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STUDIES OF MUCOPROTEINS

IX. ON THE SUSCEPTIBILITY TO ALKALI AND TO HYDROXYLAMINE OF THE PREDOMINANT CARBOHYDRATE–PEPTIDE LINKAGE IN OVINE-SUBMAXILLARY-GLAND GLYCOPROTEIN

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SUMMARY

Treatment of ovine-submaxillary-gland glycoprotein (OSM) with 0.1 N NaOH at 100° released about 89% of the carbohydrate groups (*N*-acetylneuraminyl (α,2 → 6)*N*-acetylgalactosamine; NANA → GalNHAc) according to first-order reaction kinetics; $k = 2.37 \cdot 10^{-1}$ min^{-1} (half-time 2.9 min). The remaining prosthetic groups were liberated much more slowly; the first-order rate constant was $1.89 \cdot 10^{-3}$ min^{-1}. The carbohydrate–peptide linkages were also cleaved by NH_2OH at pH 12.2 and 37° in a pseudo-monomolecular reaction, $k = 2.3 \cdot 10^{-3}$ min^{-1}. The alkaline conditions on their own released the carbohydrate groups at a lower rate, $k = 0.6 \cdot 10^{-3}$ min^{-1}. The disaccharides liberated were recovered quantitatively, mainly in form of their oxime which had protected them against destruction by alkali. Concomitant with the release of the disaccharides, hydroxamates were formed. Removal of the terminal negatively-charged *N*-acetylneuraminic acid (NANA) residues from OSM prior to NH_2OH-treatment considerably increased the reaction rates: $k_{NH_2O^-} = 7 \cdot 10^{-3}$ min^{-1}, $k_{OH^-} = 1.6 \cdot 10^{-3}$ min^{-1}. When about 89% of the carbohydrate groups had been liberated, the reaction was practically completed. OSM displayed no reducing power when tested with *o*-dinitrobenzene or Benedict's reagent under controlled conditions using GalNHAc as standard.

The data presented provide further proof for the previous conclusion that the bulk of the disaccharide groups in OSM are joined through a glycosidic–ester linkage to the peptide. 11% of the groups are apparently linked in another fashion; the possibility of an *O*-glycosidic linkage to serine residues for this fraction is discussed. The amide-*N* of OSM is low, 2.6 μmoles/100 mg OSM. It was shown that crystalline NANA, as was previously found for bound NANA, liberates NH_3 under the acid conditions used in the amide determination of proteins.

Abbreviations: OSM and BSM, ovine- and bovine-submaxillary-gland glycoprotein respectively; NANA, *N*-acetylneuraminic acid; NANA → GalNHAc, *N*-acetylneuraminyl (α,2 → 6)-*N*-acetylgalactosamine.

INTRODUCTION

In a recent article[1] we have summarized the evidence showing that in glycoproteins, defined as conjugated proteins containing one or more heterosaccharide residues of relatively low degree of polymerization and lacking a repeating unit, the heterosaccharide is bound covalently to the functional group of the side-chain of a component amino acid residue. In glycoproteins of animal origin two types of carbohydrate–peptide linkage have been reported so far. In ovalbumin a single heterosaccharide, consisting of 3 *N*-acetylglucosamine and 5 mannose residues, is linked most likely *N*-glycosidically to an asparaginyl residue; involved in the linkage are the hemiacetal OH-group of acetylglucosamine and the amide amino group. Since neither an enzyme nor a chemical reagent is available which acts specifically on this type of linkage, its presence is inferred mainly from the analysis of short glycopeptides, obtained by enzymic digestion of ovalbumin, and from a comparison of the chemical reactivity of these glycopeptides with model compounds of known structure (for literature see ref. 1). YAMASHINA AND MAKINO[2] have recently prepared from ovalbumin, by pronase[3] action followed by partial hydrolysis and dinitrophenolation of the resulting amino acid-carbohydrate fragment, a compound yielding on acid hydrolysis DNP-aspartic acid, ammonia and glucosamine in the molar ratio 1.00:1.13:1.07. Since the compound was non-reducing and moved towards the anode on electrophoresis, it conformed to the aspartylglycosylamine structure $O=\overset{R}{\overset{|}{C}}—NH\cdot R'$ (most probably *N*-(L-β-aspartyl)-2-deoxy-2-acetamido-glucopyranosylamine), first proposed by JOHANSEN, MARSHALL AND NEUBERGER[4], though the Japanese authors favoured the structure of the isomeric imido ester, $R'\cdot O—\overset{R}{\overset{|}{C}}=NH$.

In glycoproteins prepared from the submaxillary glands of ox and sheep a more direct approach to the elucidation of the carbohydrate–peptide link was possible because in this case the linkage proved to be susceptible to a chemical reagent acting specifically on one type of bond only. OSM, as prepared in our laboratory, has an average molecular weight, for various preparations studied, of $(1.0 \pm 0.2)\cdot 10^6$. About 40% of the molecule is carbohydrate composed nearly exclusively of equimolar amounts of *N*-acetylneuraminic acid and *N*-acetylgalactosamine. Structural analysis revealed these components to be present as the simple disaccharide *N*-acetylneuraminyl (2 → 6) *N*-acetylgalactosamine[5], several hundreds of which are distributed along the polypeptide chain[1]. Treatment of OSM with lithium borohydride (in tetrahydrofuran), a reagent known to effect selectively a reductive cleavage of esters, decreased the recoverable dicarboxylic acids by 64.1 μmoles (*i.e.* by 88 % of the total 72.7 μmoles of dicarboxylic acids) and the protein-bound disaccharides by 63.5 and 64.8 μmoles respectively, depending on the disaccharide component assayed (*i.e.* by about 80 % of the total 80 μmoles of disaccharides), all figures referring to 100 mg OSM. Of the released disaccharides only 31.6 % of theory were recovered when assayed by the orcinol method[6]. On treatment of OSM with 0.01 N $Ba(OH)_2$ at 80° for 4 h 81.5 % of the disaccharide residues were released from the protein[6].

