would not depend upon its substrate level alone but upon the prevailing ATP level as well. Under conditions of excess energy with high ATP levels, the microsomal nucleoside diphosphatase will be inhibited by ATP resulting in an accumulation of nucleoside diphosphates. These conditions would be favorable for the formation of nucleoside triphosphates and in turn for biosynthesis of polysaccharide, nucleic acid, and proteins. On the other hand, under conditions of energy deprivation with decreased ATP concentration an unimpeded nucleoside diphosphatase may favor hydrolysis of UDP, IDP, and GDP. This depletion of nucleoside diphosphate level coupled to the decreased ATP concentration will prevent the formation of nucleoside triphosphates by nucleoside diphosphate kinase, thus halting various synthetic reactions dependent upon these specific nucleoside triphosphates. This explanation is in line with the suggestion of Atkinson (1966) that modulation of enzymatic activities by adenine nucleotides is related to the control of energy metabolism.

References

Atkinson, D. E. (1966), *Ann. Rev. Biochem. 35*, 85.
Bessman, M. J. (1963), *Methods Enzymol. 6*, 163.
Ernster, L., and Jones, L. C. (1962), *J. Cell Biol.* 15, 563.
Gibson, D. M., Ayenger, P., and Sanadi, D. R. (1955), *Biochim. Biophys. Acta 16*, 538.
Hohorst, H. J., Kreutz, F. H., Bucher, T. (1959), *Biochem. Z. 332*, 18.
Kornberg, A. (1950), *J. Biol. Chem. 182*, 779.
Mandel, P. (1964), *Progr. Nucleic Acid Res. Mol. Biol. 3*, 299.
Mandel, P., Wintzerith, M., Klein, P. N., and Mandel, L. (1963), *Nature 198*, 1000.
Marchetti, M., Puddu, P., and Caldarera, C. M. (1962), *Biochim. Biophys. Acta 61*, 826.
Marsh, B. B. (1959), *Biochim. Biophys. Acta 32*, 357.
Nordlie, R. C., and Arion, W. J. (1965), *J. Biol. Chem. 240*, 2155.
Novikoff, A. B., and Heus, M. (1963), *J. Biol. Chem. 238*, 710.
Plaut, G. W. E. (1955), *J. Biol. Chem. 217*, 235.
Schramm, V. L., and Morrison, J. F. (1968), *Biochemistry 7*, 3642.
Yamazaki, M., and Hayaishi, O. (1965), *J. Biol. Chem. 240*, PC 2761.
Yamazaki, M., and Hayaishi, O. (1968), *J. Biol. Chem. 243*, 2934.

Preparation of Glycopeptides from Bovine Submaxillary Mucin by Chemical Degradation*

Fred Downs and Ward Pigman

ABSTRACT: The disaccharide side chains (85% of them) of bovine submaxillary mucin were cleaved by alkali through a β-elimination reaction. The resulting peptide contained unsaturated amino acids at most of the original serine and threonine positions. Hydrolytic scission of the 2-aminopropenoic acid residues was carried out by heating at 100° for 1 hr at pH 2.2, but cleavage did not occur at 2-amino-3-butenoic acid residues. Gel filtration of this material resulted in the separation on Sephadex G-25 of a glycopeptide fraction which was shown to have a molecular weight of 3000. This glycopeptide accounted for 40% by weight of the original bovine submaxillary mucin and had a relative amino acid composition equivalent to bovine submaxillary mucin and alkali-treated bovine submaxillary mucin; it still contained one disaccharide side chain. This suggests the existence of repeating sequences of about 28 amino acids in bovine submaxillary mucin.

Bovine submaxillary mucin is a glycoprotein with a molecular weight of about 4×10^5. It contains about 70% carbohydrate mainly in the form of disaccharides consisting of a sialic acid and *N*-acetylgalactosamine unit (Gottschalk, 1960; Tsuiki *et al.*, 1961; Tettamanti and Pigman, 1968). The disaccharides are attached by *O*-glycosidic linkages to most of the threonine and serine residues (Tanaka and Pigman, 1965; Bertolini and Pigman, 1967). The disaccharide side chains undergo a β-elimination reaction on treatment with alkali, and the serine and threonine units are converted into 2-aminopropenoic acid and 2-amino-3-butenoic acid units (Tanaka and Pigman, 1965).

