Action of Alkali on Bovine and Ovine Submaxillary Mucins*

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SUMMARY
Treatment of solutions of bovine and ovine submaxillary mucins with alkaline sodium borohydride at 45° for 10 hours converted essentially all of the carbohydrate chains to dialyzable materials. However, 17 to 25% of the hexosamine was not reduced to the hexosaminitol and probably was present as a glycopeptide.

The Hestrin hydroxylamine method for the determination of esters was applied to bovine, ovine, and porcine submaxillary mucins and to ovalbumin and serum α-glycoprotein. The only materials having ester content were preparations of bovine submaxillary mucin. The characteristics of the acyl function corresponded solely to that of the O-acetyl groups of the constituent sialic acids. After treatment of the bovine mucin with hydroxylamine, the constituent sialic acid could be almost completely removed by neuraminidase.

In earlier reports, Gottschalk, Murphy, and Graham (1, 2) provided evidence that the majority of the prosthetic groups of ovine submaxillary mucin and bovine submaxillary mucin (3) were bound to the protein core by an O-glycosidic ester linkage to the terminal carboxyl groups of aspartic and glutamic acids. After comparisons of the amino acid compositions of the mucins from different animals and of the glycopeptides formed by enzymatic degradation of bovine submaxillary mucin, Hashimoto, Tsuiki, Nisizawa, and Pigman (4, 5) demonstrated that the amount of dicarboxylic amino acids in bovine submaxillary mucin available for O-glycosidic ester linkage was far too small, and therefore suggested that the carbohydrate side chains were bound to the protein core by an O-glycosidic linkage to serine and threonine.

Recently, Harbon et al. (8) also reported similar evidence for ovine submaxillary mucin, and Anderson et al. (9), for the bovine submaxillary mucin and other products.

Recently, Bhavanandan et al. (10, 11) reported that the carbohydrate groups of ovine submaxillary mucin were bound to the protein core by O-glycosidic linkage to the hydroxyl group of serine and threonine. Evidence for the glycosidic ester linkage in the ovine submaxillary mucin could not be shown and was therefore excluded (12). The question of the O-glycosidic ester linkage in bovine submaxillary mucin remained to be elucidated. The purpose of the present investigation was to elucidate the question of the presence or absence of the O-glycosidic ester linkage in bovine and ovine submaxillary mucins and also to study the effect of alkali on ovine and bovine submaxillary mucins under more drastic conditions so that the carbohydrate side chains would be completely dialyzable.

EXPERIMENTAL PROCEDURE
Materials

BSM-P was prepared by the method described by Tsuiki, Hashimoto, and Pigman (13). BSM-0-85% was prepared in the same manner as BSM-P except that all of the mucin precipitating between 0 and 85% ethanol concentration was collected.

OSM-T and BSM-T were prepared according to a modified
method developed by Tettamanti and Pigman (14), involving treatment with hydroxyapatite. In the purification procedure of this method, major and minor mucin fractions are isolated.

PSM was prepared in this department by Dr. M. de Salegui (15), and BSM-G was prepared by Dr. V. L. N. Murty according to the procedure described by Gottschalk and Graham (16). OSM-C (17) was kindly supplied by Dr. D. Carlson (Western Reserve University). Neuraminidase from Vibrio cholerae was purchased from General Biochemicals and was supplied in 0.05 M acetate buffer, pH 5.5, containing 1% sodium chloride and 0.1% calcium chloride.

N-Acetylmuramic acid, galactosamine hydrochloride, and glucosamine hydrochloride were purchased from Calbiochem. Acetohydroxamic acid was kindly supplied by Dr. S. Seifter. Acetyl-α-D-glucopyranoside was purchased from Matheson, Coleman, and Bell and was used as the standard in the alkaline hydroxylamine reaction. The molar optical density values of the ferric hydroxamate chromogen from the glucose derivative did not differ significantly from the values obtained with acetohydroxamic acid.