Hydroxylamine is another chemical reacting with esters, though not as selectively as lithium borohydride. In the present paper the susceptibility of the carbohydrate–peptide linkage in OSM to hydroxylamine was tested by determining the decrease

of the peptide-bound disaccharide groups and by analysing quantitatively and qualitatively the released groups. The formation of hydroxamic acids in this reaction was also investigated. Since hydroxylamine reacts not only with esters but also with acid amides, an exact knowledge of the amide content of OSM was essential. We have, therefore, re-determined the amide nitrogen of OSM after complete removal of the interfering NANA, applying the method of JOHANSEN, MARSHALL AND NEUBERGER[7]. In connection with this determination we have examined the liberation of ammonia from crystalline NANA on acid treatment. Finally the kinetics of the decrease of peptide-bound disaccharide residues on treating OSM with 0.1 N NaOH at 100° were studied, and some work was done on the cleavage of peptide bonds in OSM by 0.05 N NaOH at 100°.

EXPERIMENTAL

Materials

The sugars used were as described previously[5,8].

Glucose oxime (m.p. 140–140.5° uncorr.) was prepared according to WOHL[9] and recrystallized according to WOLFROM AND THOMPSON[10].

Crystalline pyrrole-2-carboxylic acid was obtained by the method of ODDO[11].

Phosphotungstic acid was purified by the procedure of VAN SLYKE AND RIEBEN[12].

Hydroxylamine hydrochloride was a Fluka A.G. preparation.

Sephadex G25 was a product of Pharmacia, Uppsala, Sweden.

Cellulose casings (18/32) for dialysis were obtained from the Visking Corporation, Chicago.

Resins used were Dowex-50-X4 (50–100 mesh) and Dowex-1-X2 (50–100 mesh).

OSM was prepared according to the procedure described for BSM by MURPHY AND GOTTSCHALK[13].

Crystalline neuraminidase (EC 3.2.1.18) from *Vibrio cholerae* culture filtrates was generously supplied by Dr. G. L. ADA, The Walter and Eliza Hall Institute of Medical Research, Melbourne.

Methods

Measurements of pH were made with a Radiometer type TTT1b equipped with type-GK-2021B glass electrode.

N-Acetylneuraminic acid was determined by the resorcinol method of SVENNERHOLM[14] using crystalline NANA as standard.

Hexosamine was determined according to RONDLE AND MORGAN[15]. The carbonate–bicarbonate buffer of IMMERS AND VASSEUR[16] was used in this procedure (see STRANGE[17]). Prior to assay the material was hydrolysed with 4 N HCl at 100° for 10 h in sealed tubes, unless otherwise stated; the hydrolysate was dried *in vacuo*. Sodium chloride, present in some samples at a final concentration of 0.05 M, had no effect on the colour formation, as shown in appropriate controls.

Amino acid determinations were carried out after hydrolysis of the protein with 6 N HCl for 24 h at 105° using the ninhydrin reagent of MOORE AND STEIN[18]; DL-leucine was the standard.

Ammonia was estimated after precipitation with phosphotungstic acid according to JOHANSEN, MARSHALL AND NEUBERGER[7] unless otherwise stated.

Gel filtration: Sephadex-G25 was allowed to swell in 0.05 M NaCl and the fines

were removed by repeated decantation. The suspension was slurried into the column and a large volume of 0.05 M ammonium bicarbonate was passed through the gel bed. All filtrations were carried out in this bicarbonate solution. OSM was recovered from the column in 98.5 % yield.

Column fractions were tested for protein or polypeptide by the modified Folin method[19], for sialic acid by the resorcinol method[14], for anhydro *N*-acetylhexosamine derivatives[8] and pyrrole-2-carboxylic acid by the Ehrlich reagent in the cold[8], and for free hydroxylamine by the Ni^{2+}–diacetylmonoxime-spot test[20].

Reduction of sugars with $NaBH_4$ was performed in 0.1 M borate buffer (pH 9.0) at 24° for 6–7 h. A twice molar excess of $NaBH_4$ (in same borate buffer) was added each hour. Under these conditions *N*-acetylglucosamine and GalNHAc were no longer detectable by the Morgan–Elson reaction[21] after 5 h. Excess reagent was decomposed by dilute HCl and the borate removed by repeated evaporation with methanol.

OSM and NANA —> GalNHAc respectively were treated with neuraminidase in 0.005 M Ca^{2+} at pH 5.5 and 35° for 20 h. 20000 Units of the enzyme were used for 50–100 mg OSM and 20 mg of the disaccharide respectively[22].

Paper chromatography: Whatman No. 1 paper was used with the solvents (a) *n*-butanol–pyridine–water (6:4:3, v/v), (b) *n*-propanol – pyridine – water (6:4:3, v/v), (c) *n*-butanol – acetic acid – water (12:3:5, v/v). Location reagents for sugars were periodate–benzidine[23], Elson–Morgan and Morgan–Elson reagents[24] and aniline hydrogen phthalate[25]. For amino compounds the ninhydrin reagent was used.

High-voltage paper electrophoresis was carried out at pH 3.7 in acetic acid–pyridine buffer, 35 V/cm, 2 h, 20° according to KATZ, DREYER AND ANFINSON[26].

Alkali treatment of OSM:

(a) For a kinetic study of the decrease in prosthetic groups on mild alkali treatment the following procedure was adopted: 1.0 ml of a solution containing 100 mg dried OSM in 15 ml distilled water and adjusted to pH 7 was filled into each of a set of test tubes. To these tubes, preheated in a boiling-water bath to $>95°$, was added 1.0 ml each of 0.2 N NaOH, also preheated to $>95°$. The tubes were kept for specified times in a boiling-water bath; after withdrawal they were immediately cooled in a dry ice–acetone mixture, quickly acidified to pH 4–5 by adding a predetermined volume of 4 N HCl and allowed to freeze. When all samples had been processed in this way, they were thawed, adjusted to pH 7–8 with 1 N NaOH and submitted to treatment with $NaBH_4$ in borate buffer as described. The samples were then neutralized and, after addition of conc. HCl to give a final concentration of 4 N HCl, they were hydrolysed at 100° for 10 h and analysed for hexosamine. A control tube containing a mixture of 1.0 ml OSM solution and 1.0 ml of 0.2 N NaOH, both not preheated, was immediately acidified to pH 4–5 and further treated as above.

