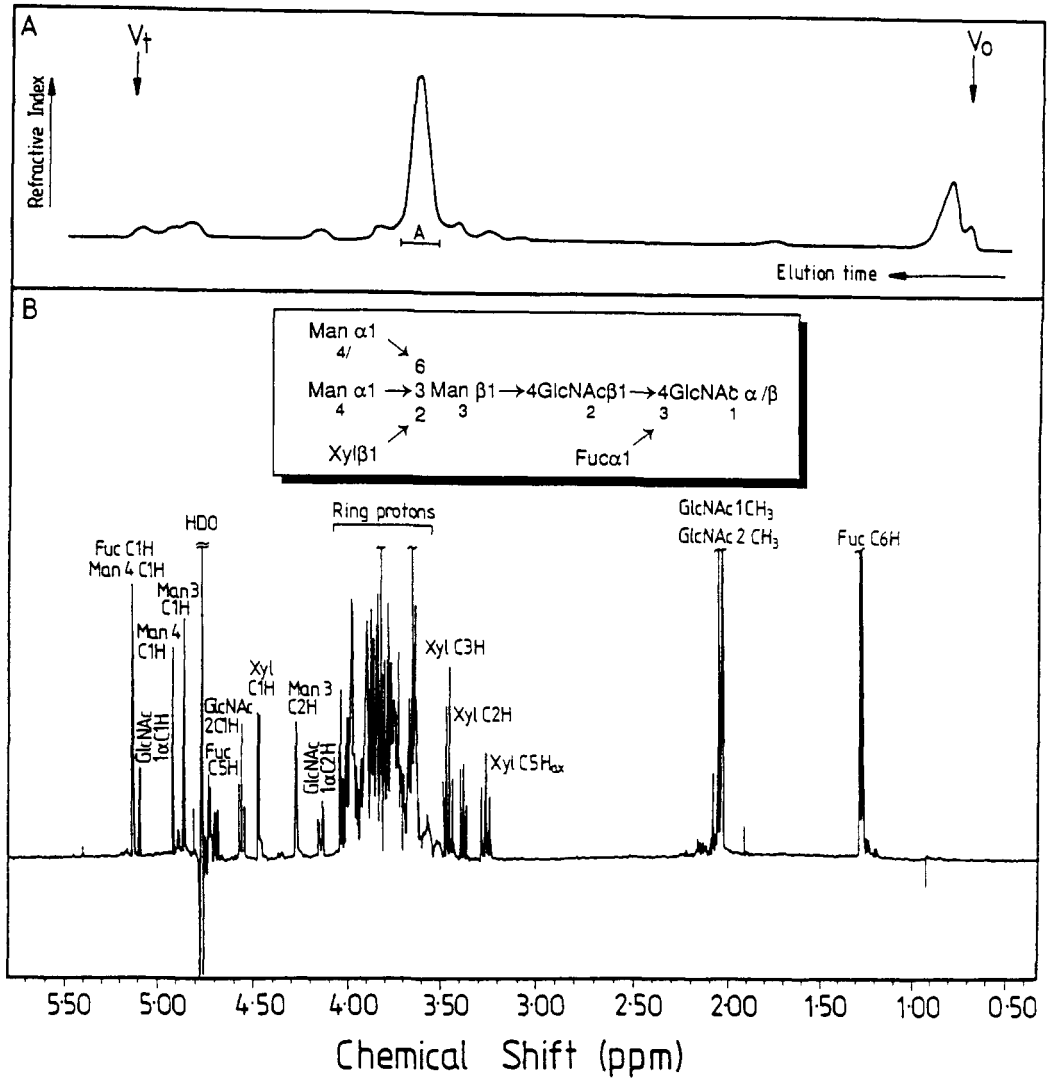


FIGURE 8: Gel-filtration chromatogram (A) of the unreduced oligosaccharides recovered from horseradish peroxidase after hydrazinolysis under conditions to release both N- and O-linked oligosaccharides. Fraction A was pooled as indicated and analyzed by one-dimensional 500-MHz ^{1}H-NMR. From an analysis of the spectrum (shown in part B) the primary sequence of the oligosaccharide in fraction A is deduced to be as indicated.

on eukaryotic glycoproteins (with the exception of O-linked mannosyl chains) are present. Model glycoamino acid compounds containing an oligosaccharide attached to a single amino acid residue were deliberately not chosen since in terms of precise reaction mechanism, peptide reaction products, and "matrix" effects these compounds are not necessarily representative of glycoproteins. The principal objective of this study was to assess the hydrazinolysis reaction for use against glycoproteins.

