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A MOUSE SUBMANDIBULAR SIALOMUCIN CONTAINING BOTH *N*- AND *O*-GLYCOSYLIC LINKAGES

PATRICIA A. DENNY AND PAUL C. DENNY
Department of Basic Sciences, School of Dentistry, University of Southern California, Los Angeles, CA 90089-0641 (U.S.A.)

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ABSTRACT

A sialomucin from mouse submandibular glands was treated with mild base–Me_2SO. This treatment cleaves *O*-glycosylically linked oligosaccharides, but preserves the integrity of the protein core. After treatment with mild base–Me_2SO, 49.2% (by weight) of the oligosaccharides were removed from the polypeptide; they were composed of residues of 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, sialic acid, and D-galactose. These oligosaccharides were linked *O*-glycosylically *via* 2-acetamido-2-deoxy-D-galactose. Chromatography of the base–Me_2SO-treated mucin on Sephacryl S-300 indicated that the protein core, with its base-resistant oligosaccharides, is a single, high-molecular-weight species. The mild-base-resistant linkages remaining on the protein core (50.8% of the total carbohydrates by weight) also contained D-mannose. The presence of these mild-base-resistant linkages, and the formation of 2-acetamido-2-deoxy-D-glucitol following treatment with M NaOH–M $NaBH_4$, confirmed the presence of *N*-glycosylic linkages.

INTRODUCTION

The salivary-mucus glycoproteins from mammals share similar chemical compositions. The linkage of the oligosaccharides to the protein core in salivary mucins has been characterized as a glycosidic linkage involving 2-acetamido-2-deoxy-D-galactose to the 3-hydroxyl groups of seryl and threonyl residues[1,2]. The β-elimination reaction has been routinely used to establish this type of linkage.

We have isolated, and electrophoretically purified to apparent homogeneity[3], a sialomucin from female-mouse submandibular glands. The mouse-submandibular sialomucin differs from most other salivary mucins by the presence of D-mannose and the higher proportion of 2-acetamido-2-deoxy-D-glucose than of 2-acetamido-2-deoxy-D-galactose.

The presence of small proportions of D-mannose is unusual in mucus glycoproteins[1]. Traditionally, D-mannose has been associated with *N*-glycosylically linked oligosaccharides in which 2-acetamido-2-deoxy-D-glucose is attached to asparagine.

This *N*-glycosylic linkage is not susceptible to the mildly basic conditions necessary to cleave the glycosidic bond[4].

The *β*-elimination reaction of Downs *et al.*[5], involving treatment with mild base–dimethyl sulfoxide, yields no diffusible protein upon dialysis. This procedure seems a particularly valuable approach for studies of the protein core and of linkages that are resistant to treatment by mild base.

The high proportion of 2-acetamido-2-deoxy-D-glucose, and the presence of D-mannose in the mouse submandibular sialomucin, prompted us to investigate the structure of this glycoprotein. Utilizing the method of Downs *et al.*[5], we present evidence for glycosidically linked oligosaccharides, free from D-mannose, and also for a base-resistant linkage. Upon further study, this base-resistant linkage appeared to be *N*-glycosylic in nature.

EXPERIMENTAL

Preparation of material. — Electrophoretically pure sialomucin was prepared from the submandibular glands of female mice. The sialomucin was isolated by a previously reported procedure[3] involving chromatography on Sephacryl S-200, acidic dialysis, and preparative, poly(acrylamide)-gel electrophoresis.

Radioactive labeling of the sialomucin. — The sialomucin was radioactively labeled by reductive alkylation, either with ^{14}C-formaldehyde[6] or sodium boro-(^{3}H)hydride[7]. This procedure labels the 6-amino group of lysine in the protein core. The specific activity of the ^{14}C-labeled mucin was 1.43 nCi/μg, and 86.4 nCi/μg for the ^{3}H-labeled mucin. Both labeled mucins co-migrate with unlabeled sialomucin in analytical, poly(acrylamide)-gel electrophoresis and molecular-exclusion chromatography.