(b) The susceptibility to mild alkali of peptide linkages in OSM was tested as follows: A solution of about 20 mg OSM in 20 ml of 0.05 N NaOH was heated at 100° for 30 min, ice-cooled, neutralized with 5 N acetic acid and dialysed against three changes of 200 ml water for 72 h. The retentate was lyophilized and weighed; the dialysates were dried in a rotatory evaporator below 30°. A solution of about 10 mg OSM in 10 ml of 0.05 N sodium acetate was dialysed and further processed as above. The dried retentates and dialysates were each dissolved in 10 ml water and aliquots were analysed for NANA, hexosamine and amino acids, the latter two after

hydrolysis of the aliquots in sealed tubes at 104° with 4 N HCl for 10 h and with 6 N HCl for 24 h respectively. One aliquot of the dialysate was treated with $NaBH_4$ prior to acid hydrolysis for hexosamine determination.

Hydroxylamine treatment of OSM: An alkaline solution of hydroxylamine was prepared by adjusting 1 vol. of 4 M $NH_2OH \cdot HCl$ solution to pH 12.2 with 20 N NaOH and diluting to 2 vol. with water. A solution of OSM was prepared by gently stirring the lyophilized material in water until dissolved and centrifuging the solution at 28000 × *g* at 4° for 20 min. A mixture of 10 ml of OSM solution (about 2 mg OSM/ml) and 13 ml of alkaline hydroxylamine solution was adjusted to pH 12.2 and made up to a volume of 26 ml. The mixture was maintained at pH 12.2 and 37°, occasional additions of alkali being necessary. Samples of 5.0 ml were withdrawn at intervals, adjusted to pH 7 with glacial acetic acid and either frozen until processed or applied directly to a Sephadex-G25 column (2.3 × 35 cm, void volume 50 ml). Fractions of 5.0 ml were collected automatically and tested for protein, NANA, hydroxylamine and sodium ions. Two NANA peaks were obtained; the first peak, also containing protein, comprised Fractions 10–17 (pool A), the second peak comprised Fractions 18–25 (pool B). Hydroxylamine and most of the sodium ions emerged from the column after the second peak. Pool A was analysed for hexosamine and NANA. Pool B was assayed for NANA and submitted to paper chromatography and high-voltage paper electrophoresis. In control experiments hydroxylamine was omitted.

A complete NANA and hexosamine balance of NH_2OH-treated OSM was carried out in 9-h experiments. For this purpose 50 mg OSM, dissolved in 8 ml water, was mixed with 8 ml of an alkaline solution of hydroxylamine prepared from $NH_2OH \cdot HCl$ and NaOH. This mixture, molar with respect to NH_2OH, was adjusted to pH 12.2. 5 ml of the mixture was immediately neutralized and frozen. The remaining mixture was kept for 9 h at pH 12.2 and 37°; 5-ml samples of this solution, after neutralization, and the frozen sample were then fractionated separately at 4° on a Sephadex-G25 column as described. The resulting pools A and B were both analysed for NANA and hexosamine. Pool B was also examined paper-chromatographically and submitted to high-voltage paper electrophoresis.

To exclude any salt effect on the NH_2OH reaction, OSM was also treated with a solution of NH_2OH prepared from $(NH_2OH)_2 \cdot H_2SO_4$ and an equivalent amount of $Ba(OH)_2$. After removal of the precipitate the NH_2OH content of the solution was determined according to YASHPHE, HALPERN AND GROSSOWICZ[27]. The elution pattern from the Sephadex column is shown in Fig. 1.

Appropriate reference compounds for chromatography of the sugars released from OSM by the action of NH_2OH were prepared as follows. Glucose, GalNHAc and NANA → GalNHAc were each treated for 3 h with alkaline NH_2OH solution under the conditions described for OSM (in the presence of 1 M NaCl; pH 12.2). The resulting solution was processed as above and finally passed over a Dowex-50 (H^+ form) column. Chromatography of the Dowex-50 effluents in Solvent b and location with periodate–benzidine reagent showed for each sugar derivative a spot at an R_F value higher than that of the untreated sugar. The spot was initially white but quickly turned yellow. The ratio of the R_F value of the NH_2OH-treated sugar to that of the untreated sugar was 1.2 for glucose, 1.10 for GalNHAc and 1.15 for NANA → GalNHAc. The same result was obtained when the sugars were treated with a salt-free solution of NH_2OH (see preceding paragraph) at pH 8.5 for 2 h at 25° followed by

drying *in vacuo*. The product thus obtained could be used directly for chromatography. In the same solvent system authentic glucose oxime showed a spot of $R_{glucose} = 1.2$.

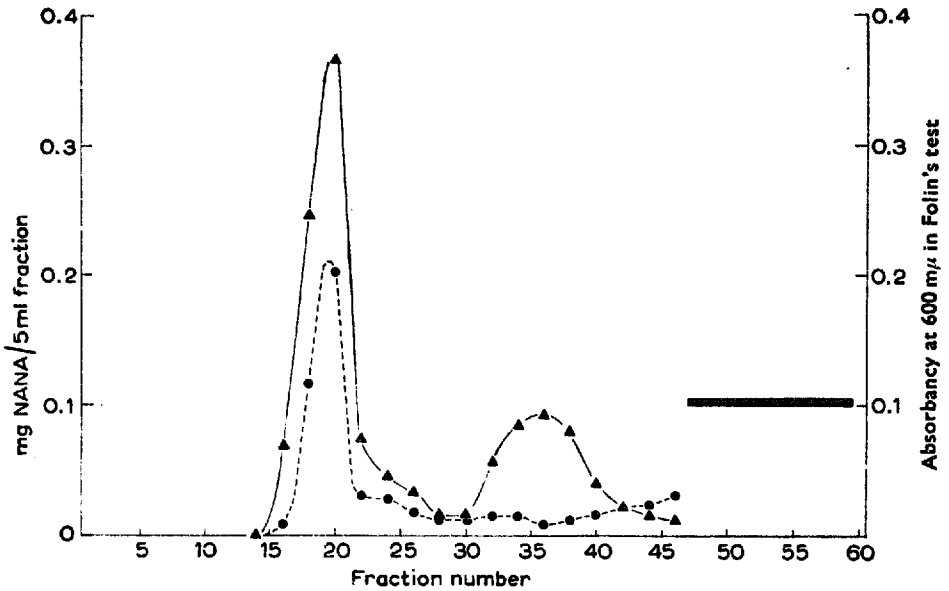

Fig. 1. Elution pattern from a Sephadex-G25 column (38 × 3 cm) of a 5-ml sample of NH_2OH-treated OSM (0.45 M NH_2OH; pH 12.2; 37°; 9 h). Elution carried out with 0.05 M NH_4HCO_3 at 4°. Fractions of 5 ml were collected. ▲—▲, NANA content in mg/5 ml fraction; ●—●, absorbancy at 600 mμ in the Folin test (0.1 ml of fraction in final volume of 2.85 ml). The bar denotes fractions containing NH_2OH.