Methods

Hexosamines were determined by the Elson-Morgan method as modified by Boas (18). Samples were hydrolyzed for 1 hour in 6 N HCl in sealed tubes at 100°. Standard hexosamine treated in the same manner resulted in no diminution of optical density values when compared with the unhydrolyzed standard. Sialic acid was determined by the resorcinol method of Svennerholm (19) with the modification of Miettinen and Takki-Neuraminidase at 37° for 22 hours at pH 5.5. After the incubation period, each sample was heated to 65° for 5 min, cooled in an ice bath, and ultrafiltered at 5°. The nonfilterable and filtrable portions were collected, made up to known volumes, and assayed for sialic acid.

Enzymatic Removal of Sialic Acid—A 5-mg portion of BSM-T, OSM-C, or de-O-acetylated BSM-T was treated with 500 units of neuraminidase at 37° for 22 hours at pH 5.5. Enzymatic treatment with hydroxylamine—A solution of 37.5 mg in 5 ml was treated for 5 min with 0.5 N NaOH at room temperature. The reaction mixture was adjusted to pH 6.4 and ultrafiltered at 4° in a cellulose dialysis sac approximately 6 mm in diameter. The ultrafiltration procedure, including washings, required approximately 48 hours. The filterable and nonfilterable portions were collected, made up to known volumes, and assayed for sialic acid.

RESULTS

Alkaline Hydrolysis at 45°—Figs. 1 and 2 show the rate of cleavage of the major components of BSM-P and OSM-T, respectively, at 45° in 0.1 N NaOH and 0.3 N NaBH₄. Max-
FIG. 1. The rate of cleavage of the main components of bovine submaxillary mucin by alkaline sodium borohydride at 45°C. N-Acetylneuraminic acid; N-acetylhexosamine; N-acetylgalactosamine was determined as free base according to the Elson-Morgan procedure and reported as N-acetylhexosamine by using the appropriate factor. "Before dialysis" signifies that the hexosamine analyses were performed on the total reaction mixture and reported as N-acetylhexosamine by using the appropriate factor.

FIG. 2. The rate of cleavage of the main components of ovine submaxillary mucin by alkaline sodium borohydride at 45°C. N-Acetylneuraminic acid; N-acetylhexosamine; N-acetylgalactosamine was determined as free base according to the Elson-Morgan procedure and reported as N-acetylhexosamine by using the appropriate factor. "Before dialysis" signifies that the hexosamine analyses were performed on the total reaction mixture and reported as N-acetylhexosamine by using the appropriate factor.

Minimum removal of the carbohydrate components was achieved after 10 hours of treatment with alkaline sodium borohydride. For each mucin the rates of release of N-acetylhexosamine and N-acetylneuraminic acid were approximately equal, and about 90 to 95% was made dialyzable. For BSM, however, only 75% of the total N-acetylhexosamine was apparently reduced by the sodium borohydride before dialysis in the total reaction mixture, as was shown by analysis of N-acetylhexosamine before dialysis (Fig. 1). Similarly, for OSM, only 83% of the N-acetylhexosamine was reduced in the total reaction mixture, although 90 to 95% was dialyzable. Analysis of the total reaction mixture of BSM and OSM after treatment with alkaline sodium borohydride for 14 hours and 12 hours, respectively indicated that 79% and 75% of the total serine and threonine were lost. The results suggest the formation of approximately 25% and 15% of dialyzable glycopeptide in BSM and OSM, respectively, unless some of the hexosamine residues are present in oligosaccharide side chains longer than disaccharides.

Determination of Esters—In Fig. 3 is shown the rate of formation of the ferric hydroxamate chromogen after exposure of several mucin samples to alkaline hydroxylamine for various lengths of time. Maximum color was formed within 2 min of exposure of all BSM samples to alkaline hydroxylamine at room temperature, with negligible increase in chromogen formation thereafter up to 60 min. For the PSM and OSM-T samples, no ferric hydroxamate chromogen could be detected. The amount of chromogen formed with the OSM-C sample was just at the limit of detection of the chromogen.