The net release of both N- and O-linked oligosaccharides from bovine serum fetuin as a function of reaction temperature at constant reaction time (8 h) is shown in Figure 2A and that of N- and O-linked oligosaccharides from several of the other standard glycoproteins in parts B and C of Figure 2, respectively. It is clear from these data that intact O-linked oligosaccharides are released during the hydrazinolysis reaction with a lower temperature dependence than N-linked oligosaccharides. Once released, O-linked oligosaccharides are susceptible to a degradation reaction (which obviously has a higher temperature dependence than their release). N-Linked oligosaccharides are much more stable. Furthermore, the release of oligosaccharides from the different standard glycoproteins has a remarkably similar temperature dependence, suggesting that the reaction is essentially *independent* of the primary sequence of either oligosaccharide or protein. All hydrazinolyses in this study were performed under considerable molar excess of hydrazine (32 M) over glycoprotein ($\leq$10 μM). Under these reaction conditions, both release of N- and O-linked oligosaccharides, and subsequent degradation of O-linked oligosaccharides is pseudo-first-order with respect to glycoprotein, as shown by the linearity of plots of log K_R against $1/T$ (see Figure 3). For all such plots, $r^2 \geq 0.91$ (from regression analysis), and this is therefore taken as strong evidence in favor of the reactions for all glycoproteins being pseudo-first-order with respect to glycoprotein. On the basis of this order, the release of N- and O-linked oligosaccharides and degradation of O-linked oligosaccharides can be analyzed in terms of the Arrhenius equation (ignoring second- and higher-order coefficients) and the activation energy and Arrhenius constant for each reaction determined (see Table II). Using these experimentally determined constants, three-dimensional plots of the mole fraction of intact released N- and/or O-linked oligosaccharides as a function of reaction temperature (°C) and reaction time (hours) for *each* glycoprotein can be generated. These plots are similar for all standard glycoproteins carrying N-linked oligosaccharides and for the three carrying O-linked oligosaccharides, and 2-D contour plots are therefore shown only for bovine serum fetuin