In Solvent c the NH_2OH-treated and the original sugar had the same R_F value. The products resulting from NH_2OH-treatment of GalNHAc and NANA —> GalNHAc could not be crystallized. However, the identical behaviour in paper chromatography and in the distinct colour formation with periodate–benzidine of crystalline glucose oxime and of the compound resulting from NH_2OH-treatment of glucose as described above and the similar behaviour in these assays of NH_2OH-treated GalNHAc and NANA —> GalNHAc make it very probable that the products were indeed oximes. It may be mentioned that glucose oxime prepared under mild alkaline conditions gave in Solvent b in addition to the main spot a weak spot of lower R_F trailing to the main spot. On eluting and rechromatographing the material moved to the position of the main spot.

Tests for hydroxamic acids in NH_2OH-treated OSM

(1) An aliquot of pool A was treated with $FeCl_3$ according to SCHWEET[28]. The resulting reddish solution was examined spectrophotometrically in a Beckman Model DU spectrophotometer.

(2) 1-ml aliquots of Pool A were submitted to the procedure described by YASHPHE, HALPERN AND GROSSOWICZ[27]. In this procedure the hydroxamates are oxidized with iodine to nitrite which in turn is allowed to react with sulfanilic acid. The resulting diazonium compound is coupled with α-naphthylamine to give a coloured derivative. Oxidation with iodine at pH's 2.3 and 3.7 allows differentiation between free NH_2OH and free NH_2OH plus hydroxamates. Spectrophotometric readings are made at 530 mμ.

RESULTS

Determination of amide-N in OSM

Step 1: 100 mg dried OSM was dissolved in 16.5 ml of 0.005 M $CaCl_2$ solution. To 10 ml of this solution were added 10000 units of crystalline *Vibrio cholerae* neuraminidase. After incubation of the mixture at 37° for 8 h in the presence of toluene another 10000 units of the enzyme were added and digestion continued for 18 h. The digest, after addition of 1.1 ml 1 N H_2SO_4, was heated in a water bath at 80° for 50 min and then ice-cooled. This procedure resulted in the removal of >98% of the total sialic acid. Since it was found in model experiments with DL-asparagine and L-glutamine that even under these mild acid conditions more than 10% of the amide-*N* is liberated as NH_3, 1.1 ml of the above hydrolysate was analysed directly, *i.e.* without any further acid hydrolysis, for NH_3 according to JOHANSEN *et al.*[7].

Step 2: The remainder was dialysed against 2 l of distilled water in the presence of toluene at 2° for 24 h. The cellophane bag was emptied quantitatively and concentrated in a freeze-dryer to a volume of 8.5 ml. 1-ml aliquots were hydrolysed in 1 N HCl (final concentration) at 100° for 3 h. A blank was kept under same acidic conditions at 20° for 30 min. Hydrolysates and blank were dried at 4° in the vacuum of a desiccator and redissolved in 2.0 ml water. Precipitation of ammonia by phosphotungstic acid and steam-distillation at alkaline pH of the ammonia contained in the precipitate were carried out according to JOHANSEN *et al.*

Analytical results: NH_3-N released in Step 1: 4.2 μg/100 mg OSM (corrected for blank). NH_3-N released in Step 2: 32.4 μg/100 mg OSM (corrected for blank). Total amide-N = 36.6 μg/100 mg OSM = 2.6 μmoles/100 mg OSM = 0.32% of total N.

Formation of ammonia from N-acetylneuraminic acid on treatment with mineral acid

0.5 mg crystalline NANA was heated in 1.0 ml 1 N HCl at 100° for various periods of time. After cooling the mixture was analysed for NH_3 at 4° by the microdiffusion method of TRACEY[29]. The results obtained are shown in Table I.

TABLE I

AMMONIA* PRODUCED FROM NANA BY 1 N HCl AT 100°

Time of hydrolysis (h)	*NH_3-N in per cent of total N of NANA*		
	1	2	3
1	13.8	—	12.5
1.5	18.0	19.5	—
2	19.7	21.4	18.1
3	22.8	22.7	20.7
5	—	24.7	22.8

* Measured by microdiffusion at 4° (TRACEY[29]).

Kinetics of the decrease in protein-bound hexosamine on mild alkaline treatment of OSM

In preliminary experiments it was observed that treatment of OSM with 0.1 and 0.2 N NaOH for 15 and 10 min respectively at 100° resulted in a decrease of bound hexosamine (taken as indicator for bound disaccharide residues) by about 88% of the total. The residual hexosamine was lost at a much lower rate. This indicated, in

agreement with earlier findings[6], that a minor fraction of the disaccharides was bound to the protein in a type of linkage more resistant to mild alkali than the bulk of the disaccharides. To obtain an exact estimate of this minor fraction, the decrease of hexosamine content of OSM on treatment with 0.1 and 0.2 N NaOH respectively at 100° was determined in intervals over a period of 120 min (for details see *Methods*).

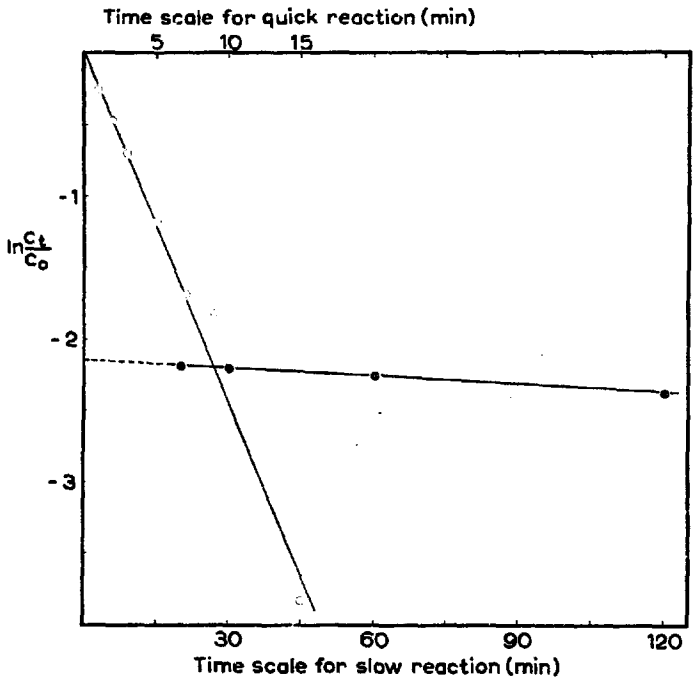

Fig. 2. Decrease with time of protein-bound hexosamine of OSM on treatment with 0.1 N NaOH at 100°. ●—●, slow reaction; O—O, quick reaction (corrected for 11% of bound hexosamine reacting very slowly; see text).

