SERINE AND THREONINE GLYCOSIDIC LINKAGES IN BOVINE SUBMAXILLARY MUCIN*

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Gottschalk et. al. (1960, 1961 a, 1961 b, 1963 a, 1963 b) provided evidence for ovine submaxillary mucin that the aspartic and glutamic acids of the peptide core are linked to oligosaccharide side chains mainly by ester bonds. Hashimoto and Pigman (1962, 1963) suggested, after comparisons of the amino acid compositions of mucins from several different animals and of the glycopeptides formed by enzymatic degradation of bovine submaxillary mucin that a glycosidic linkage through the hydroxyl groups of serine and threonine is more probable for this mucin. The presence of such a serine linkage was suggested for chondroitin sulfate by Anderson, Hoffman and Meyer (1968) because a disappearance of serine occurred after treatment with alkali; this was ascribed to a beta-elimination reaction from an O-substituted serine with the creation of a double bond and a loss of serine.

In the present work, the reductive cleavage of bovine submaxillary mucin by alkaline sodium borohydride is reported. The premise was that if the hydroxyl groups of serine or threonine are involved in glycosidic linkage, reduction of the double bonds by alkaline borohydride may occur after the beta elimination reaction and that a conversion to alanine or to α-aminobutyric acid would result. In this communication it is reported that treatment of bovine submaxillary mucin with sodium borohydride caused an extensive conversion of serine to alanine and a small conversion of threonine to α-aminobutyric acid.

EXPERIMENTAL: Seventy five mg. of purified bovine submaxillary mucin (Tsuiki et al., 1961) was reacted at 5° with 15 ml. of 0.3 M sodium borohydride dissolved in 0.1 N NaOH solution in a test tube. After treatment for different periods as indicated in Table I, one or two drops of n-octanol was added

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Table I. Amino Acid Composition of Bovine Submaxillary Mucin after Treatment with Alkaline $NaBH_4$ and Dialysis
(Mg. per 100 mg. original mucin)

Amino Acid	Treatment Time (Hours)				
	Control	24	72	144	216
Alanine	4.25	4.86	5.36	6.31	7.02
α-Aminobutyric acid	-	-	0.04	0.22	0.33
Aspartic acid	1.39	1.31	1.34	1.35	1.42
Glutamic acid	3.67	3.61	3.73	3.63	4.03
Glycine	5.12	5.23	5.33	5.18	5.29
Isoleucine	0.97	0.93	1.10	1.03	1.03
Leucine	1.87	1.88	2.26	2.05	1.99
Methionine	-	-	-	-	0.09
Phenylalanine	0.37	0.34	0.39	0.37	0.27
Tyrosine	0.20	0.20	0.15	0.17	0.17
Proline	5.14	5.64	5.48	5.35	5.13
Serine	6.34	4.70	4.00	2.94	2.59
Threonine	6.71	6.04	5.59	4.44	4.12
Valine	3.30	3.39	3.39	3.38	3.51
Arginine	2.19	3.14	2.57	2.85	2.83
Histidine	0.05	0.06	0.07	0.09	0.08
Lysine	0.20	0.27	0.22	0.22	0.21
Total	41.77	41.60	41.02	39.55	40.10
Protein*	34.91	34.81	33.90	33.04	33.51
N-Acetylhexosamine**	22.60	14.80	11.80	7.39	6.45
N-Acetylsialic acid***	27.05	20.17	18.35	13.46	11.11
L-Fucose****	1.85	1.39	1.14	0.80	0.64
D-Galactose*****	3.60	2.55	2.06	1.68	1.81

*As serum albumin, Lowry et al., (1951).
**Boas (1953). ***Miehttinen et al., (1959).
****Dische et al., (1948). *****Schöenberger et al., (1958).

and the solution was neutralized to pH 5.3 with 1 N acetic acid to remove the excess borohydride. The solution was transferred to a cellophane bag and dialyzed under toluene at 5° exhaustively against deionized water. The dialyzate was lyophylized. Portions were dissolved and analyzed for amino acids, protein, N-acetylhexosamine, N-acetylsialic acid, fucose and galactose.

The absolute concentration of each component with reference to the original mucin was determined by removing 3.0 ml. aliquots from another reaction mixture (15 ml.) at the same time intervals. These aliquots were made up to 15 ml. after neutralization and dialysis. The protein concentrations determined

by the Lowry-Folin method were used as the basis for the correction of the analyses at the various times to the original amount of mucin. The amino acid analysis was carried out as reported previously (Hashimoto and Pigman, 1962). The hydrolyzed sample from 0.75 mg. of mucin substance in 1.0 ml. of pH 2.2 buffer was analyzed by the Spinco accelerated method, after some modifications required because of high pressure. A column 0.6x10-13cm. of resin Type 50A was used for analysis of the basic amino acids at 51° and 20-23 psi, and a column 0.9x64cm. of a mixture of resin Type 150A and 50A(1:1) was used for the neutral and acidic amino acids at 38-40 psi. Elution of the latter column was with pH 3.28 buffer at 51° and with pH 4.25 buffer at 55°.

RESULTS AND DISCUSSION: The results obtained are shown in Table I in which compositions are given as mg. undialyzable component remaining from 100 mg. of original mucin. After treatment of the mucin with alkaline borohydride and dialysis, the protein content remained almost unchanged, but the carbohydrate components were progressively lost. This result indicated that the linkages between the protein and carbohydrate components were cleaved. The amino acids, except alanine, serine and threonine, remained in their original amounts. An increase in alanine, and a decrease in serine and threonine occurred. After 72 hours a new peak was found between cysteine and valine in the chromatograms. This peak corresponded to that of known α-aminobutyric acid. In a control experiment, serum albumin was treated similarly to the mucin, but after the treatment no significant change was found in the serine, threonine and alanine components and no α-aminobutyric acid was formed.