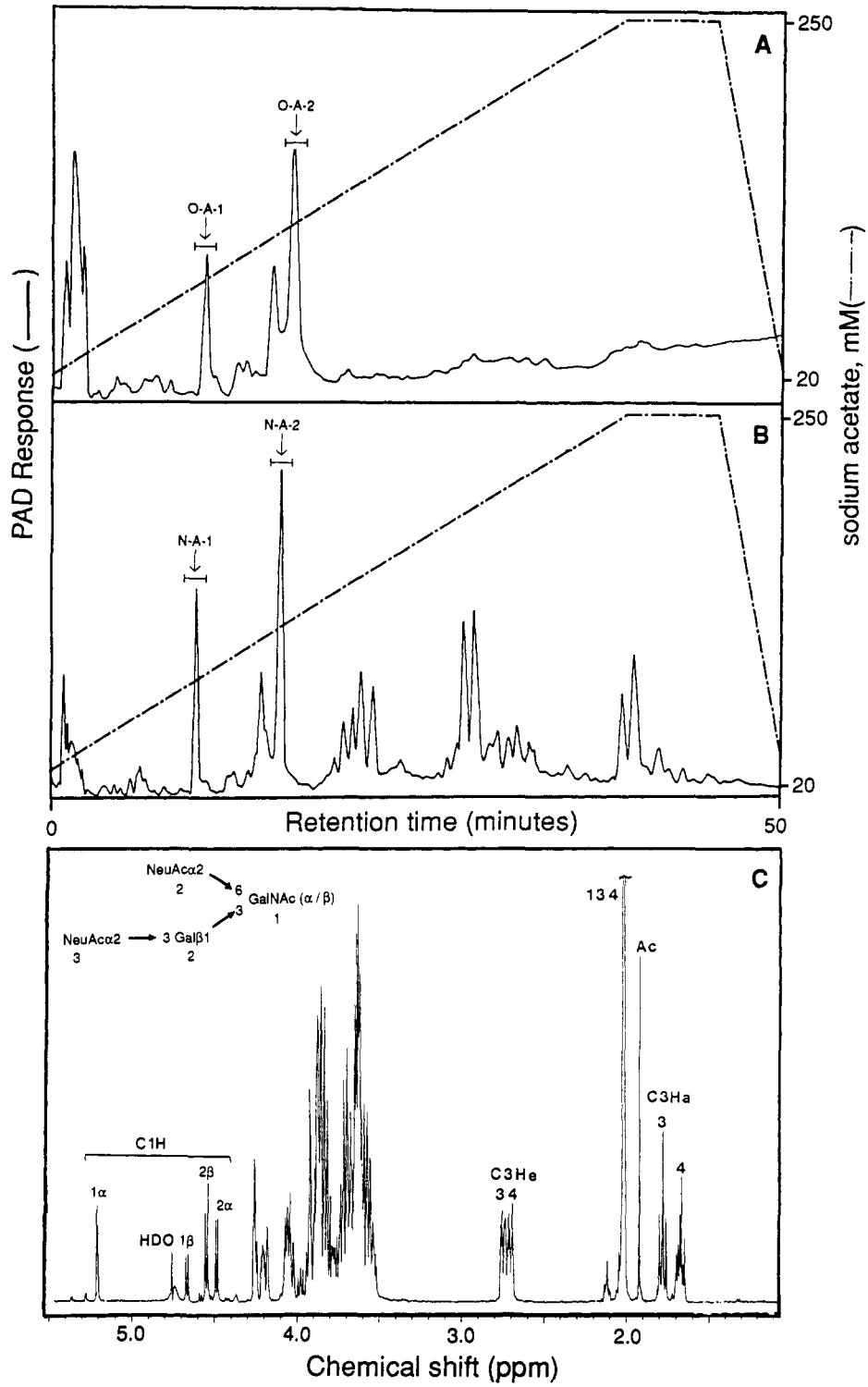


FIGURE 9: HPAEC–PAD chromatograms of the oligosaccharides released from bovine serum fetuin using the "O-only" reaction conditions (A) and "N+O" reaction conditions (B). Fractions O-A-1, O-A-2, N-A-1, and N-A-2 were pooled as indicated, desalted, and individually subjected to ^{1}H-NMR analysis. The ^{1}H-NMR spectrum for N-A-2 is shown (C).

(Figure 4A,B). The net result of this analysis is a definition of two sets of standard reaction conditions. Namely, one involving a hydrazinolysis reaction of 60 °C for 5 h during which ≥90% of O-linked oligosaccharides are released but <10% of N-glycosidic bonds are broken, and a second involving a hydrazinolysis reaction of 95 °C but 4 h in which ≥85% of both O- and N-linked oligosaccharides are released. These will be referred to as 'O-only' and 'N+O' reaction conditions.

The sensitivity of m_n and m_o to even very small changes in E^{ACT} and A is considerable for values of E^{ACT} and A comparable to those obtained. In order therefore to confirm the experimental validity and accuracy of these standard reaction conditions, oligosaccharides were released under these conditions from each of the standard glycoproteins. The yield, structural integrity, and content of individual oligosaccharides was assessed in the recovered oligosaccharide pools. Yields were estimated by quantitation of individual monosaccharides in the intact glycoprotein and in the recovered oligosaccharide pool and are summarized in Table III. Since this method would not distinguish unreduced oligosaccharides from peptide-associated material or other conjugates, a time course of oligosaccharide release was performed on four of the standard