When linear regressions were fitted to the experimental figures of the slow reaction by the method of least squares and estimates made of the intercepts with the ordinate, values of −2.14282 and −2.25758 were obtained for the reactions with 0.1 and 0.2 N NaOH respectively. These values corresponded to 11.7% and 10.5% respectively of the total hexosamine. Since theoretically the intercepts should coincide, a mean value of 11% was taken for that fraction of total hexosamine which was very slowly released from the protein on mild alkali treatment. When the experimental figures of c_0 and c_t in the quick reaction (0.1 N NaOH) were corrected for these 11%, the straight line shown in Fig. 2 was obtained. The first order rate constant, calculated from the first 7 min of the reaction, was $k = 2.37 \cdot 10^{-1}$/min. The error introduced by neglecting the decrease of hexosamine due to the slow reaction is not appreciable. The first-order rate constants for the slow reaction were found to be $1.89 \cdot 10^{-3}$/min in 0.1 N NaOH and $3.35 \cdot 10^{-3}$/min in 0.2 N NaOH at 100°.

Fragmentation of OSM by 0.05 N NaOH

When 21.7 mg of dried OSM was heated in 20 ml of 0.05 N NaOH at 100° for 30 min and then processed as described under *Methods*, it was found that 9.5% of the original hexosamine and 9.9% of the original NANA content of OSM did not pass through the cellophane membrane. In the dialysate 3.5% of the total hexosamine

was recovered, 70% of which was not reducible by $NaBH_4$ *i.e.* was still bound to a protein fragment. Only 1.2% of the hexosamine released survived the impact of alkali with an unimpaired reducing group. The resorcinol assay registered a recovery of 28% of the total NANA; however, alkali-degradation products of NANA are known to contribute to the colour measured in the assay[30].

TABLE II

QUANTITATION OF DIALYSABLE PEPTIDES AFTER ALKALI TREATMENT OF OSM

OSM, heated in 0.05 N NaOH at 100° for 30 min, was dialysed. Results are expressed as μmoles per 100 mg dried OSM.

Ninhydrin value* of hydrolysed** native OSM	574
Correction for 5.6% proline content of OSM	+49
Correction for contribution of hexosamine, present in the hydrolysate**, to the ninhydrin value	−62
Amino acids in native OSM	561
Ninhydrin value of hydrolysed** dialysate, obtained from alkali-treated OSM	182
Correction for proline assuming 1/3 of total proline to be present in the dialysate	+16
Correction for contribution of hexosamine, present in the dialysate	−2
Amino acids in dialysate	196 = 34.9% of total

* DL-Leucine was used as standard.
** Hydrolysis conditions: 6 N HCl, 104°, 24 h.

The degree of fragmentation of the polypeptide chain(s) of OSM, as assessed by dialysability of the fragments resulting from the above alkali treatment, is shown in Table II. Contributing to the ninhydrin values of the acid hydrolysates of both the original OSM and the dialysate from the alkali-treated OSM is the ammonia liberated from NANA during acid hydrolysis. Since, however, the proportion of material reacting with resorcinol is similar in both samples the final result may not be affected significantly. Two corrections applied in Table II involve assumptions lacking experimental evidence. First, it was assumed that the amino groups of the 17% hexosamine destroyed when OSM, for amino acid analysis, was hydrolysed with 6 N HCl at 104° for 24 h, did not contribute to the ninhydrin value. Second, one-third of the proline content of OSM was assumed to be present in the dialysate. Because of these uncertainties the balance was checked by weight determination. When the dry weight of the non-dialysable glycopeptides (9.3 mg) was corrected for the weight of their NANA and GalNHAc content (0.74 mg), it was found that 66% of the protein moiety of the original OSM was retained inside the cellophane bag, *i.e.* 34% was lost on dialysis. In this calculation the only assumption was that alkali treatment of OSM had removed half of the acetyl groups of the disaccharide residues. Any error in this assumption would but slightly affect the final result since the carbohydrate content of the non-dialysable fraction is only 7.9%.

Chromatography (Solvent a) of the hydrolysate of the non-dialysable fraction revealed galactosamine as the major component reacting with the Elson–Morgan

reagent. Galactosamine in trace amounts was also seen when the hydrolysate of the dialysable material was chromatographed.

Test for reducing power of OSM

The following solutions were prepared and tested: A, OSM solution with a GalNHAc content of 1.9 μmoles/ml; B GalNHAc solution containing 2.1 μmoles/ml; C, mixture of equal parts of A and B.

0.1-ml samples of A, B and C were mixed with 0.2 ml of NaOH-KCl buffer (pH 12.2) and one drop of a saturated solution of *o*-dinitrobenzene in ethanol. The mixtures were heated in a boiling-water bath. A gave no colour in 60 sec and only a very faint colour after 2 min. B and C gave a pink colour within 30 sec becoming intense at 45 sec. Benedict's reagent, diluted 1:5, was reduced (in a boiling-water bath) by B in 3 min; A did not reduce even in 4 min.

Kinetics of the decrease in protein-bound prosthetic groups on treatment of OSM with NH_2OH at pH 12.2

In preliminary experiments OSM was treated with 1 M NH_2OH at pH 8 and 37° (see ref. 31), but no appreciable release of prosthetic groups was observed. At pH 12.2 and 37° the reaction proceeded at measurable rate and these conditions were used in subsequent experiments. Under similar conditions in the absence of NH_2OH, prosthetic groups were also released from OSM, but at a lower rate.