Using model serine peptides, Patchornik and Sokolovsky (1964) have shown that 2-aminopropenoic acid residues are cleaved by mild acids at the amino group, and the "NH_2-terminal group" is pyruvic acid. Harbon *et al.* (1968) found that the β-elimination product from

* From the Department of Biochemistry, New York Medical College, New York, New York 10029. *Received October 28, 1968.* This work is a part of a Ph.D. dissertation by Downs (1968). It was supported by a grant from the U. S. Public Health Service, National Institute for Arthritis and Metabolic Diseases (AM-04619).

bovine submaxillary mucin could be cleaved by mild acids to liberate pyruvic acid. Treatment of BSM[1] with alkali followed by mild acid hydrolysis would be expected to break the core peptide into small peptides composed of the amino acids between the carbohydrate side chains attached to the serine residues. The present paper describes the isolation of glycopeptides obtained from BSM by these processes. The work is described in more detail elsewhere (Downs, 1968).

Experimental Procedure

Materials. BSM was isolated and purified by the method of Tettamanti and Pigman (1968). All of the material used in this work was their major mucin. This material was similar in chemical and physical properties to preparations described earlier (Tsuiki *et al.*, 1961). Sodium borohydride and palladium chloride were obtained from K & K Laboratories, Plainview, N. Y.; the $PdCl_2$ was purified according to the method of Tanaka and Pigman (1965). Phenylhydrazine hydrochloride was recrystallized twice from 95% ethanol prior to use. 1-Dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) and standard dansylamino acids were purchased from Mann Research Laboratories. Materials for the thin-layer chromatography came from Brinkmann Instruments, Westbury, N. Y., and the Sephadex G-25 from Pharmacia Fine chemicals.

Analysis. Hexosamines were determined by the Elson–Morgan method as modified by Boas (1953). Ratios of glucosamine to galactosamine were determined with the amino acid analyzer (Hashimoto *et al.*, 1964). Assays for sialic acid were made by the resorcinol method of Svennerholm (1957) as modified by Miettinen and Takki-Luukkainen (1959). Proteins were assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. Amino acid analyses were performed on the Beckman Model 120B amino acid analyzer (Spackman *et al.*, 1958) using samples of about 1 mg. Hydrolysis was carried out with 6 N HCl at 110° for 22 hr. After hydrolysis, solutions were taken to dryness in a rotary vacuum evaporator and then were dissolved in 0.2 N sodium citrate buffer (pH 2.2).

Degradation of BSM. A solution of 3.33 mg/ml of BSM in 150 ml of 0.2 N NaOH was incubated under nitrogen at 45° for 4 hr. The β-elimination reaction was stopped by immersing the reaction flask in an ice bath and neutralizing with glacial acetic acid. The material was dialyzed in cellophane bags for 12 hr against 0.1 N sodium acetate–acetic acid (pH 7.0) and then against several changes of glass-distilled water for 4 days at 4°. After centrifugation to remove a small insoluble precipitate which formed during the reaction, the material was lyophilized. The resultant material will be referred to as β-E-BSM. It was completely excluded from the Sephadex G-25 column described below.

To cleave the peptide backbone at 2-aminopropenoic acid residues (Patchornik and Sokolovsky, 1964),