Base treatment in dimethyl sulfoxide (Me_2SO). — A lyophilized sample containing 450 μg of sialomucin, and a small amount of radiolabeled mucin for quantitation of recoveries, was dissolved in Me_2SO and aqueous KOH to give a final concentration[5] of 1:1 (v/v) Me_2SO and 0.17M KOH. Following a 2-h incubation at 45°, the base was neutralized with 0.5M HCl, and the sample was applied directly to a column (0.9 × 48 cm) of Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ 08854). Fractions were eluted with water at room temperature, at a flow rate of 6.5 mL/h. Those fractions containing both radioactivity and sialic acid at the void volume were determined and pooled (protein core), and those containing sialic acid only (chains) were combined. Both solutions (of the core and the chains) were lyophilized to dryness, and their corresponding carbohydrates were identified and quantitated. Amino acids from the core were also quantitated.

Base treatment and reduction. — Native mucin was treated[8] with 0.05M KOH–M sodium borohydride ($NaBH_4$) for 15 h at 45°, to preserve the glycosidically linked sugar as the alditol for quantitation. The sample was made neutral, and desalted on a column (0.9 × 26 cm) of Sephadex G-25. All fractions containing sialic acid were pooled, and lyophilized to dryness.

The lyophilized, protein-core fraction from the column of Sephadex G-50 was dissolved in M NaOH–M $NaBH_4$, to cleave the base-resistant linkage[9,10]. The core material was incubated for 6 h at 100°, cooled in ice, the base neutralized with glacial acetic acid, and the neutral sample applied directly to the column of Sephadex G-25 previously described. All fractions between the void and the included volumes were pooled, and lyophilized to dryness. It was necessary to *N*-reacetylate the reduced oligosaccharides prior to methanolysis[11]. The methyl glycosides were *N*-reacetylated, per(trimethylsilyl)ated, and the products analyzed by g.l.c. Hexosaminitol standards were prepared by treating *N*-acetylated amino sugars with 0.05M NaOH–M $NaBH_4$ for 1 h at 45° [8]. The standards were made neutral, and the boric acid was removed by several additions and distillations of methanol[12]. The standards were then treated by the routine methods employed for g.l.c. analysis.

Analytical methods. — Preparation of sugars for, and analysis by, g.l.c. were conducted by the method described previously[3]. Additionally, a column of 3% of OV-17 on Gas Chrom Q was used to separate 2-acetamido-2-deoxy-D-glucitol from 2-acetamido-2-deoxy-D-glucose[10]. Amino sugars in the core were additionally quantitated with an amino acid analyzer. The hexosamines prepared for the latter method were hydrolyzed with 4M HCl for 6 h at 100°.

Amino acids in the core fraction were analyzed as before[3], except that samples were hydrolyzed for 24 h only.

Sialic acid was determined fluorometrically by a modification of the method of Hof *et al.*[13], using *N*-acetylneuraminic acid as the standard.

The integrity of the core fraction was examined by chromatography on Sephacryl S-300 (Pharmacia). Both the ^{14}C-labeled core and the ^{3}H-labeled, native mucin were applied to a column (1.5 × 86 cm) equilibrated with 0.1M potassium phosphate (pH 7.5)–2.0M guanidine · HCl. Fractions were eluted at room temperature, at a flow rate of 27 mL/h. Radioactivity was quantitated by liquid scintillation spectrometry.

RESULTS

Base treatment in Me_2SO. — The elution profile of the glycoprotein (that had been treated with base–Me_2SO) when chromatographed on Sephadex G-50 is shown in Fig. 1. There is no significant breakdown of the core after a 2-h treatment with base–Me_2SO, but there is partial removal of sialic acid. Treatment times of 5 h yielded slightly degraded, core units, as evidenced by the redistribution of 19% of the radiolabel into the column elution-volume.

When the mucin treated for 2 h was applied to a column of Sephacryl S-300 (see Fig. 2), the protein core was eluted as a compact peak behind that for the untreated, native mucin. This indicates that the core fraction contains a single, high-molecular-weight species, diminished in size by the removal of carbohydrates. Other methods of base treatment to cleave glycosidically linked, carbohydrate chains were attempted[8,14], but these failed to preserve the integrity of the protein core.