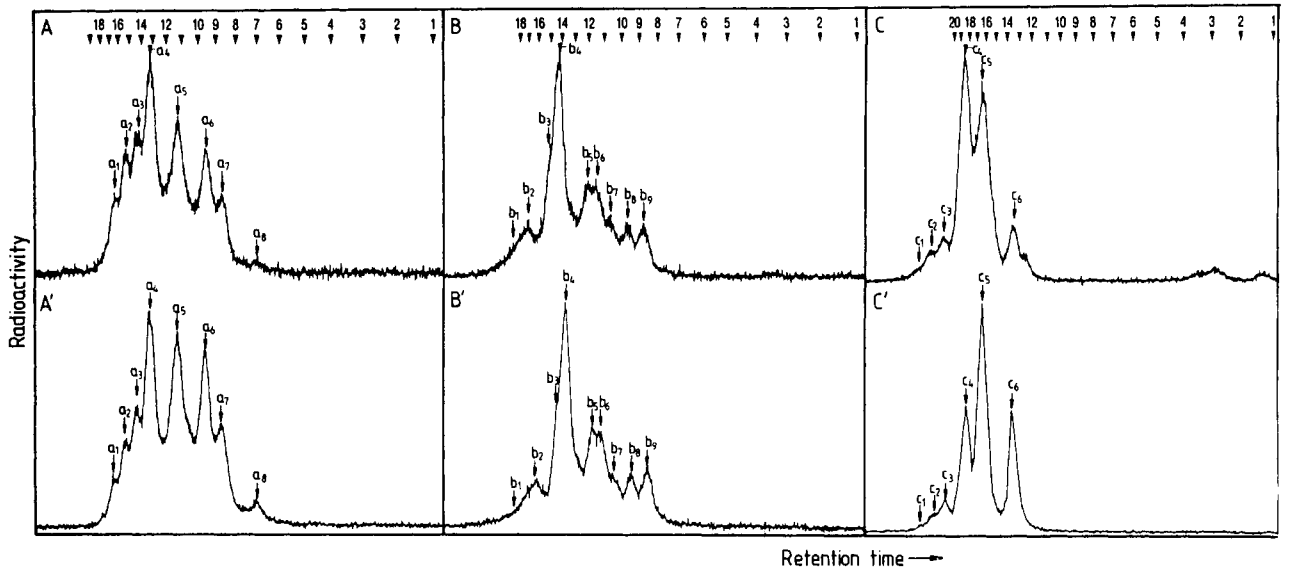


FIGURE 10: P4 gel-filtration chromatograms of the desialylated radiolabeled oligosaccharides recovered from hen egg ovalbumin (A, A'), porcine thyroglobulin (B, B') and human α_1-acid glycoprotein (C, C') using hydrazinolysis under conditions to release both N- and O-linked oligosaccharides (A, B, C) and exhaustive incubation with PNGase F (A', B', C').

Table V: Comparison of the O-Linked Oligosaccharide Alditol Pools Recovered from Fetuin by Hydrazinolysis (O-Only) and Reductive β-Elimination[a]

	reductive β-elimination (%)[c]	O-only hydrazinolysis (%)[c]
D[b]	4.1	4.8
D-A1	16.8	15.9
D-A2	60.1	57.6
T	1.4	1.8
T-A1	2.1	2.6
T-A2	15.5	17.3

[a] Alditol pools were recovered as described under Materials and Methods. [b] D = Gal-GalNAc; D-A1 and D-A2 = mono- and disialylated Gal-GalNAc, respectively; T = Gal-GlcNAc(Gal)GalNAc; T-A1 and T-A2 = mono- and disialylated Gal-GlcNAc(Gal)GalNAc. [c] Relative percentages were determined as described under Results and are calculated after excluding any recovered N-linked oligosaccharide alditols (which are separated from D and T during gel-filtration chromatography). The content of N-linked oligosaccharide alditols was particularly noticeable in the alditol pool obtained by reductive β-elimination.

glycoproteins with quantitation of released oligosaccharide by coreduction in the presence of the glucose-7 standard. These data, summarized in Figure 5, indicate that a reaction time of >4 h at 95 °C does not lead to significantly increased release of N- and O-linked oligosaccharides and that a reaction time of >5 h at 60 °C does not lead to significantly increased release of O-linked oligosaccharides. Under these reaction conditions, the percent monosaccharide recoveries when corrected for any losses postrelease (during chromatography and workup as determined using radiolabeled oligosaccharide standards) indicate essentially quantitative release of N- and O-linked oligosaccharides in the N+O reaction condition and of O-linked oligosaccharides in the O-only condition.

A primary method for the evaluation of oligosaccharide structure is ^{1}H-NMR (Van Kuik et al., 1985; Vliegenthart et al., 1983). NMR analysis was therefore performed on the total pool of oligosaccharides recovered from bovine serum fetuin after release under N+O reaction conditions (Figure 6A). The general complexity of the spectrum prevents a detailed analysis of all the resonances; however, the general form of the spectrum, and in particular the anomeric region (4.3–5.4 ppm), is very similar to that of the major isolated desialylated oligosaccharide (Figure 6C). The only major additional peaks (labeled in Figure 6A) are from sialic acid linked both $\alpha2\rightarrow3$ and $\alpha2\rightarrow6$. The intensity of these peaks relative to the anomeric resonances of the outer-arm galactose residues (≈4.4 ppm, labeled in Figure 6C) indicates almost full sialylation of the oligosaccharides. These is a little free sialic acid present (sharp peaks at ≈2.2 ppm, not labeled). These peaks increase in intensity with time over periods of several weeks, suggesting that some autocleavage is occurring, but are initially at very low levels. The α anomeric resonance from the reducing terminal GlcNAc (≈5.1 ppm, labeled in Figure 6C) can be seen, although the β anomeric resonance is obscured by the water peak. The intensity of this peak, relative to that of the other anomeric peaks, suggests that the reducing terminus is intact. The major desialylated N- and O-linked oligosaccharides recovered from this pool of fetuin oligosaccharides (Figure 6B) are likewise structurally intact (Figure 6C,D and Table IV). The ^{1}H-NMR spectrum (Figure 6D) of the unreduced Gal($\beta1\rightarrow3$)GalNAc is relatively complex. This spectrum is highly reproducible from different fetuin preparations, is also obtained on treatment of O-A-2 and N-A 2 (Figure 9 and later) with neuraminidase, and more importantly is also similar to that obtained with authentic Gal($\beta1\rightarrow3$)GalNAc. The complexity of this spectrum would appear to arise from conformational heterogeneity, the principal factor being contributions from both α and β anomers, since reduction of this disaccharide yields a much simplified ^{1}H-NMR spectrum (Figure 6E). Fraction B can therefore be identified as the disaccharide galactose$\beta(1\rightarrow3)$*N*-acetyl-galactosamine. There is also some evidence from the spectrum for the occurrence of some deacetylation (reduced intensity of the -NHCOCH3 resonance at approximately 2.0 ppm) which increases with time. A full spectral analysis, together with other data on the chemical stability of galactose($\beta1\rightarrow3$)-*N*-acetylgalactosamine, will be presented elsewhere.