For each mucin the rates of release of N-acetylhexosamine and N-acetylneuraminic acid were approximately equal, and about 90 to 95% was made dialyzable. For BSM, however, only 75% of the total N-acetylhexosamine was apparently reduced by the sodium borohydride before dialysis in the total reaction mixture, as was shown by analysis of N-acetylhexosamine before dialysis (Fig. 1). Similarly, for OSM, only 83% of the N-acetylhexosamine was reduced in the total reaction mixture, although 90 to 95% was dialyzable. Analysis of the total reaction mixture of BSM and OSM after treatment with alkaline sodium borohydride for 14 hours and 12 hours, respectively indicated that 79% and 75% of the total serine and threonine were lost. The results suggest the formation of approximately 25% and 15% of dialyzable glycopeptide in BSM and OSM, respectively, unless some of the hexosamine residues are present in oligosaccharide side chains longer than disaccharides.

FIG. 3. The rate of ferric hydroxamate chromogen formation of mucin samples after treatment with alkaline hydroxylamine at room temperature. X-X, BSM-P and BSM-T; O-O, BSM-G; O-—O, OSM-G; O, OSM-C; O-—O, OSM-T. Two-milligram samples were used.

FIG. 4. The rate of ferric hydroxamate chromogen formation after treatment of the following samples with alkaline hydroxylamine at room temperature. X-X, orosomucoid; O-O, ovalbumin; C-C, ovomucoid. Two-milligram samples were used. Orosomucoid was generously supplied by Dr. Karl Schmidt, Boston University; ovalbumin was generously supplied by Dr. Leon Cunningham, Vanderbilt University.
submaxillary mucins and of porcine submaxillary mucin are given in Table I. It is interesting that BSM prepared according to the method of Gottschalk and Graham (16) contained the least amount of ester of the BSM samples assayed.

The nature of the acyl function from BSM was determined as follows. The various mucin samples were treated with alkaline hydroxylamine for 5 min at room temperature, and the ferric hydroxamate chromogen was developed with ferric chloride. An aliquot of the reaction mixture was placed on a Sephadex G-25 column as described above. The limit of detection of the ferric hydroxamate chromogen was developed with ferric chloride. An excess ferric chloride used in the development of the chromogen. The slower moving band was yellow and was apparently followed. The various mucin samples were treated with alkaline hydroxylamine for 5 min at room temperature, and the ferric hydroxamate chromogen was eluted with water, made up to a known volume, and spectrophotometrically compared with an aliquot of the original reaction mixture diluted to the same volume as the eluted band, the optical densities at 540 μm were essentially identical. These results were further substantiated by the fact that essentially all of the ferric hydroxamate chromogen of the various preparations of bovine submaxillary mucin after alkaline hydroxylamine treatment was found to be ultrafilterable, as shown in Table II. This behavior of the chromogen is consistent with an identification of the acyl group as the O-acetyl group of some of the sialic acid units in the original BSM preparations.

In contrast to the behavior of the hydroxylamine-treated BSM samples on Sephadex G-25 columns, the ferric hydroxamate chromogen derived from ethylated bovine serum albumin separated into two distinct bands on the Sephadex columns. The slower moving band was yellow and was apparently excess ferric chloride used in the development of the ferric hydroxamate chromogen. The faster moving band possessed the characteristic color of the ferric hydroxamate chromogen. When 0.1 M acetate buffer, pH 5.3, was used as the eluent, this band was observed to elute with the void volume. Bovine submaxillary mucin samples eluted with the same buffer after hydroxylamine treatment gave the same results on a Sephadex G-25 column as described above.