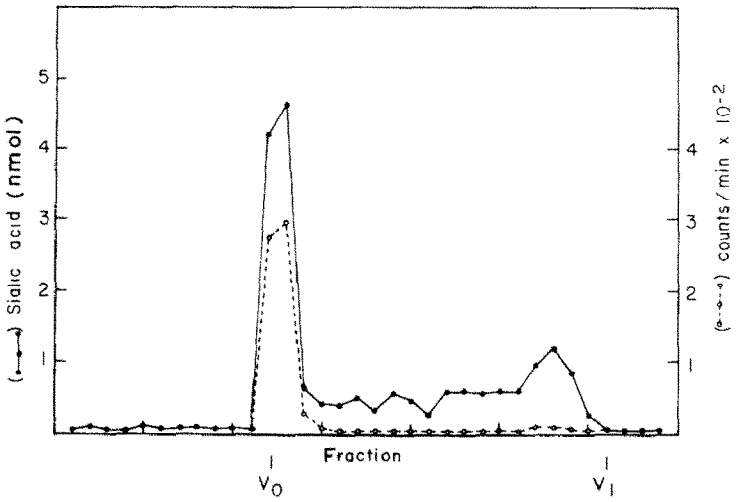

Fig. 1. Sephadex G-50 chromatography of mucin following 2-h, base–Me_2SO treatment. [Sialic acid, which is expressed as nmol per fraction, is shown by the solid line, and ^{14}C (in counts per min) is given by the broken line. The fractions containing both the radioactive peak and the major sialic acid peak in the void volume (V_0) were those pooled for the protein-core fraction. Those fractions subsequent to the void volume and containing sialic acid, were combined for the chain fraction. The included volume is indicated by V_I.]

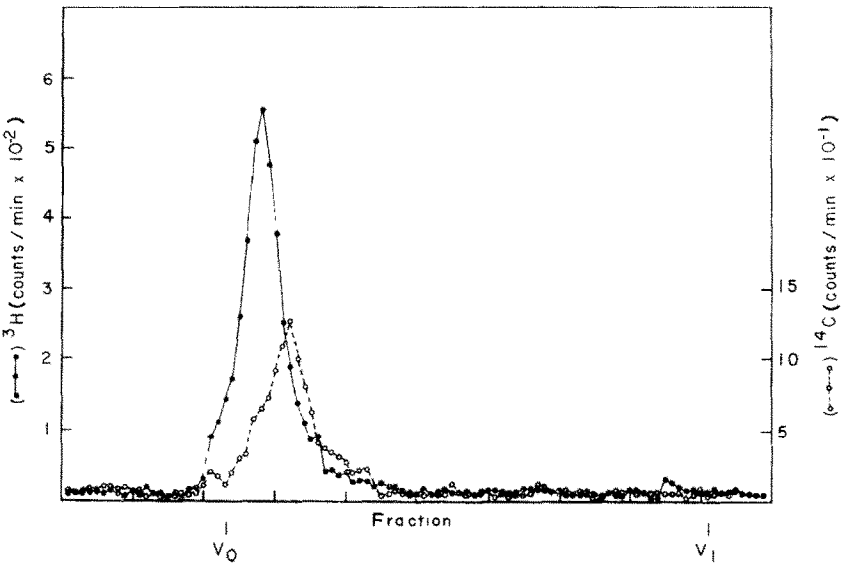

Fig. 2. Chromatography of mucin on Sephacryl S-300 following 2-h, base–Me_2SO treatment. [Untreated, ^3H-labeled, native mucin is expressed as counts per min, and is given by the solid line. Mucin, which was treated for 2 h with base–Me_2SO, was ^{14}C-labeled, and is indicated by the broken line. The void volume is expressed as V_0, and the included volume, by V_I.]

TABLE I

CARBOHYDRATE COMPOSITION OF NATIVE MUCIN, AND THE RESULTING FRACTIONS SUBSEQUENT TO THE β-ELIMINATION REACTION[a,b]

Component	*Native mucin*	*Conditions of Downs* et al.[5], *and G-50 chromatography*	
		Chains	*Protein core*
D-Galactose[c]	26.2	31.5	31.1
D-Mannose[c]	9.6	0	20.8
Sialic acid[c,d]	28.4	30.4	15.0
GlcNAc[c,d]	20.4[e]	15.6	24.9[e]
GalNAc[c,d]	15.4[e]	22.5[f]	8.2[e]

[a]Experiment repeated 3 additional times. [b]The values are reported in mol/100 mol of carbohydrate. [c]Determined as methyl glycosides by g.l.c. [d]Expressed as the *N*-acetyl derivative. [e]Value confirmed by the amino acid analyzer. [f]The value for GalNAc was corrected for decomposition that occurred during the β-elimination reaction.