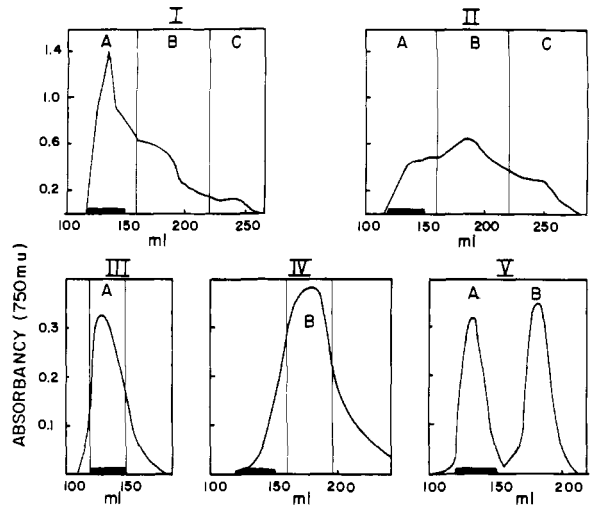

FIGURE 1: Fractionation studies. (1.I) Fractionation of 100 mg of alkali-treated bovine submaxillary mucin which was subjected to mild acid hydrolysis (pH 2.2, 1 hr, 100°) and separation on a column (1.8 × 100 cm) of Sephadex G-25. The dark bar at the base of each trace refers to the void volume. The samples were applied to the column under gravity, and elution was performed with glass-distilled water. The elution pattern was followed by Lowry protein analysis (Lowry *et al.*, 1951), and the ordinate is expressed as absorbance at 750 mμ. The vertical lines show how the fractions were separated. All tubes in each fraction were pooled. The same column and chromatographic procedure was used in all elution patterns. (1.II) Gel filtration of fraction A after a second mild hydrolysis (pH 2.2, 1 hr, 100°). (1.III) Repeated gel filtration of fraction A. (1.IV) Repeated gel filtration of pooled fractions B. (1.V) Gel filtration of a mixture of A and fraction B.

100 mg of β-E-BSM in 50 ml of glass-distilled water was adjusted to pH 2.2 with 1 N HCl, and the solution was heated at 100° for 1 hr. The flask was cooled in an ice bath, and the solution was neutralized with 0.3 N NaOH and lyophilized. The product was subjected to gel filtration on a 2.0 × 100 cm column of Sephadex G-25 (coarse). A total of 100 mg of the above material was applied as a 3.0-ml aliquot to the column. The column was eluted with glass-distilled water at 20 ml/hr and 4–5-ml fractions were collected. The patterns are shown in Figure 1. The exclusion volumes for 3.0-ml aliquots were less than 30 ml and are shown by the bars in the patterns in Figure 1.

The eluted material was divided into three fractions, A, B, and C as indicated in Figure 1.I. Fraction A of Figure 1.I was subjected to another hydrolysis at pH 2.2 for 1 hr at 100°. This material was lyophilized and passed again through Sephadex G-25 as shown in Figure 1.II. Fraction A of Figure 1.II was passed through the column again as shown in Figure 1.III. Fractions B of Figure 1.I and 1.II were pooled and passed through the column again with the results shown in Figure 1.IV. A mixture of A and B was rechromatographed as shown in Figure 1.V. It should be noted that fractions A and B refer to the material eluted between the vertical lines in Figure 1.III and 1.IV. Fraction C, from Figure 1.I and 1.II, was pooled. It contained only 10% by weight of the amino acids in

[1] BSM, bovine submaxillary mucin.

the β-E-BSM, and they were in the same proportions as fractions A and B, and contained much salt. It was discarded.

Reduction of Unsaturated Amino Acid Residues. The unsaturated residues of the alkali-treated BSM (β-E-BSM), fractions A and B of Figure 1, and the phenylhydrazine derivative of fraction B (described below) were reduced by a new modification of the Tanaka–Pigman method (1964, 1965). A solution (1 ml) containing 1–10 mg/ml of these fractions in water and 0.1 ml of 0.08 M $PdCl_2$ were placed in a test tube (18 × 150 mm) and stirred vigorously with a magnetic stirring bar. $NaBH_4$ (2 ml of 0.661 M) in 0.1 N NaOH and 2.0 ml of 0.016 M $PdCl_2$ in 0.8 N HCl were added simultaneously, but slowly, from two burets while the solution was vigorously stirred. The reduction was complete in about 1 min. The salts were separated in the G-25 column.

Conversion of the Terminal α-Keto Acid of Fraction B into the α-Amino Acid. To samples of 1.0–3.0 mg of the material forming fraction B (Figure 1) dissolved in 0.5 ml of water, 0.1 ml of 100 μmole/ml of phenylhydrazine hydrochloride in water was added. The mixture was allowed to stand at 4° for 48 hr during which time a yellow precipitate formed. Ethanol (1 ml) was added to the mixture to solubilize the precipitate before reduction of the entire mixture as described previously. The material was then hydrolyzed for 22 hr at 110° with 6 N HCl. The hydrolysate was filtered to remove the palladium black before amino acid analysis was performed. Attempts to remove the palladium black prior to hydrolysis resulted in lower recoveries of the amino acids.

Samples for NH_2-Terminal Analysis. Samples of the above-reduced material were concentrated to about 1 ml on a rotary evaporator, *in vacuo;* some coagulation of the colloidal palladium occurred. The solution was placed on a Sephadex G-25 column (1 × 20 cm) and eluted with approximately 100 ml of water. The palladium was retained on the top portion of the column. The effluent was reduced to a volume of 2.0 ml and desalted on a Sephadex G-25 column (1 × 20 cm) using glass-distilled water as a solvent. The dansylation procedure described below was then performed on the desalted fraction.