Similar analysis was performed on oligosaccharides recovered after release under N+O conditions from bovine pancreatic RNase B (Figure 7) and horseradish peroxidase (Figure 8). In the case of ribonuclease, the unfractionated pool of oligosaccharides gave a ^{1}H-NMR spectrum (Figure 7D) very similar to the difference spectrum (Figure 7C) between ribonuclease B (Figure 7A) and ribonuclease A (Figure 7B), which is the nonglycosylated form of RNase B. This supports the nonselective release of intact oligosaccharides

Table VI: Identification of Reducing Terminal Monosaccharide Alditols[a] in the Oligosaccharide Populations Obtained from Standard Glycoproteins under Standard Reaction Conditions

standard glycoprotein	reaction condition	% [^{3}H] recovery after gel filtration[b]	% [^{3}H]GlcNAc$_{OL}$[c]	% [^{3}H]GalNAc$_{OL}$	% [^{3}H]GalOL	% [^{3}H]Glc$_{OL}$	% [^{3}H]X$_{OL}$
BSF	O-only[d]	>95%	4	83	9	4	nd[e]
	N+O	>95%	52	43	4	nd	nd
IgA	O-only	>95%	6	79	10	5	nd
	N+O	>95%	29	63	7	1	nd
pogen	O-only	>95%	8	80	11	1	nd
	N+O	>95%	36	59	5	nd	nd
RNase B	N+O	>95%	94	nd	nd	5	2
PTG	N+O	>95%	94	nd	nd	3	3
ovalb	N+O	>95%	97	nd	nd	3	nd
HRP	N+O	>95%	90	nd	nd	9	1
α_1-AGP	N+O	>95%	86	4	nd	8	2

[a] Identification of reducing terminal alditols was achieved as described under Materials and Methods. [b] This refers to the percentage of radioactivity recovered after applying the glycosidase digestions product(s) to a P4 gel-filtration column. [c] This refers to the percentage of radioactivity recovered in a given monosaccharide alditol after applying the radioactivity recovered from the P4 gel-filtration chromatography to a Shodex sugar SP1010 HPLC column. Abbreviations are as follows: GlcNAc$_{OL}$ = *N*-acetylglucosaminitol; GalNAc$_{OL}$ = *N*-acetylgalactosaminitol; Gal$_{OL}$ = galactitol; Glc$_{OL}$ = glucitol; X$_{OL}$ = unidentified alditol. All percentages are stated to the nearest integer and are in each case the average of two separate analyses. [d] O-only refers to hydrazinolysis reaction conditions of 60 °C for 5 h; N+O refers to hydrazinolysis conditions of 95 °C for 4 h. [e] nd = not detectable.

Table VII: Alteration[a] of the Charge of Standard Acidic Alditols during the Hydrazinolysis Reaction[b]

structure of alditol	molar percent[c] deacidification
N-acetyl-D-glucosaminitol 3-sulfate	1.4 ± 0.5
N-acetyl-D-glucosaminotol 6-sulfate	11.3 ± 2.1
D-galactitol 6-sulfate	2.1 ± 0.3
N-acetyl-D-glucosaminitol 6-phosphate	21.3 ± 4.1
D-mannitol 6-phosphate	3.1 ± 1.2
NeuNAc[d](α2→3)Gal(β1→3)GalNAc$_{OL}$	2.2 ± 1.8
NeuNAc(α2→6)Gal(β1→4)GlcNAc$_{OL}$	1.9 ± 1.9