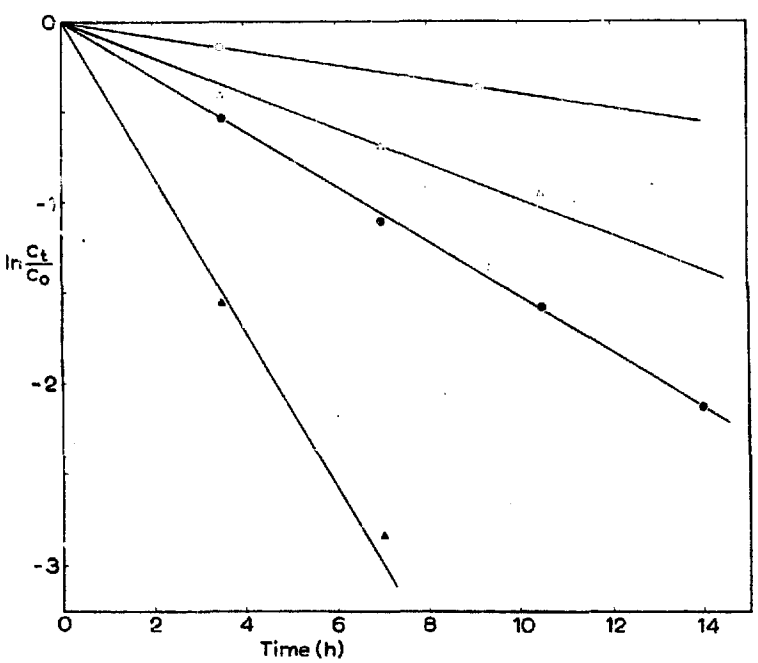

Fig. 3. Decrease with time of protein-bound hexosamine of OSM at pH 12.2 and 37° (○—○); at pH 12.2 and 37° in the presence of 1 M NH_2OH (●—●); NANA-free OSM at pH 12.2 and 37° (△—△); NANa-free OSM at pH 12.2 and 37° in the presence of 1 M NH_2OH (▲—▲).

For an estimate of the rate constant of the decrease in protein-bound prosthetic groups determination of the hexosamine content of the protein fraction (see *Methods*) gave consistent results. Fig. 3 shows the decrease with time of the protein-bound hexosamine on treatment of OSM at pH 12.2 and 37° in the presence and absence of

NH_2OH. Since the alkali experiments described in the preceding section had shown that 11 % of the prosthetic groups are bound to the protein through a linkage fairly resistant to 0.2 N NaOH at 100°, it was assumed that NH_2OH at pH 12.2 and 37° would not act on this linkage (see below). The experimental hexosamine values were, therefore, corrected for these 11 %. The straight lines in Fig. 3 indicate first-order reaction kinetics for the NH_2OH reaction and the control reaction in alkali alone. The first-order rate constants computed from separate experiments were found to be $k_{NH_2O^-} = 2.3 \cdot 10^{-3}$/min and $k_{OH^-} = 0.6 \cdot 10^{-3}$/min.

In the NH_2OH experiments the sum total of the NANA content of the protein fraction (pool A, see *Methods*) and of the released prosthetic groups (pool B) at any time interval tested, closely agreed with the NANA content at 0 h, *i.e.* there was a near quantitative recovery of the released sialic acid (see Table III). In some experiments more NANA than hexosamine was found in pool B probably due to cleavage of NANA residues from the protein fraction during processing.

TABLE III

RECOVERY OF NANA AFTER TREATMENT OF OSM WITH NH_2OH AND FRACTIONATION ON SEPHADEX-G25*

Conditions: 1 M NH_2OH, pH 12.2, 37°.

Time (h)	*μmoles NANA/5 ml sample*		
	Pool A	*Pool B*	*Total*
0	2.72	0	2.72
3.5	1.62	1.14	2.76
7.0	0.99	1.88	2.87
10.5	0.67	2.20	2.87
14.0	0.37	2.42	2.79

* For technical details see *Methods*.

In order to eliminate a possible charge effect of the NANA residues on the NH_2OH reaction, some experiments with NANA-free OSM were performed. A solution of neuraminidase-treated OSM containing about 2 mg glycoprotein/ml was adjusted to pH 1.5 with HCl, kept for 1 h at 70° and dialysed exhaustively. Sialic acid assay showed removal of >97 % of the NANA of OSM. The remaining glycoprotein was treated at pH 12.2 and 37° with and without 1 M NH_2OH as described above for native OSM. The results are included in Fig. 3. Again first-order reaction kinetics obtained, $k_{NH_2O^-} = 7 \cdot 10^{-3}$/min and $k_{OH^-} = 1.6 \cdot 10^{-3}$/min. The hexosamine remaining bound to the protein after 7 h and 14 h treatment with NH_2OH was 15.8 % and 11.3 % respectively of the original. These figures seem to support the above assumption that about 11 % of the prosthetic groups of OSM are bound to the polypeptide through a type of linkage resistant to NH_2OH.

To ascertain that the material of pool B (see *Methods*) was in fact prosthetic group and not glycopeptide, identification of the carbohydrate by chromatography and electrophoresis was undertaken. Preliminary paper chromatograms of pool B of NH_2OH-treated OSM revealed that the prosthetic group was present mainly as the oxime; a minor portion coincided in R_F value with the unsubstituted prosthetic group. Sugar oximes can be easily distinguished from glycopeptides by their charac-

teristic colour reaction with periodate–benzidine (see *Methods*). The differentiation between free prosthetic groups and glycopeptides would have to be based mainly on R_F values since a positive Morgan–Elson reaction may not be restricted to a free prosthetic group (NANA → GalNHAc) only. It is quite conceivable that the alkali treatment at 100° required for this reaction would split the ester linkage in a glycopeptide. For this reason any free disaccharides were converted prior to paper chromatography, to their oximes under conditions (0.3 M NH_2OH, pH 8, 25°, 2–3 h) known not to release prosthetic groups from OSM (see above). Chromatography of the vacuum-dried material in Solvent b showed an intense yellow spot coincident in R_F value (0.40) with the oxime of the disaccharide. In addition a spot of slightly lower R_F value was observed, very weak after 3 h treatment of OSM with NH_2OH, but intensifying with time of treatment. NANA determinations on the eluates of the two spots in an 9-h experiment (see below) registered 89 % of the NANA content of the applied material in the main spot and 12 % in the weak spot. A similar chromatogram was obtained with Solvent c.

High-voltage paper electrophoresis of pool B showed an intense spot (periodate–benzidine reagent) moving towards the anode with a mobility of 0.85 of that of a NANA standard. A weak spot moving slowly from the origin towards the cathode was also seen and, as in the chromatogram, increased in intensity with time of NH_2OH treatment.