NH_2-Terminal Studies. Dansylation with 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl) of fraction B and the reduced, phenylhydrazine-treated fraction B was carried out generally by the procedure of Gray (1967) and Gray and Hartley (1963). To a solution of 100 nmoles of the reduced peptide (Table II, 4c) in 0.1 ml of water were added 0.4 ml of 0.5 M $NaHCO_3$ and 0.5 ml of DNS-Cl in acetone (20 mg/ml). The clear yellow solution was incubated at room temperature overnight. Sometimes it was necessary to add a small quantity of water to obtain a clear solution. The sample was then dried in a vacuum desiccator over sodium hydroxide. The dansyl-peptide was dissolved in 6 N HCl and hydrolyzed for 8–10 hr at 110°. After hydrolysis, the sample was taken to dryness and dissolved in 0.2 ml of acetone. Approximately 5–10 nmoles of the dansylated amino acid from the peptide was subjected to thin-layer chromatography on a silica gel G plate. Standard dansylamino acids (2 or 3 nmoles) were used for identification. Standards were obtained commercially, or were prepared.

Identification of the dansylalanine as the terminal group was made by the method of Morse and Horecker (1966). Plates (20 × 20 cm) of silica gel G were used. The dansyl derivatives were located with ultraviolet light. The DNS-amino acids appeared yellow, while dansylsulfonic acid, a product of the hydrolysis of the dansyl chloride reagent, was blue-green.

The general solvent system for the separation of DNS-amino acids has been described (Morse and Horecker, 1966). A solvent system which gave good separation of DNS-alanine, DNS-α-aminobutyric acid, and the other DNS-aliphatic amino acids was chloroform–amyl alcohol–acetic acid (70:30:0.2, v/v).

Determination of Molecular Weight. Sedimentation equilibrium studies were performed with a Beckman Model E analytical ultracentrifuge at 20°. The studies were performed by the meniscus depletion method of Yphantis (1964) and Van Holde (1967). A 1.0-mg/ml solution in 0.2 N NaCl was centrifuged at a speed of 44,720 rpm using a double-sector cell and a time of 16 hr.

Results

Action of Alkali at 45°. Bovine submaxillary mucin was treated at 45° with 0.2 N NaOH for 4 hr. These conditions are similar to those developed by Bertolini and Pigman (1967), except for the absence of sodium borohydride, to cause the removal of the disaccharide side chains by a β-elimination reaction (Figure 2). Under these conditions, 15% of the hexosamine and sialic acid residues were not eliminated, but longer times were not used in order to minimize the cleavage of peptide bonds.

The reaction was carried out under nitrogen to limit chromogen formation. In the presence of oxygen, the reaction mixture became highly colored (yellow). Most of the chromogen material was removed during dialysis and seemed to be formed from the carbohydrate moieties. A small insoluble precipitate which formed during the reaction was recovered by centrifugation. The precipitate accounted for 2–3% of the original mucin. This material was soluble in 6 N HCl, and after hydrolysis, it had 1.5% of the threonine and serine of the native mucin. Small amounts of hexosamine and the basic amino acids were also present. Amino acid analysis of the hydrolyzed material revealed unknown peaks which did not correspond to known amino acids.

The nondialyzable material (β-E-BSM) was lyophilized and analyzed for protein, hexosamine, and sialic acid. The recovery of protein was 85%. The results of these analyses are shown in Table I (column 2). The results are expressed as mmoles/100 g of β-E-BSM by weight. The alkali treatment removed approximately 85% of the carbohydrate side chains leaving a product with an enriched protein content. The protein content of β-E-BSM was about 80% compared with 35% for native BSM.