**Table I**

<table>
<thead>
<tr>
<th>Sample analyzed</th>
<th>Native Ultrafiltrable</th>
<th>Nonfiltrable</th>
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</thead>
<tbody>
<tr>
<td>BSM-P</td>
<td>89.0</td>
<td>0</td>
</tr>
<tr>
<td>BSM-T (major)</td>
<td>90.0</td>
<td>0</td>
</tr>
<tr>
<td>BSM-T (minor)</td>
<td>91.5</td>
<td>0</td>
</tr>
<tr>
<td>BSM-0-85%</td>
<td>75.0</td>
<td>0</td>
</tr>
<tr>
<td>BSM-G</td>
<td>27.5</td>
<td>0</td>
</tr>
<tr>
<td>OSM-C</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>OSM-T (major)</td>
<td>&lt;5.0</td>
<td>0</td>
</tr>
<tr>
<td>OSM-T (minor)</td>
<td>&lt;5.0</td>
<td>0</td>
</tr>
<tr>
<td>PSM</td>
<td>&lt;5.0</td>
<td>0</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>&lt;5.0</td>
<td>0</td>
</tr>
<tr>
<td>Ethylated bovine serum albumin</td>
<td>&lt;5.0</td>
<td>28.8</td>
</tr>
</tbody>
</table>

Enzymatic Removal of Sialic Acid—As shown above, the ester content (as O-acetyl) of BSM was 88 μmoles/100 mg of mucin and was negligible for OSM. Since BSM-T has 107 μmoles of sialic acid per 100 mg, the sum of O-acetyl and N-acetyl (including N-glycolyl) is 195 μmoles, and the O-acetyl is 41% of the total acetyl. This is in general agreement with the isolation work of Blix and Lindberg (28).

After BSM was treated with sodium hydroxide, analysis of the ultrafiltrable portion indicated that not more than 1% of the total liberated acyl groups was glycolic acid. These results are in agreement with those obtained by Faillard (29), who found that alkali did not cause a liberation of the glycolyl groups from BSM.

It was decided to test the effect of the removal of the O-acetyl groups from BSM on the extent of removal of sialic acid residues by neuraminidase. Gottschalk (30) had shown that they were only partially removed. The data of Table III shows that after treatment of BSM with neuraminidase and subsequent ultrafiltration, 41% of the original sialic acid was retained with the mucin and 49% was filtrable. The sialic acids of OSM and de-O-acetylated BSM were almost completely removed under these conditions. The latter two products had no measurable amounts of O-acetyl groups by the hydroxylamine method. Intact BSM-T, however, after enzyme treatment, had sialic acids with O-acetyl groups in both the ultrafiltrable and nonfiltrable fractions. The recoveries were 63% of the O-acetyl groups in the nonfiltrable and 29% in the filtrable fraction. Evidently, as

**Table II**

<table>
<thead>
<tr>
<th>Sample analyzed</th>
<th>Ultrafiltrable</th>
<th>Nonfiltrable</th>
<th>Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSM-P</td>
<td>87.5</td>
<td>88.0</td>
<td>0</td>
</tr>
<tr>
<td>BSM-T</td>
<td>58.0</td>
<td>81.0</td>
<td>0</td>
</tr>
<tr>
<td>BSM-G</td>
<td>97.5</td>
<td>27.5</td>
<td>0</td>
</tr>
<tr>
<td>BSM-0-85%</td>
<td>74.0</td>
<td>72.5</td>
<td>0</td>
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**Table III**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sialic acid</th>
<th>Recovered</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Nonfiltrable</td>
<td>Ultrafiltrable</td>
</tr>
<tr>
<td>BSM-T</td>
<td>40.8</td>
<td>40.2</td>
</tr>
<tr>
<td>OSM-C</td>
<td>2.4</td>
<td>90.6</td>
</tr>
<tr>
<td>De-O-acetylated BSM-T</td>
<td>5.0</td>
<td>85.0</td>
</tr>
</tbody>
</table>
TABLE IV  
N-Acetylhexosamine released from mucin samples by assay procedure of Morgan-Elson method as modified by Reissig et al. (23) and by Aminoff et al. (24)