The intensification of the minor spot with time of NH_2OH action together with the behaviour of this material in the electric field would be consistent with deacetylation of an acetamido group, as present in the disaccharide, producing a zwitterion. This interpretation is supported by the further observation that application of pool B (9 h treatment with NH_2OH) on a Dowex-50 (H^+ form) column resulted in the retention of 11 % of the material as assessed by NANA determination. The retention would indicate the presence of a charged amino group.

Further evidence for the identification of the material of pool B as prosthetic group was obtained by neuraminidase action on this material. Chromatography of the neuraminidase-treated material revealed two spots of comparable intensity coinciding with NANA and the oxime of GalNHAc respectively. There was an additional but faint spot coinciding with galactosamine standard.

When at the later stages of the NH_2OH-treatment peptide material was traced[32] in pool B by paper chromatography, it did not react with periodate–benzidine.

Carbohydrate balance of OSM after treatment with NH_2OH at pH 12.2

In several experiments a complete carbohydrate balance of OSM before and after 9 h treatment with NH_2OH was made. Table IV summarizes the results of two representative experiments, one with 1 M NH_2OH in the presence of 1 M NaCl, the other with 0.45 M NH_2OH in the absence of inorganic salts (see *Methods*).

Demonstration of the presence of hydroxamate in NH_2OH-treated OSM

The presence of hydroxamate was demonstrated by two independent methods:

(1) Pool A gave with $FeCl_3$ a red colour with absorption maximum at 505 mμ. Butyl acetate when treated with 1 M NH_2OH at pH 12.2 and 37° for 15 min gave with $FeCl_3$ a similar colour with an absorption peak at 505–510 mμ.

(2) The absorption spectrum obtained by application of Method 2 (see *Methods*)

to pool A coincided with that of a standard prepared from sodium nitrite accordingly. In several experiments the hydroxamate determined corresponded to 42–53 % of that expected from the disaccharide content of pool B.

TABLE IV

CARBOHYDRATE BALANCE OF OSM AFTER HYDROXYLAMINE TREATMENT (pH 12.2 AND 37°) AND FRACTIONATION ON SEPHADEX-G25*

Time (*h*)	NH_2OH (*M*)	*μmoles NANA/5 ml sample*			*μmoles hexosamine/5 ml sample*		
		Pool A	*Pool B*	*Total*	*Pool A*	*Pool B*	*Total*
0	1.0	10.90	0	10.90	11.04	0	11.04
9		3.84	6.92	10.76	4.40	6.86	11.26
0	0.45	11.04	0	11.04	10.50	0	10.50
9		6.88	4.09	10.97	6.97	4.04	11.01

* For technical details see *Methods*.

DISCUSSION

The present work confirmed by two independent approaches previous results and the conclusion that in OSM the multiple prosthetic groups of the structure *N*-acetylneuraminyl (2 → 6) *N*-acetylgalactosamine are linked to the polypeptide chain by two different types of linkage. The majority of the prosthetic groups is joined through an *O*-glycosidic-ester type of linkage to the non-peptide bonded carboxyl groups of aspartyl as well as glutamyl residues. The evidence for the ester-type of linkage is the following: (a) $LiBH_4$-treatment of OSM resulted in the loss of 88% of the total 72.7 μmoles of monoamino-dicarboxylic acids per 100 mg OSM (molar ratio of aspartyl to glutamyl residues in OSM is 3.1:4.2). An equimolar quantity of prosthetic groups, representing about 80 % of the total was released concomitantly[6]. (b) Treatment of OSM with NH_2OH at pH 12.2 and 37° resulted in the release of prosthetic groups according to first-order reaction kinetics concomitant with the formation of hydroxamates. The released disaccharides were quantitatively recovered. Even after removal from OSM of the terminal NANA units the negative charge of which depressed the reaction rate, NH_2OH did not remove more than 88.7 % of the protein-bound hexosamine. (c) On treatment of OSM with 0.1 N NaOH at 100° two types of reactions were discernible: a fast reaction involving the removal of 89 % of the disaccharide residues and proceeding according to first-order reaction kinetics ($k = 2.37 \cdot 10^{-1}$/min; half-time 2.9 min); a slow reaction involving the residual disaccharide residues also proceeding according to first-order reaction kinetics ($k = 1.89 \cdot 10^{-3}$/min; half-time 367 min). The evidence for the engagement of the hemiacetyl group of GalNHAc in the ester linkage is (a) the failure of OSM to reduce *o*-dinitrobenzene in alkali and Benedict's reagent under standard conditions; (b) the previous observation that after $LiBH_4$-treatment of OSM the recovered disaccharides had their reducing groups intact, *i.e.* these groups were shielded against excess reducing agent by their linkage to boron in the intermediate boron complex[1].

The quantitative recovery of the disaccharides released on NH_2OH-treatment, even when 63 % of their total was liberated, was most linkely due to oxime formation of the released disaccharides. Oximes are more alkali-stable than the corresponding

unsubstituted sugars. Though the oximes of the disaccharides were not obtained crystalline, the conditions to which the released disaccharides were exposed for many hours and the close similarity in chromatographic behaviour and colour formation with periodate–benzidine between the disaccharide derivative and an authentic oxime leave little doubt on their identity. The incompleteness of oxime formation in the NH_2OH experiments would indicate that a fraction of the released disaccharides was not long enough exposed to NH_2OH for oxime formation to take place, a further argument in favour of a glycosidic linkage.

The main effect of alkali in the NH_2OH reaction seems to be the withdrawal of a proton from NH_2OH, *i.e.* $NH_2OH \rightleftharpoons NH_2O^- + H^+$ (see ref. 33). The specific effect of NH_2OH on the release of the prosthetic groups is seen from a comparison of the first-order rate constants of the reactions at pH 12.2 and 37° in the presence and absence of NH_2OH: $k_{NH_2O^-} = 2.3 \cdot 10^{-3}$/min and $k_{OH^-} = 0.6 \cdot 10^{-3}$/min. The difference in the two reaction rates becomes even more marked after removal of the terminal negatively charged NANA residues: $k_{NH_2O^-} = 7 \cdot 10^{-3}$/min and $k_{OH^-} = 1.6 \cdot 10^{-3}$/min.