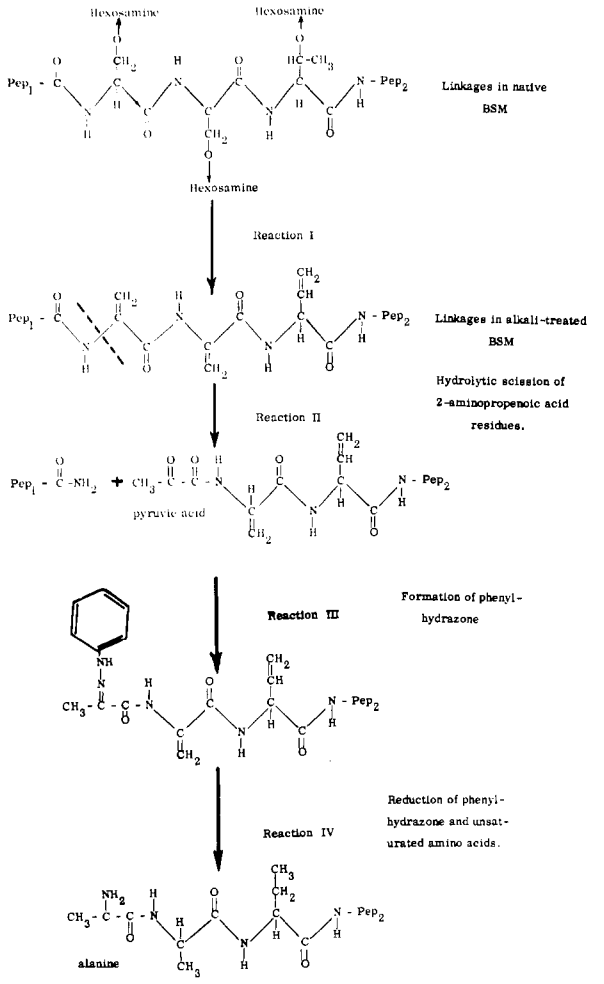

FIGURE 2: The cleavage of bovine submaxillary mucin into glycopeptides. Reaction I, native bovine submaxillary mucin was treated with 0.2 N NaOH for 4 hr at 45°. The carbohydrate side chains were removed by the β-elimination reaction with alkali leaving the unsaturated amino acids in the protein core (β-E-bovine submaxillary mucin). Reaction II, β-E-bovine submaxillary mucin was hydrolyzed at pH 2.2 for 1 hr at 100°; Figure 1 shows the fractionation of this material on Sephadex G-25 column. Reaction III, fraction B (Figure 1) was treated with phenylhydrazine hydrochloride for 48 hr at 4°. Reaction IV, is the reduction of the phenylhydrazone of fraction B using $PdCl_2$ and $NaBH_4$.

The loss of the carbohydrate constituents and the considerable decrease of serine and threonine which occurs as a result of the β-elimination reaction made it desirable to find a basis for comparison of the relative amino acid composition of the β-E-BSM with that of the original BSM. Proline in each product was given a relative value of 3.0, and the other amino acids expressed as micromoles relative to 3.0 proline residues (Table II). The same method of comparison is used for all subsequent reaction products (Figure 2).

The relative amino acid composition of the β-E-BSM is closely similar to the native mucin, as can be seen by comparing columns 1a and 2a (Table II). The loss of serine and threonine is expected because their conversion into 2-aminopropenoic and 2-amino-3-butenoic acid residues as shown in Figure 2. The 2-aminopropenoic acid and 2-amino-2- or 3-butenoic acid residues in peptide linkage are converted into pyruvic acid and α-ketobutyric acid, respectively, during acid hydrolysis (Patchornik and Sokalovsky, 1964; Harbon *et al.*, 1968).

Hydrolytic Scission. Hydrolytic scission of the alkali-treated product by acid should occur at the amino group of the 2-aminopropenoic acid residues (Figure 2, reaction II). The cleavage would result in the formation of glycopeptides which have a pyruvic acid residue at one end and an amide group at the carboxyl-terminal end. This reaction was carried out by heating at 100° for 1 hr at pH 2.2 as suggested by Patchornik and Sokolovsky (1964). Under these conditions even the peptide bonds of aspartic acid which are known to be sensitive to acid hydrolysis, appear to be resistant. Similar procedures have been employed by Kochetokov *et al.* (1967) and Harbon *et al.* (1968) in structural studies of glycoproteins.

After the mild acid hydrolysis of alkali-treated BSM (β-E-BSM), the hydrolysate was lyophilized and separated on a Sephadex G-25 column. The results of the gel filtration is shown in Figure 1. The effluent was divided into three fractions, A, B, and C. Fraction A was excluded (Figure 1.I) and when subjected to an additional acid treatment was converted into the fractions B and C (Figure 1.II); 25% was not carried to fraction B. Later work by Mr. John Moschera has shown that a G-50 column is better and gives separation of three distinct peaks.