Fig. 1 shows the changes of some of the amino acid components on the basis of μ moles per 100 mg. of the mucin with reference to several of the amino acids. The aspartic and glutamic acids, valine and glycine remained virtually constant. As the serine decreased, the alanine showed an approximately corresponding increase. However, the recovery of α-aminobutyric acid was far less than expected from the molar decrease of threonine.

In order to determine whether some of the α-aminobutyric acid was dialyzable, the total reaction mixture prepared without dialysis was subjected to amino acid analysis. For this purpose, 5.0 ml. aliquots were taken from a reaction mixture, prepared as before, after 30 minutes and 144 hours; the 30 minute-sample was used as control. Both samples were ad-

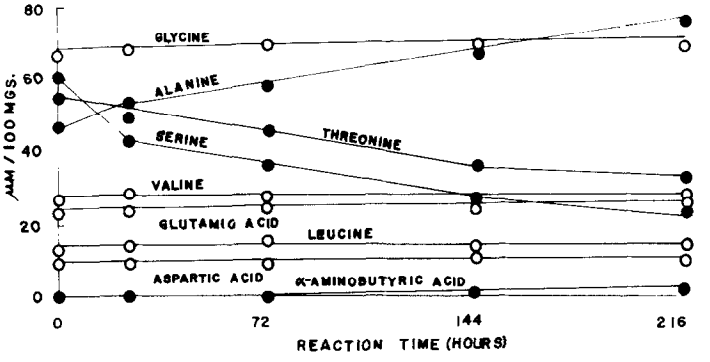

Fig. 1. Change of content (μ moles per 100 mgs.) of serine and threonine with reference to those of several other amino acids in bovine submaxillary mucin after the alkaline $NaBH_4$ treatment.

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justed to pH 5.3, and then to pH 7.0 with 0.1 N NaOH. The solutions were evaporated to dryness at 50°. The dry material was evaporated with methanol several times to remove boric acid and dissolved in a known volume of water. The control showed nearly the same amino acid composition as in Table I. The results for the control as mg./100 mg. of mucin were: alanine (4.15), α-aminobutyric acid (0.0), serine (5.78) and threonine (6.44). The 144 hour-treated sample showed a decrease of serine and threonine, an increase of alanine and the formation of α-aminobutyric acid. But no corresponding change in the amounts of threonine and α-aminobutyric acid were found. The results at 144 hours as mg./100 mg. were: alanine(5.53), α-aminobutyric acid(0.11), serine(2.66) and threonine(3.92). These results suggest that the discrepancy between the decrease of threonine and the formation of α-aminobutyric acid is the result of an incomplete reduction of dehydro-α-amino-butyric acid.

Fig. 2 compares the percentage decrease of serine and threonine with that of the carbohydrate components. The rate and total decrease of serine was nearly the same as those of sialic acid, fucose and galactose. However, the loss of hexosamine was greater than that of the other sugar components.

The slope of these curves indicates an initial rapid cleavage during the first 24 hours followed by a slower reaction. These results are compatible with

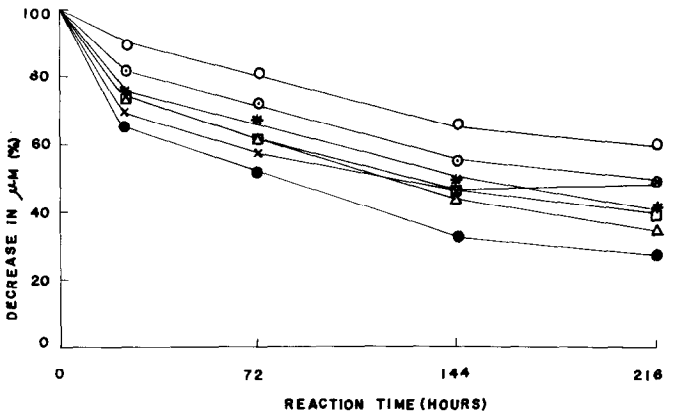

Fig. 2. Correlation between decrease of serine and threonine and the carbohydrate components of bovine submaxillary mucin after alkaline $NaBH_4$ treatment. ▢ (serine), ○ (threonine), ◎ (serine+threonine) ✽ (N-acetylsialic acid), △ (L-fucose), ✕ (D-galactose), ● (N-acetylhexosamine).

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a structure for bovine submaxillary mucin for which most of the carbohydrate units are disaccharides side chains connected to the protein core by N-acetyl-2-amino-2-deoxy-D-galactosidic linkages to the hydroxyl groups of serine and threonine (Hashimoto et al., 1963). Upon treatment with alkali the disaccharides were removed by a β-elimination reaction and the serine and threonine residues were converted to unsaturated derivatives. The serine derivative was reduced by sodium borohydride to alanine, but the threonine derivative was only partially reduced. However, for the threonine residue two types of double bonds are possible, only one enolic as for the serine derivative. For complete conversion of threonine to α-aminobutyric acid, another type of reduction will be required; this is now under study.

These results do not eliminate the possibility of other bonds between carbohydrate and protein, but indicate that such bonds are a minor type. In fact the initial lability of the side chains during the first 24 hours (Fig. 2) is compatible with some ester bonds as suggested by Gottschalk. The more rapid release of hexosamine than of sialic acid may be related to the presence of a secondary type of side chain attached by glucosamine rather than by galactosamine units.

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