In agreement with the mechanism of NH_2OH action on esters, NH_2OH-treatment of OSM resulted in the formation of hydroxamates. Their presence in the protein fraction was demonstrated by their colour reaction with $FeCl_3$ and by the production of nitrite when the protein fraction was oxidized with iodine. A quantitative correlation between the prosthetic groups released and hydroxamates formed could not be expected because the NH_2OH treatment of OSM had to be carried out at pH 12. It is known[31] that hydroxamates of amino acids undergo hydrolysis at pH values above 9. GALLOP *et al.*[34] observed that 30 % of the hydroxamate initially formed from the γ-methyl ester of poly-L-glutamic acid by hydroxylamidation was lost on subsequent dialysis at pH > 10 due to hydrolysis. In the case of hydroxamate formed from polyanhydroaspartic acid (polysuccinimide) the loss, due to hydrolysis on subsequent dialysis at pH 9 5, was even 66 %. In view of this experience a recovery of hydroxamate in NH_2OH-treated OSM to the extent of 50 % of theory appears to be reasonable, Though NH_2OH acts also on acid amides, an *N*-glycosidic linkage of the disaccharides to asparaginyl or glutaminyl (see INTRODUCTION) can be ruled out. The amide-*N* of OSM was found to be 2.6 μmoles as compared with 80 μmoles of disaccharides/100 mg OSM.

The quantitative recovery of the disaccharides liberated from OSM by NH_2OH contrasts with a recovery of only 32 % of the disaccharides detached from OSM on treatment for 10 h with $LiBH_4$ in tetrahydrofuran. It seems likely that during the long period of refluxing at the boiling point of the solvent (65°) some decomposition of the boron intermediate complex takes place liberating free prosthetic groups. Such a decomposition may be effected by an alkaline medium generated by the reaction of $LiBH_4$ with traces of water, unavoidably introduced into the system by OSM. Once liberated, the disaccharides will be destroyed with time. Thus it was observed that of NANA → GalNHAc, when submitted to the same treatment, only 42.7 % and 35.2 % were recovered (assayed by NANA determination) after 5 h and 10 h respectively[13].

The presence of a second type of linkage between carbohydrate and protein in OSM is suggested by the abrupt change in the rate of decrease in protein-bound hexosamine on mild alkali treatment when about 89 % of the total hexosamine has been released (Fig. 2) and by the completion of the hydroxylamine reaction with

OSM when about the same amount of hexosamine has been removed. $LiBH_4$ released only 80 % of the prosthetic groups; however, in this case incomplete solubility of the material in tetrahydrofuran may have been a limiting factor. The most probable type of linkage of this minor fraction of prosthetic groups is an *O*-glycosidic linkage to serine (and threonine). Such a linkage would be resistant to NH_2OH, but would be cleaved slowly in alkali by the mechanism of β-elimination (WITKOP[35], JONES *et al.*[36], HARTLEY AND JEVONS[37]). More direct evidence for the presence of this type of linkage was provided when alkali-pretreated OSM was submitted to hydrazinolysis and the resulting mixture of hydrazides was fractionated by preparative electrophoresis followed by application to coupled columns of Dowex-50 (H^+ form) and Dowex-1 (formate form). On elution of the latter column with 0.4 N formic acid a glycopeptide was obtained yielding on acid hydrolysis glycine, serine, alanine, galactosamine and valine in the molar ratios 1:1:3:1:1 (MURPHY[38]). The analysis strongly suggests that in this glycopeptide GalNHAc was attached to serine by an *O*-glycosidic linkage resistant to pretreatment of OSM with 0.01 N NaOH (80°, 4 h) and with anhydrous hydrazine (100°, 4 h). The galactosamine recovered in this glycopeptide amounted to about 15–20 % of the hydroxylamine-resistant portion (see above).

The re-determination of the amide-N of OSM after complete removal of NANA and employing the phosphotungstic acid method of JOHANSEN *et al.*[7] confirmed our earlier results[39]. It is noteworthy that crystalline NANA when submitted to the conditions of amide hydrolysis by HCl loses 22 % of its N as NH_3. SPIRO AND SPIRO[40] reported a value of 25 % under stronger acidic conditions. When NANA is bound in glycoproteins, acid hydrolysis for amide cleavage converts a larger proportion of NANA nitrogen to NH_3 as calculated from published data: 42 % in OSM (see ref. 39), 54 % in BSM (see ref. 13), 58 % in orosomucoid[41] and in fetuin[40]. The reason for this differential behaviour will be discussed elsewhere[42].

The evaluation of the experiments concerned with the cleavage of peptide bonds in OSM on treatment with 0.05 N NaOH at 100° for 30 min is difficult since no comparable data for carbohydrate-free proteins are available and since the passage of peptides and glycopeptides through a cellophane membrane is determined by many factors, size being only one of them. It seems, however, to be the experience of protein chemists that fragmentation of considerable degree occurs when proteins are treated, for instance, with 0.2 N NaOH at 100° for 2 h. WARNER[43] found that heating ovalbumin in 0.28 N NaOH at 68° for 3 h resulted in the formation of 12 % free amino groups referred to the maximum amount attainable under standard conditions of acid hydrolysis, *i.e.* under these mild alkaline conditions one out of eight peptide bonds was split. GIBBONS AND ROBERTS[44] reported recently that treatment of bovine cervical glycoprotein with 0.05 N Na_2CO_3 at 100° for 20 min rendered half the material dialysable. A common feature of the submaxillary and cervical glycoproteins is their high content of α-amino-β-hydroxy amino acids[45]. One may expect such proteins to be very susceptible to alkali.

The susceptibility of most of the disaccharide–peptide linkages in OSM to $LiBH_4$, hydroxylamine and very mild alkali proves this linkage beyond reasonable doubt to be of the ester type. Based on our results with $LiBH_4$-treated BSM, as prepared in our laboratory, we concluded that also in this glycoprotein the majority of the disaccharides is bound through a glycosidic–ester linkage to aspartyl and glutamyl residues of the polypeptide. We do not wish to contradict the results from Pigman's

laboratory on the carbohydrate–peptide linkage in BSM since from a comparison of the composition and biological activity of his preparation and our material it is obvious that different species of glycoproteins produced by the same type of bovine glands have been submitted to analysis in the respective laboratories[46].

It is interesting that in glycoproteins of animal origin the monosaccharide unit linking the heterosaccharide to the polypeptide chain was found so far to be an *N*-acetylhexosamine residue, this was shown for BSM (see ref. 8), OSM (see ref. 5), ovalbumin[2,47], γ-globulin[48] and α_1-acid glycoprotein[49].

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