[a] Alteration of the charge was measured by separating products after the hydrazinolysis reaction by high-voltage paper electrophoresis, recovering the radioactivity in any acidic and any deacidified products, and measuring the relative percentage radioactivity recovered in deacidified product as compared to still acidic starting material. [b] Only the more vigorous standard hydrazinolysis reaction conditions of 95 °C for 4 h were investigated. [c] Molar percent deacidification is expressed as the average ± 1 sd of 7 separate analyses. [d] Abbreviations are as follows: NeuNAc = *N*-acetylneuraminic acid; Gal = galactose; GalNAc$_{OL}$ = *N*-acetylgalactosaminitol; GlcNAc$_{OL}$ = *N*-acetylglucosaminitol.

during hydrazinolysis. Sequences of oligosaccharides in individual fractions (Figure 7F) deduced from NMR analysis are supported by direct mass determinations using laser desorption mass spectrometry (Table IV). In the case of horseradish peroxidase, a single major oligosaccharide was recovered (Figure 8A), and only this oligosaccharide (and not the unfractionated pool) was therefore analyzed (Figure 8B).

Oligosaccharides were released from bovine serum fetuin under each standard reaction condition and fractionated by HPAEC–PAD (Figure 9). The major O-linked oligosaccharide, identified by its content of *N*-acetylgalactosamine (data not shown), was analyzed by a combination of ^{1}H-NMR (Figure 9) and laser desorption mass spectrometry (Table IV), from which the structure is deduced to be as shown.

Collectively, these data indicate that to the limits of detection of ^{1}H-NMR and laser desorption mass spectrometry, intact N- and O-linked oligosaccharides are recovered under the standard reaction conditions. To probe further for any hydrazine-induced structural artifacts, oligosaccharide pools recovered from porcine thyroglobulin, hen egg ovalbumin, and human α_1-acid glycoprotein by hydrazinolysis under N+O reaction conditions were compared by chromatographic analysis to the oligosaccharide pools recovered from these same glycoproteins using PNGase F. This study was not performed on bovine serum fetuin, human serum IgA, and plasminogen since these also contain O-linked oligosaccharides or on horseradish peroxidase since the major oligosaccharide structure on this glycoprotein is not released by PNGase F.

The P4 chromatograms of the desialylated oligosaccharide alditols from ovalbumin and porcine thyroglobulin are virtually identical, whether the oligosaccharides are released using PNGase F or hydrazine (Figure 10). In the case of α_1-acid glycoprotein, the oligosaccharide pool recovered by hydrazinolysis is enriched in larger structures compared to that recovered using PNGase F (Figure 10). This is interpreted as indicating some selectivity of release of oligosaccharides from α_1-acid glycoprotein by PNGase F, since further release of these oligosaccharides cannot be induced either by addition of more PNGase F or by prolonging the incubation with PNGase F (Patel et al., in preparation). In any case, this selectivity of release is inconsistent with hydrazine-induced *degradation* of these oligosaccharides. Similarly, a comparison of the O-linked oligosaccharides recovered from fetuin using hydrazinolysis ("O-only") and reductive β-elimination indicate a close similarity between the two pools of oligosaccharides (see Table V). Finally, the predominant reducing termini were *N*-acetylglucosaminitol and/or *N*-acetylgalactosaminitol in pools of oligosaccharides recovered under either N+O or O-only conditions (see Table VI).

The conservation of certain acidic substituents during the N+O reaction conditions was studied using model compounds. "Matrix" and peptide effects were approximated by the inclusion of bovine serum albumin. The data, summarized in Table VII, indicate that only substituents at the C-6 position of *N*-acetylglucosamine are significantly labile. The sialic acid linkage itself appears to be stable under these reaction conditions, but it is expected that certain *N*- and *O*-acyl substitutions to sialic acid may not be. The stability of various other sialic acid linkages involving different forms of sialic acid are currently being investigated using model compounds.

Overall, the total analysis presented above indicates that the standard reaction conditions defined herein can be successfully used to cleave quantitatively and allow subsequent recovery of intact and unreduced O- or N- and O-linked oligosaccharides. Numerous studies involving a variety of hydrazinolysis reaction conditions have been reported, and hydrazine-induced structural artifacts have often been identified (Michalski et al., 1984; Van Kuik et al., 1987; Bendiak & Cumming, 1985; Saed & Williams, 1980). On the basis of the analysis presented here, it is suggested that previously