The treatment of the mouse sialomucin with base–Me_2SO for 2 h results in substantial loss of 2-acetamido-2-deoxy-D-galactose. Porcine sialomucin contains only *O*-glycosylic linkages. For comparison, the porcine mucin was subjected to the same conditions of treatment with base–Me_2SO. A similar loss of 2-acetamido-2-deoxy-D-galactose was observed. The quantity of this amino sugar lost corresponded approximately to the number of *O*-glycosylic linkages in the porcine mucin. There was, however, no recovery of identifiable derivatives from the missing 2-acetamido-2-deoxy-D-galactose. The carbohydrate chains released from the mouse submandibular sialomucin by treatment with base–Me_2SO, and isolated by chromatography on Sephadex G-50, were subsequently reduced[8] with 0.05M KOH–M $NaBH_4$ in an attempt to recover the linkage hexosamine as its alditol, but no identifiable product was formed. Sodium borohydride was also used concurrently with base–Me_2SO in an attempt to reduce the linkage hexosamine immediately. This procedure afforded numerous by-products (g.l.c.); however, we were unable to duplicate any of the by-products following a variety of treatments of hexosamine standards.

Carbohydrate chains. — The proportion of oligosaccharides removed from the polypeptide after base–Me_2SO treatment was 49.2% by weight (see Table I). These base-labile chains were composed of residues of D-galactose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, and sialic acid. Whereas D-mannose is found in the native mucin, it was absent from the chains released by the base–Me_2SO treatment. D-Galactose and sialic acid are abundant in the chains. The ratio of 2-acetamido-2-deoxy-D-glucose to 2-acetamido-2-deoxy-D-galactose has been reversed. In the native mucin, this ratio is 1.33:1, whereas, in the chains, the ratio has decreased to 0.69:1, and this indicates an enrichment of 2-acetamido-2-deoxy-D-galactose in the chains.

TABLE II

CHANGES IN COMPOSITION FOLLOWING BASE TREATMENTS[a,b]

Component	*Native mucin*		*Protein core*		
	Untreated	*Conditions of Carlson*[8]	*Conditions of Downs* et al.[5]	*Loss*	*Conditions of Lee and Scocca*[9]
Threonine[c]	28.9		15.1	13.8	
Serine[c]	9.3		3.1	6.2	
GalNAc[c,d,e]	31.6	18.9	8.5	23.1	
GalNAc-OH[d,e,f]		12.7			
GlcNAc[c,d,e]	41.9		27.9	14.0[g]	23.1
GlcNAc-OH[d,e,f]					4.5

[a]The values are reported in mol/100 mol of native amino acids. [b]Unless indicated, all values represent recoveries. [c]Determined by amino acid analysis. [d]Expressed as the *N*-acetyl derivative. [e]Determined by g.l.c., following methanolysis. [f]Abbreviations: GalNAc-OH, 2-acetamido-2-deoxy-D-galactitol; GlcNAc-OH, 2-acetamido-2-deoxy-D-glucitol. [g]Recovered with *O*-glycosylic oligosaccharides.

Protein core. — Nearly 51% of the total weight of the carbohydrates remained attached to the core (see Table I). The mild-base-resistant carbohydrates remaining on the core after the 2-h, base–Me_2SO treatment were D-galactose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, sialic acid, and D-mannose. The value for D-galactose is similar to the value in the chains, whereas sialic acid is diminished two-fold. The presence of D-mannose is exclusive to the core oligosaccharides. The ratio of 2-acetamido-2-deoxy-D-glucose to 2-acetamido-2-deoxy-D-galactose in the core fraction is dramatically enhanced to over four-fold the ratio in the chains, namely, 3.03:1.

The amino acid analysis of the core was in good agreement with that of the native mucin, except for a 48% diminution in threonine and a 67% diminution in serine (see Table II). The core additionally shows a 73% diminution in 2-acetamido-2-deoxy-D-galactose, as revealed both by g.l.c. and by amino acid analysis. A 5-h, base–Me_2SO treatment showed no additional decomposition of this hexosamine in the core.