The amino acid composition of fraction A is shown in Table I as mole/100 g of fraction A. The relative amino acid composition of fraction A, shown in Table II, is closely similar to those of the other materials reported in the table including the initial BSM. After reduction of fraction A, 2.3 residues of α-aminobutyric acid were observed for 3.0 proline residues. This amount represented an 85% yield in the reduction of the 2.8 theoretical residues of the 2-amino-3-butenoic acid units. An increase of 0.5 residue of alanine was also found, which corresponds to a 13% reduction of the 3.7 residues of 2-aminopropenoic acid which should be present. The low recovery of serine units as alanine can probably be explained by the prior loss of 2-aminopropenoic acid residues during acid hydrolysis (Figure 2, reaction II).

The amount of hexosamine in fraction A (Table II) remained relatively constant, whereas that of the sialic acid decreased as a result of the mild acid hydrolysis (Tettamanti and Pigman, 1968).

Fraction C corresponds to approximately 10% of the alkali-treated BSM. This material was eluted after fraction B from the Sephadex G-25 column; 47% of the sialic acid was recovered in this fraction.

The glycopeptides of fraction B were the most intensively studied. Fraction A represented higher molecular weight glycopeptides whereas fraction C consisted of fragments of a low molecular weight. A direct estimation of the molecular weight of glycopeptides of fraction B was performed by Dr. N. Payza using the equilibrium sedimentation method of Yphantis (1964)

TABLE I: The Amino Acid and Carbohydrate Composition of Native BSM and the Products Obtained from the Chemical Degradation.[c]

	Native BSM	Alkali-Treated BSM	Alkali-Treated BSM and Acid-Hydrolyzed Fractions from Gel Filtration on Sephadex G-25	
Amino Acid	1	2	A	B
Lysine	2.0	5.2	3.1	4.9
Arginine	14.4	19.5	15.3	25.4
Aspartic acid	8.1	20.1	17.8	18.0
Threonine[a]	47.1	29.7	34.3	32.4
Serine[b]	65.1	54.3	51.2	53.7
Glutamic acid	21.8	52.4	54.2	61.2
Proline	35.9	86.0	93.8	95.6
Glycine	59.1	140.0	142.3	162.1
Alanine	41.1	94.4	95.8	117.2
Half-cystine	0.4			
Valine	22.4	54.5	55.9	60.4
Methionine	0.2			
Isoleucine	5.4	14.8	12.5	12.3
Leucine	13.4	31.7	33.7	37.5
Tyrosine	0.5			
Phenylalanine	1.2			
Hexosamine	114.0	45.0	50.0	33.5
Sialic acid	114.6	39.4	8.5	32.6

[a] Corrected for destruction by 2.7% of 22-hr hydrolysis value (8). [b] Corrected for destruction by 10.0% of 22-hr hydrolysis value (8). [c] The composition is given as mmoles/100 g of dry material.

and Van Holde (1967) and gave a value of 3000 ± 10%. The calculated weight of 2800 based on the residues found in 4c of Table II is in good agreement with the physical determination. The linearity of the log C *vs.* $x^2 - C^2$ plot indicates molecular homogeneity (Van Holde, 1967).

The yield of fraction B was 40% of the protein core of the alkali-treated BSM (β-E-BSM). This fraction thus represents a major portion of the protein core of β-E-BSM, when it is considered that a substantial portion was discarded in the purification step shown in Figure 1.IV. This yield might have been still higher if all of fraction A had been subjected to further acid treatment to produce fraction B. It should also be noted that the chemical composition of fraction B corresponded very closely to fraction A. The amino acid composition of fraction B is given in Table I as mmoles/100 g. The relative amino acid composition is essentially the same as for the other products shown in Table II. The number of residues relative to 3.0 of proline is 21.7, whereas the original BSM has 28.1. Thus, 6.5 residues were converted into unsaturated amino acids and after the acid hydrolysis one of these should be pyruvic acid at the "N-terminal" end of the peptides produced by the cleavage.

The sialic acid and hexosamine values given for this material (Table II, 2a) were calculated on the same basis as for the amino acids described above and 1.0 residue of hexosamine and 1.0 residue of sialic acid were present for each 3 moles of proline.