reported hydrazinolysis reaction conditions may have been excessively vigorous and certainly none could have led to significant recoveries of O-linked oligosaccharides. The mechanistic details of the hydrazinolysis reaction for the release of N-linked oligosaccharides have been investigated, though the reaction mechanism is still the subject of debate (Tang & Williams, 1983; Takasaki & Kobata, 1982; Bendiak & Cumming, 1985; Inoue & Kitajima, 1985). The data presented here shed little further light on this point other than to establish that the reaction is first-order with respect to glycoprotein. One can only speculate on the mechanism of release, and in particular the surprising stability of unreduced O-linked oligosaccharides containing the reducing terminal disaccharide galactose(β1→3)*N*-acetylgalactosamine. Such oligosaccharides are notoriously labile, particularly to even mildly alkaline conditions. One would suggest that the hydrazone derivative (Bendiak & Cumming, 1985; Tang & Williams, 1983) which is likely to be the primary product of the hydrazine-induced cleavage of O-linked oligosaccharides from peptide under anhydrous conditions is relatively stable even in the presence of a powerful Lewis base (hydrazine) in the relative absence of alkali (OH^-) and that it is further reactions of this hydrazone form that lead to subsequent degradation of released O-linked oligosaccharides.

The "standard" reaction conditions apply to the standard glycoproteins used in this study and to several additional ones (human serum IgG, IgM, fibrinogen, rat brain Thy-1). That hydrazinolysis (or any chemical) method for cleaving N- and Oglycosidic bonds could be general (by which is meant that yield of intact unreduced N- and O-linked oligosaccharides is essentially independent of the primary structure of both the oligosaccharide being cleaved and the protein to which it is attached) is not considered surprising. In the case of all N-linked oligosaccharides, the same bond (i.e., GlcNAc-β1→Asn) is being cleaved, and in the case of all O-linked oligosaccharides the bond being cleaved is either GalNAc-α1→serine or threonine. Substituents beyond the N- or O-glycosidic bond may not significantly affect its reactivity, and the access of hydrazine, which is a relatively small chemical, may not be significantly influenced by steric factors. While these reaction conditions do appear to apply equally well to a broad range of serum and other glycoproteins from various species, further studies are in progress to investigate more fully their true generality.

A final aspect of the hydrazinolysis reaction is the potential for the differential use of the two standard reaction conditions to facilitate the selective recovery and identification of O-linked oligosaccharides. The data shown in Figure 9 and Tables III and IV indicate that the discrimination between the two reaction conditions is sufficient to allow, by comparative analysis, a separate 'mapping' of O- and N-glycosylation. This is clearly shown in Figure 9. O-Linked oligosaccharides were recovered from bovine serum fetuin using the O-only conditions with little recovery of N-linked oligosaccharides (Figure 9A). Release under N+O conditions leads to recovery of both N- and O-linked oligosaccharides (Figure 9B). In subsequent papers, we compare more fully the hydrazinolysis conditions defined here with the use of PNGase F (for release of N-linked oligosaccharides) and with the use of reductive β-elimination (for release of O-linked oligosaccharides). The use of hydrazine for release of oligosaccharides from defined glycopeptides, mucins, O-mannosylated yeast-derived glycoproteins, various classes of glycolipid, and saponins is currently under investigation, as is the use of the reaction conditions defined here for the sequential release of first O- and then N-linked oligosaccharides from the same aliquot of a glycoprotein.

ACKNOWLEDGMENT

We are grateful to Dr. Antonis Ioannides for valuable discussions, to Dr. David Harvey for performing laser desorption mass spectrometric analyses, to Dr. Ian Scragg for preparing the ribonucleases A and B, and to Rad Mitrovic for expert technical assistance. We also acknowledge the helpful comments of Professor J. F. G. Vliegenthart.

REFERENCES

Anamula, R. R., & Taylor, P. B. (1991) *Eur. J. Biochem. 195*, 269–280.

Ashford, D., Dwek, R. A., Welply, J. K., Amatayakul, S., Homans, S. W., Lis, H., Taylor, G. N., Sharon, N., & Rademacher, T. W. (1987) *Eur. J. Biochem. 166*, 311–320.

Bayard, B., & Montreuil, J. (1974) *Colloq. Int. CNRS 221*, 209–218.

Bendiak, B., & Cumming, D. A. (1985) *Carbohydr. Res. 144*, 1–12.

Bundi, A., & Wutrich, K. (1979) *Biopolymers 18*, 285–298.

Carlsson, D. M. (1968) *J. Biol. Chem. 243*, 616–626.

Chaplin, M. F. (1982) *Anal. Biochem. 123*, 336–341.

Damm, J. B. L., Kamerling, J. P., Van Dedem, G. W. K., & Vliegenthart, J. F. G. (1987) *Glycoconjugate J. 4*, 129–144.

Field, M. C., Dwek, R. A., Edge, C. J., & Rademacher, T. W. (1989) *Biochem. Soc. Trans. 17*, 1033–1035.

Fukuda, M. (1989) *Methods Enzymol. 179*, 17–29.