<table>
<thead>
<tr>
<th>Sample</th>
<th>N-Acetylhexosamine released</th>
<th>Reissig modification</th>
<th>Aminoff modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMS-T</td>
<td>0</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>OSM-C</td>
<td>0</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>BSM-P</td>
<td>0</td>
<td>42.6</td>
<td></td>
</tr>
<tr>
<td>BSM-T</td>
<td>0</td>
<td>49.8</td>
<td></td>
</tr>
<tr>
<td>BSM-0.85%</td>
<td>0</td>
<td>47.1</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. The infrared spectra of de-O-acetylated bovine submaxillary mucin and native bovine submaxillary mucin.

shown earlier by Cassidy, Jourdian, and Roseman (31) and Faillard (29), sialic acid residues containing O-acetyl groups can be removed by neuraminidase. The original BSM contained 107 μmoles of sialic acid and 88 μmoles of O-acetyl groups per 100 mg. After treatment with neuraminidase, 57 μmoles of the O-acetyl groups and 43 μmoles of the sialic acid remained non-
filtrable. These results suggest that every sialic acid residue in neuraminidase-treated BSM still bears at least one O-acetyl group. Presumably, the resistance of these sialic acid residues to the action of neuraminidase may be due to the position of the O-acetyl groups on the sialic acid residues or to changes in the secondary structure of BSM caused by the alkali.

Release of N-Acetylhexosamine from BSM and OSM under Conditions of Morgan-Elson Method—In previous studies (5, 11, 12), the alkaline cleavage of the carbohydrate side chains of ovine submaxillary mucin has been followed by the Morgan-Elson method, as modified by Reissig et al. (23) or by Aminoff et al. (24). In another study (10), the Morgan-Elson reaction was apparently used under similar circumstances but the modifications or conditions of the assay were not reported. Since alkali treatment is involved in these procedures, the extent of β elimination was measured by determinations of the loss of N-acetylhexosamine under the conditions of these methods.

Table IV shows the amount of N-acetylhexosamine released by the assay procedure of the Reissig modification and the Aminoff modification of the Morgan-Elson method. It is apparent that the Reissig method may be used for the determination of free N-acetylhexosamine in the presence of bound N-acetylhexosamine in mucin samples under conditions set forth by Reissig et al. However, the pH of the assay solution must be carefully controlled. Theished method cannot be used with such materials.

Infrared Analysis of BSM—The infrared spectrum of native BSM and PSM has been previously reported (32). The infrared spectrum of native BSM shows a very distinct absorption band in the 1725 cm⁻¹ vicinity, whereas the infrared spectrum of PSM was devoid of this absorption band. Absorption in this vicinity has been attributed to a η- hydroxyl group of an ester or ketone. Since there were two possible types of ester bonds (O-glycosidic ester to the protein core or the O-acetyl groups of the sialic acid residues, or both) in the BSM molecule, a sample of hydroxylamine-treated (de-O-acetylated) BSM was subjected to infrared analysis in order to observe whether any changes in the spectrum resulted from the treatment.

Fig. 5 shows a comparison of portions of the infrared spectrum of native BSM and de-O-acetylated BSM. No absorption in the 1725 cm⁻¹ region is present in the case of de-O-acetylated BSM. This absorption seems to be a measure of the O-acetyl content of the mucins. An absorption band in the 1635 cm⁻¹ region which was observed in the native BSM was also greatly diminished in the de-O-acetylated BSM, exposing some minor absorption bands. The absorption in the 1635 cm⁻¹ region has also been attributed to the η-hydroxyl frequency of esters. Johnson and Chilton (33) also interpreted the absence of an absorption band in the 1725 cm⁻¹ region as indicating absence of O-acetyl groups.

DISCUSSION

The complete removal of the carbohydrate groups of bovine and ovine submaxillary mucins by means of alkaline sodium borohydride cannot be achieved without degradation of the protein core. Alkaline hydrolysis of bovine submaxillary mucin at 5° (6) or 45° (7) permitted the removal of 60 to 65% of the carbohydrate side chains before any appreciable amount of dialyzable peptides was produced. For ovine submaxillary mucin this was 50 to 55% removal of the side chains. After extensive treatment, approximately 25% and 15% apparently dialyzable glycopeptide were produced from bovine and ovine submaxillary mucins, respectively.