NH_2-terminal analysis of fraction B using the dansyl-chloride procedure described by Gray (1967; Gray and Hartley, 1963) was unsuccessful, a result supporting the concept that these glycopeptides have a pyruvic acid as the "N-terminal" group.

The phenylhydrazone of the fraction B glycopeptide was formed as shown in reaction III of Figure 2. This phenylhydrazone was then reduced to the corresponding α-amino acid as shown in reaction IV of Figure 2. The NH_2-terminal amino acid formed was identified using the dansyl-chloride procedure of Gray. Alanine was the only dansylamino acid formed. The identification was made by thin-layer chromatography. Further evidence supporting this result is the amino acid analysis which will be discussed below.

Reduction of fraction B lead to the formation of 2.7 residues of α-aminobutyric acid and a total of a 95% recovery of the original threonine units. The recovery of the serine units converted into 2-aminopropenoic acid residues was much lower. Thus, of the original 5.5 serine residues, 3.4 were lost in the β-elimination reaction. Reduction of fraction B produced only 0.9 residue of alanine. Since it is known from other work (Downs, 1968) that some, at least, of the serine and threonine residues are present as di- or tripeptides of the type Ser-Ser-Ser, Thr-Thr-Thr, or mixtures of the two, the terminal 2-aminopropenoic residues may have been cleaved off during the acid hydrolysis step. This possibility is under current investigation. This explanation would also account for the presence of 26.1 amino

TABLE II: The Relative Amino Acid and Carbohydrate Composition of Native BSM, Alkali-Treated BSM, and the Fractions Obtained by the Gel Filtration of Acid-Hydrolyzed Alkali-Treated BSM.[c]

		Alkali-Treated BSM		Alkali-Treated BSM and Acid-Hydrolyzed Fractions A and B from Gel Filtration on Sephadex G-25				
				Fraction A		Fraction B		
Amino Acid	Native BSM 1a		Reduced Sample 2b		Reduced Sample 3b		Reduced Sample 4b	Phenylhydrazine and Reduced 4c
Lysine	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.2
Arginine	1.2	0.7	0.7	0.5	0.5	0.8	0.8	0.8
Aspartic acid	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.6
Threonine	3.9	1.0	1.0	1.1	1.1	1.0	1.0	1.0
Serine	5.5	1.9	1.8	1.8	1.8	1.8	1.7	1.8
Glutamic acid	1.8	1.8	1.8	1.7	1.7	1.9	1.9	2.0
Proline	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Glycine	4.9	4.9	4.7	4.6	4.4	5.2	5.1	5.2
Alanine	3.4	3.3	4.8	3.1	3.6	3.7	4.6	5.1
2-Aminobutyric acid			2.1		2.3		2.7	2.7
Valine	1.9	1.9	1.9	1.8	1.7	1.9	2.0	2.0
Isoleucine	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.9
Leucine	1.1	1.1	1.2	1.1	1.1	1.2	1.2	1.2
Total residues	28.1	21.0	24.4	19.8	23.0	21.7	25.2	26.1
Hexosamine	9.4	1.5		1.6		1.0		
Sialic acid	9.4	1.3		0.3		1.0		
Calculated molecular weight	7440							2800
% recovery of								
Serine[a]			60		42		46	58
Threonine[b]			80		85		95	95

[a] Sum of serine plus increase of alanine from reduction of dehydroserine. [b] Threonine plus α-aminobutyric acid from reduction of dehydrothreonine. [c] Proline in each product was given a relative value of 3.0 and the other amino acids and carbohydrates are expressed as μmoles relative to 3.0 proline residues. The amino acid values were obtained by hydrolysis in 6 N HCl at 110° for 22 hr.

acid residues in the glycopeptide (4c) as compared with 28 in the repeating unit of the original mucin (1a).

Amino acid analysis of fraction B after the phenylhydrazine and reduction treatments (reactions III and IV of Figure 2) showed the same amount of α-aminobutyric acid as for the reduced fraction B described above. Alanine showed an increase of 0.5 residue (compare columns 4b and 4c, Table II) per mole of glycopeptide, a 50% conversion of the terminal pyruvic acid into the amino acid. Since the optimal conditions for the phenylhydrazone formation were not determined, the incomplete recovery may have arisen from this cause.