Nature of the linkages. — The mild-base-lability of linkages to the protein core indicates the presence of *O*-glycosylic bonds. The loss of 2-acetamido-2-deoxy-D-galactose and the concomitant diminution in serine and threonine corroborate the presence of this linkage[15]. There is good agreement between the loss of serine and threonine (20.0 mol/100 mol of amino acids) and the diminution of 2-acetamido-2-deoxy-D-galactose (23.1 mol/100 mol of amino acids; see Table II). In addition, there is a 55% conversion of available 2-acetamido-2-deoxy-D-galactose into 2-acetamido-2-deoxy-D-galactitol following the reductive, β-elimination reaction of native mucin by the method of Carlson[8]. These results confirm the presence, in the mouse sub-

mandibular sialomucin, of an *O*-glycosylic linkage which involves attachment of carbohydrate chains, free from D-mannose, by "reducing-terminal" 2-acetamido-2-deoxy-D-galactosyl groups to the 3-hydroxyl groups of seryl and threonyl residues.

Following base–Me_2SO treatment, the core fraction was treated[9] with M NaOH–M $NaBH_4$; this produced a peak that, in g.l.c., emerged at the same location as 2-acetamido-2-deoxy-D-glucitol. Quantitation of this peak indicated that ~4.5 mol were formed per 100 mol of amino acids (see Table II). The presence of mild-base-resistant oligosaccharides remaining on the core following base–Me_2SO treatment, and the resultant 2-acetamido-2-deoxy-D-glucitol following the strong base–borohydride treatment, substantiate the presence of *N*-glycosylic linkages involving attachment of 2-acetamido-2-deoxy-D-glucose to asparagine.

DISCUSSION

The mouse submandibular sialomucin is composed of two classes of oligosaccharide, both linked onto the same protein core. The treatment with base–Me_2SO preserved the integrity of the protein core, and provided the basis for identification of two types of oligosaccharide. One oligosaccharide fraction is mild-base-labile and is glycosidically linked. The other chain is high in content of D-mannose, and resistant to mild treatment with base; this is characteristic of a *N*-glycosylic linkage.

Chromatography of the Me_2SO-treated glycoprotein on Sephadex G-50 yielded the protein core-fraction, which was eluted in the void volume, and the glycosidically linked oligosaccharides, which were eluted subsequent to the void volume. We observed a direct relationship, in the *O*-glycosylic oligosaccharides, between an increased retention time on the G-50 column and an increased, relative concentration of sialic acid. Free sialic acid applied to G-50 under the same conditions is eluted after the included volume; this indicates a probable interaction of sialic acid with the G-50 gel. Such an interaction may explain the observed fractionation of oligosaccharides.

The 2-acetamido-2-deoxy-D-galactose content of the protein core was decreased by 73%, but none of it was recovered in the oligosaccharides removed by base–Me_2SO treatment. This suggests that 73% of the total 2-acetamido-2-deoxy-D-galactose residues in the glycoprotein are involved as the linkage sugar. The 73% loss of 2-acetamido-2-deoxy-D-galactose, or 23.1 mol/100 mol of amino acids, is consistent with the diminution in (serine plus threonine) of 20.0 mol/100 mol of amino acids in the protein core as there is a direct relationship between the loss of the amino sugar and the loss of the amino acids involved in the *O*-glycosylic linkage following the β-elimination reaction[15]. Furthermore, the observation that a 5-h, Me_2SO treatment failed to increase the decomposition of 2-acetamido-2-deoxy-D-galactose suggests that 23.1 glycosidic linkages/100 amino acid residues constitutes the maximum.

The presence of the glycosidic linkage was verified by the formation of 2-acetamido-2-deoxy-D-galactitol following treatment[8] of the glycoprotein with 0.05M KOH–M $NaBH_4$. Only 55% of the available 73% of 2-acetamido-2-deoxy-D-galactose

was converted into the alditol, supporting the contention that this procedure may not quantitatively cleave the bond[4]. This is consistent with the finding that the rate of the β-elimination reaction is dependent upon the concentration of the base, rather than on that of the borohydride[16].

The decomposition of serine in the β-elimination reaction proceeds faster than that of threonine[17,18]. In support of this, we found that the amount of serine decomposed was the same in treatment with Me_2SO for one hour as it was in two hours. More than twice as much threonine was, however, decomposed in the samples treated for 2 h than in those treated for 1 h.