Fukuda, M., Kondo, T., & Osawa, T. (1976) *J. Biochem. (Tokyo) 80*, 1223–1332.

Hase, S., Ikenaka, T., & Matsushimo, Y. (1979) *J. Biochem. (Tokyo) 85*, 989–994.

Hayes, M. L., & Castellino, F. J. (1979) *J. Biol. Chem. 254*, 8768–8780.

Inoue, Y., & Kitayima, K. (1985) *Carbohydr. Res. 135*, 342–347.

Korrel, S. A. M., Clemetson, K. J., Van Halbeek, H., Kamerling, J. P., Sixma, J.-J., & Vliegenthart, J. F. G. (1985) *Glycoconjugate J. 2*, 229–234.

Krotkiewski, H., Nilsson, B., & Svensson, S. (1989) *Eur. J. Biochem. 184*, 29–38.

Lee, Y. C., & Scocca, J. R. (1972) *J. Biol. Chem. 247*, 5753–5759.

Lee, Y. C., Lee, B. I., Tomiya, N., & Takahashi, N. (1990) *Anal. Biochem. 188*, 259–266.

Liang, C.-J., Yamashita, K., & Kobata, A. (1986) *J. Biochem. (Tokyo) 88*, 51–58.

Likhosherstov, L. M., Novikova, O. S., Piskarev, V. E., Trusikhina, E. E., Derevitskaya, V. A., & Kochetkov, N. K. (1988) *Carbohydr. Res. 178*, 155–163.

Likhosherstov, L. M., Novikova, O. S., Derevitskaya, V. A., & Kochetkov, N. K. (1990) *Carbohydr. Res. 199*, 67–76.

Michalski, J.-C., Peter-Katalinic, J., Egge, H., Paz-Parente, J., Montreuil, J., & Strecker, G. (1984) *Carbohydr. Res. 134*, 177–189.

Montreuil, J. (1982) in *Comprehensive Biochemistry* (Florkin, G., & Stotz, E. H., Eds.) Vol. 19B, Part II, pp 1–188, Elsevier Publishing Company, Amsterdam.

Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G., & Strecker, G. (1986) in *Carbohydrate Analysis: A Practical Approach* (Chaplin, M. F., & Kennedy, J. F., Eds.) pp 143–202, IRL Press, Oxford, U.K.

Nuck, R., Zimmermann, M., Sauvageot, D., Josic, D., & Reutter, W. (1990) *Glycoconjugate J. 7*, 279–286.

Parekh, R. B., Tse, A. G. D., Dwek, R. A., Williams, A. F., & Rademacher, T. W. (1987) *EMBO J. 6*, 1233–1244.

Parekh, R. B., Dwek, R. A., Thomas, J. R., Opdenakker, G., Rademacher, T. W., Wittwer, A. J., Howard, S. C., Nelson,

R., Siegel, N. R., Jennings, M. G., Harakas, N. K., & Feder, J. (1989) *Biochemistry 28*, 7644–7662.
Rademacher, T. W., Parekh, R. B., & Dwek, R. A. (1988) *Annu. Rev. Biochem. 57*, 785–838.
Saeed, M. S., & Williams, J. (1980) *Carbohydr. Res. 84*, 83–94.
Takasaki, S., & Kobata, A. (1978) *Methods Enzymol. 50*, 50–54.
Takasaki, S., & Kobata, A. (1986) *Biochemistry 25*, 5709–5715.
Takasaki, S., Mizuochi, T., & Kobata, A. (1982) *Methods Enzymol. 83*, 263–268.
Takeuchi, M., Takasaki, S., Inoue, N., & Kobata, A. (1987) *J. Chromatogr. 400*, 207–213.
Tang, P. W., & Williams, J. M. (1983) *Carbohydr. Res. 121*, 89–97.
Umemoto, J., Bhavanandan, V. P., & Davidson, E. A. (1977) *J. Biol. Chem. 252*, 8609–8614.
Van Kuik, J. A., Van Halbeek, H., Kamerling, J. P., & Vliegenthart, J. F. G. (1985) *J. Biol. Chem. 260*, 13984–13988.
Van Kuik, J. A., Sijbesma, R. P., Kamerling, J. P., Vliegenthart, J. F. G., & Wood, E. J. (1987) *Eur. J. Biochem. 169*, 399–411.
Vliegenthart, J. F. G., Dorland, L., & Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–374.
Yamashita, K., Tachibana, Y., & Kobata, A. (1978) *J. Biol. Chem. 253*, 3862–3869.
Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T., & Kobata, A. (1981) *J. Biol. Chem. 256*, 8476–8484.