These results show that the 2-aminopropenoic acid residues are more susceptible to hydrolytic scission than those of 2-amino-3-butenoic acid. This is supported by the formation of alanine as the NH_2-terminal amino acid of fraction B, after reduction of the phenylhydrazone derivative. The reduction of the 2-amino-3-butenoic acid to the corresponding amino acid in a 95% yield is also in agreement with the above observation. The probable reason for the resistance of these residues to hydrolysis is their presence as a 2-amino-3-butenoic acid residue mainly as suggested earlier (Tanaka and Pigman, 1965), rather than as 2-amino-2-butenoic acid residues which should be acid labile.

Discussion

When bovine submaxillary mucin was treated with 0.2 N NaOH at 45° for 4 hr, 84% of the carbohydrate side chains was eliminated. This product on mild acid hydrolysis gave a mixture of glycopeptides which were fractionated on Sephadex G-25. The major fractions had chemical compositions corresponding to the original BSM. The major fraction corresponding to 40% of the original β-eliminated product was shown to have a unit of pyruvic acid in the "amino-terminal" position. This was shown by converting the pyruvic acid residue into alanine by reduction of the phenylhydrazone.

This glycopeptide had one disaccharide side chain. When it was reduced, the unsaturated amino acids were converted into alanine (from the original serine units) and into 2-aminobutyric acid (from the original threonine). The recovery of threonine was high, but

two serine units were lost, presumably in the acid hydrolysis step.

The molecular weight, determined by the ultracentrifuge, was 3000 for the pyruvic acid glycopeptide. The molecular weight calculated on the basis of one unit of arginine plus lysine per glycopeptide molecule is 2800. The material acted as a homogeneous component in the ultracentrifuge.

The amino acid composition of the various glycopeptides was closely equivalent to that of the original mucin and suggest the existence of repeating amino acid sequences in the original BSM (molecular weight 4×10^5) of about 28 amino acids. Eight of the nine component serine and threonine units carry oligosaccharide units, but one of these is apparently unsubstituted. Possibly a family of repeating sequences may be present in which homologous substitution of amino acids of the same functional type may occur. These possibilities are under further investigation.

The action of trypsin on bovine and ovine submaxillary mucins, from which the sialic acid has been removed, also gives similar glycopeptides (G. Tettamanti and W. Pigman, 1969, unpublished data). Similar small peptides having the same composition as the original elastin were reported by Partridge and Davis (1950).

References

Bertolini, M., and Pigman, W. (1967), *J. Biol. Chem. 242*, 3776.

Boas, N. P. (1953), *J. Biol. Chem. 204*, 553.

Downs, F. (1968), Ph.D. Dissertation, New York Medical College, New York, N. Y.

Gottschalk, A. (1960), *Nature 186*, 949.

Gray, W. R. (1967), *Methods Enzymol. 11*, 139, 470.

Gray, W. R., and Hartley, B. S. (1963), *Biochem. J. 89*, 379.

Harbon, S., Herman, G., and Clauser, H. (1968), *European J. Biochem. 4*, 265.

Hashimoto, Y., Hashimoto, S., and Pigman, W. (1964), *Arch. Biochem. Biophys. 104*, 282.

Kochetkov, N. K., Derevitskaya, V. A., and Kara-Murza, S. G. (1967), *Carbohydrate Res. 3*, 403.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Miettinen, T., and Takki-Luukkainen, L. T. (1959), *Acta Chem. Scand. 13*, 856.

Morse, D., and Horecker, B. L. (1966), *Anal. Biochem. 3*, 297.

Partridge, S. M., and Davis, H. F. (1950), *Nature 165*, 62.

Patchornik, A., and Sokolovsky, M. (1964), *J. Am. Chem. Soc. 86*, 1206.

Spackman, D. N., Moore, S., and Stein, W. H. (1958), *Anal. Chem. 30*, 1190.

Svennerholm, L. (1957), *Biochim. Biophys. Acta 24*, 604.

Tanaka, K., Bertolini, M., and Pigman, W. (1964), *Biochem. Biophys. Res. Commun. 16*, 404.

Tanaka, K., and Pigman, W. (1965), *J. Biol. Chem. 240*, PC1487.

Tettamanti, G., and Pigman, W. (1968), *Arch. Biochem. Biophys. 124*, 41.

Tsuiki, S., Hashimoto, Y., and Pigman, W. (1961), *J. Biol. Chem. 236*, 2172.

Van Holde, K. E. (1967), News of Biochemical Instrumentation, Fractions, No. 3, Beckman Instrument Co., Palo Alto, Calif.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.