When the protein core was chromatographed on Sephacryl S-300, it migrated as a single peak. Because the core possesses mild-base-resistant oligosaccharides composed of D-mannose, D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, and sialic acid, it seemed reasonable to suspect the presence of a *N*-glycosylic linkage. The method of Lee and Scocca[9] is useful for determining the linkage of 2-acetamido-2-deoxy-D-glucose to asparagine. Although the core contained some 2-acetamido-2-deoxy-D-galactose, no 2-acetamido-2-deoxy-D-galactitol was formed following treatment with M NaOH–M $NaBH_4$. Thus, the remaining 2-acetamido-2-deoxy-D-galactose residues are probably situated within the chains, and do not serve as linkage sugars. There were 4.5 mol of 2-acetamido-2-deoxy-D-glucitol formed per 100 mol of amino acids. This value is likely to be an underestimate, and it probably represents only a 50–70% yield of 2-acetamido-2-deoxy-D-glucitol[10]. A 70% yield would increase the value to 7 mol per 100 mol of amino acids. We previously reported[3] that there are 6.75 $\pm$0.31 mol of aspartate or asparagine per 100 mol of amino acids; this suggests that every asparaginyl residue has an oligosaccharide *N*-glycosylically linked *via* 2-acetamido-2-deoxy-D-glucose.

It has been reported[19] that the strongly basic conditions used in the Lee and Scocca[9] procedure cause *N*-deacetylation. The presence of a *N*-acetyl group increases the susceptibility of a *N*-glycosylic linkage to acid hydrolysis or methanolysis[19]. A re-*N*-acetylation step was, therefore, necessary prior to methanolysis. Baenziger *et al.*[10] reported that the bond between asparagine and 2-acetamido-2-deoxy-D-glucose resists methanolysis, and thus, must be acid-hydrolyzed. Our preparation for examination in the amino acid analyzer had been acid-hydrolyzed, whereas the material used for g.l.c. analysis had been methanolyzed, and the results of the two methods of quantification for this amino sugar agreed precisely.

D-Mannose was never associated with *O*-glycosylic oligosaccharides released after base–Me_2SO treatment of the mouse mucin; instead, D-mannose remained with the protein core. That D-mannose was present in a contaminating glycoprotein, in *N*-glycosylic linkages, seems improbable. The purity of the salivary mucin was assessed previously[3] by analytical and sodium dodecyl sulfate–poly(acrylamide)-gel electrophoresis, isoelectric focusing, immunoelectrophoresis, and immunodiffusion. Now, an additional indication of purity has been demonstrated by chromatography in guanidine hydrochloride on Sephacryl S-300, before and after base–Me_2SO treat-

ment. In both cases, only a single peak resulted. It seems unfeasible that a contaminant would co-migrate under such a variety of conditions.

Other investigators[12] have isolated from the rat sublingual gland a sialomucin which also contains D-mannose. This mucin from the rat sublingual gland was treated by the method of Carlson[8] in order to release the *O*-glycosylic linkage to the protein core[20]. D-Mannose was not detected in the *O*-glycosylic carbohydrate chains, but it remained with the portion of the treated material that was unable to pass through a dialysis bag. The presence of this nondialyzable D-mannose, and the higher proportion of 2-acetamido-2-deoxy-D-glucose in the rat sublingual mucin, suggests that the D-mannose could be a component of *N*-glycosylically linked oligosaccharides.

The presence of both linkages may not be limited to rodent salivary mucins. In studies with the bovine submandibular sialomucin, base–Me_2SO treatment indicated that 10–15% of the oligosaccharides are base-resistant[5]. The authors[5] suggested that the resistant linkages may be *N*-glycosylic, as such a bond is as resistant to mild base as the peptide bond itself.

The presence of *N*- and *O*-linked glycosyl residues on a single polypeptide is not unknown. Human IgA_1 (ref. 21) and human thyroglobulin[22] are glycoproteins that contain both types of linkage. However, the female-mouse submandibular sialomucin is the first salivary mucin for which both linkages have been confirmed. All asparaginyl residues are *N*-glycosylically linked, whereas only 52% of the total seryl and threonyl residues are involved in the *O*-glycosylic linkages.

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