The apparent formation of glycopeptides, rather than complete hydrolysis of the prosthetic groups from the protein core, might be explained in terms of a decrease in acidity of the hydrogen atom which is attacked by alkali in the initiation of the β elimination reaction. Jones et al. (34) found that O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-serine was quite stable to alkali. Vercelloti and Luetzow (35) reported that an O-glycoside of serine, with an electron-withdrawing substituent on the nitrogen atom of the serine molecule, was quite alkali labile. Thus, it is possible that as the protein core of the mucins is progressively hydrolyzed to smaller and smaller peptides, the O-glycoside linkage may also become progressively less alkali-labile.

The formation of apparently dialyzable glycopeptides from BSM and OSM is based on the finding of unreduced hexosamine in the total reaction mixture, before dialysis. It has been reported (30, 36, 39) that the branching points of ovine and bovine submaxillary mucins are O-sialyl-β-(N-acetylglactosaminy)serine. Alternative explanations for the appearance of bound dialyzable hexosamine after extensive treatment with sodium borohydride are that a different type of linkage may exist...
for some of the side chains or that some side chains may be more complex than disaccharides and contain two hexosamine units. These possibilities are under investigation.

The lability of the O-glycosidic linkage in the intact mucin is also shown by the results of the Morgan-Elson reaction at pH 8.9 and pH 10.5. In the Aminoff modification, the alkalinity of the solution and the brief heating period were sufficient to cause the hydrolysis of a rather large amount of N-acetylmucosamine. The reason for the apparent liberation of approximately twice as much N-acetylmucosamine from bovine submaxillary mucin as from ovine submaxillary mucin is at present not known.

The alkaline hydroxylamine reaction has been used successfully in the demonstration of ester bonds in collagen (26). It appears from the results of the experiments with alkaline hydroxylamine and from the infrared study of the de-O-acetylated bovine submaxillary mucin that the O-glycosidic ester linkage in bovine submaxillary mucin does not exist in detectable amounts. Of the submaxillary mucins investigated, only the bovine submaxillary mucin, prepared by various different methods, showed the presence of ester linkages. However, this ester has the properties to be expected for the O-acetyl groups known to exist in BSM, and the method was used by Blix and Lindberg (28) as a measure of O-acetyl in sialic acids isolated from BSM.

BSM-G was prepared according to the method described by Gottschalk and Graham (16) by a modification of the Method of Curtain and Pye (37). This procedure involves possible exposure to alkali and may be the reason for the low O-acetyl content of BSM-G.

The hydroxylamine method thus gives a means for the determination of the O-acetyl content of mucins and for their removal. After removal of O-acetyl groups, the sialic acid residues of BSM were essentially completely removed by neuraminidase. None of the other mucins or glycoproteins tested gave any indication of ester linkages. It was also shown that, as reported by Boas (5), the hydroxylamine method cannot be used for products which undergo β elimination reactions of hexosamines.

The presence of the O-glycosidic ester linkages in the early reports by Gottschalk et al. and the absence of these linkages in ovine submaxillary mucin in later reports were explained by these investigators as arising from the age difference of the sheep at the time of slaughter. Thus, the O-glycosidic ester linkages were found in submaxillary mucin isolated from the submaxillary glands of Australian sheep which were slaughtered at an average age of 4 months. On the other hand, no evidence was found for the presence of the O-glycosidic ester linkages in mucin isolated from the submaxillary glands of German sheep which were slaughtered at about the age of 3 years. This explanation has not yet been tested because of the difficulty in obtaining the necessary glands for comparison.

Acknowledgment—We wish to express our appreciation to Dr. Frank Parker for performing the infrared analyses and for his aid in interpreting the spectra.

REFERENCES

Maurice Bertolini and Ward Pigman

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