THE LINKAGE BETWEEN SUGARS AND AMINO ACIDS IN OVINE SUBMAXILLARY GLAND MUCOPROTEIN " OSM ".

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The linkage between the dioside α, D-N-acetylneuraminyl 2 →6 N-acetylgalactosamine (Graham and Gottschalk, 1960) and the protein moiety of ovine submaxillary mucoprotein "OSM" mainly consists, according to current views (Gottschalk and Murphy, 1961; Graham et al., 1963) in ester linkages between the reducing hydroxyl group of N-acetylgalactosamine and ω carboxylic groups of aspartic and glutamic acids. It has however been emphasised (Pigman, 1962) that after digestion by proteolytic enzymes the total amount of dicarboxylic acids in the resulting glycopeptides was not sufficient to account for separate ester links between these acids and the individual diosides. In addition the kinetics of the release of prosthetic groups from the mucoprotein in alkaline medium was found to be much slower than could be expected if ester linkages were concerned by this hydrolysis. Moreover no specific effect of hydroxylamine on the rate of this reaction was observed, provided pH and ionic strength were kept constant (Harbon et al., 1963).

We have previously suggested (Harbon et al., 1963) that a β-elimination mechanism (Balou, 1954) was responsible for the release of diosides linked glycosidically to the -OH groups of serine and threonine within the protein chain. The present paper deals with additional proof for such kind of linkages in OSM, according to experiments performed with pronase-digested glycopeptides, as well as β-elimination and β-substitution experiments in alkaline medium.

OSM was purified according to the procedures of Curtain and Pye (1955) "OSM_G" and Pigman (Tsuiki, Hashimoto and Pigman, 1961) "OSMp"; sugar and amino acid compositions of both preparations were found to be essentially identical (Table I). After digestion with the bacterial proteolytic enzyme "Pronase" (pH 7, 9, Ca^{++} 0.015 M, 8 days, ratio enzyme/substrate 1/20, in presence of toluene) and dialysis of the resulting glycopeptides, 41 % of the total amino acids were removed, whereas the total amount of carbohydrates was recovered within the dialysis bag. The absence of free diosides in the glycopeptide pool was checked by dialysis and zone electrophoresis. The molar ratios between individual amino acids and sugars in 2 of these glycopeptide fractions were as follows:

1) N-acetylgalactosamine (GalNAc) 2.0, N-acetylneuraminic acid (NANA) 2.08, Gly 1.82, Ala 1.09, Ser 1.54, Thr 1.68, Pro 1.18, Val 1.45, Leu 0.0, Asp 0.21, Glu 0.38.
2) GalNAc 2.0, NANA 1.9, Gly 2.2, Ala 1.0, Ser 1.5, Thr 1.5, Pro 0.48, Val 0.15, Leu 0.30, Asp 0.30, Glu 0.27.

It appears that the amount of dicarboxylic acids in these glycopeptides is insufficient to account for individual carboxylic ester links between these acids and the diosides, whereas serine and threonine content of these fractions amounts to almost 50 % of the total amino acids still present. The fact that the diosides are individually linked to the peptide chain was confirmed by periodate oxydation of the intact glycoprotein and of the pronase-digested glycopeptides; these results appear to be in complete agreement with the data previously published by Graham and Gottschalk (1960).

Elimination of electron attracting substituents of the hydroxyl groups of threonine and serine within a peptide chain is supposed to yield unsaturated amino acids which decompose on subsequent acid hydrolysis of the protein (Photaki, 1963). In agreement with this hypothesis OSM was treated at pH 12.8 (ionic strength 1.6, 45 minutes at 70°), neutralized and dialyzed. Under these conditions (Harbon et al., 1963), 80 % of the diosides are removed

TABLE I

Amino acids and sugar composition of ovine submaxillary mucoproteins.

	OSM_G		OSM_P		alkali-treated OSM		alkali-treated OSM + Na_2SO_3	
	g/100 g OSM	molar ratio	g/100 g OSM	molar ratio	g/100 g OSM	molar ratio	g/100 g OSM	molar ratio
Cysteic acid	absent		absent		absent		4. 1	3. 8
Aspartic acid	1. 92	2. 55	1. 35	1. 92	1. 14	1. 48	1. 1	1. 3
Threonine	6. 55	9. 7	6. 5	9. 75	1. 64	2. 27	1. 64	2. 15
Serine	7. 8	13. 1	6. 9	11. 7	2. 50	4. 0	3. 1	4. 6
Glutamic acid	3. 3	4. 0	3. 43	4. 17	3. 49	3. 8	3. 75	4. 0
Proline	4. 8	7. 35	4. 38	6. 8	5. 0	7. 0	4. 30	5. 75
Glycine	6. 7	15. 75	5. 72	13. 6	6. 5	14. 2	6. 0	12. 5
Alanine*	3. 95	8. 0	3. 98	8. 0	4. 27	8. 0	4. 55	8. 0
Cystine + cysteine**	-	-	0. 22	0. 24	-	-	-	-
Valine			2. 6	4. 0	2. 4	3. 45	2. 26	3. 0
Methionine	absent		absent		-	-	-	-
Isoleucine	0. 8	1. 1	0. 72	1. 0	0. 48	0. 62	0. 52	0. 64
Leucine	2. 0	2. 7	1. 8	2. 5	1. 87	2. 67	1. 56	2. 06
Tyrosine	0. 28	0. 3	0. 15	0. 15	0. 11	0. 10	0. 13	0. 12
Phenylalanine	1. 06	1. 1	0. 95	1. 0	0. 96	1. 1	0. 83	0. 8
Lysine	1. 06	1. 3	0. 47	0. 58	0. 26	0. 30	0. 64	0. 7
Histidine	0. 15	0. 19	0. 13	0. 15	0. 13	0. 15	0. 08	0. 1
Arginine	2. 24	2. 2	2. 6	2. 4	2. 18	2. 1	0. 64	0. 6
N-acetylgalactosamine	18. 8	14. 6	18. 8	14. 6	3. 2	2. 6	3. 2	2. 6
N-acetylneuraminic acid	26. 0	14. 6	26. 0	14. 6	5. 1	2. 6	5. 1	2. 6

[*] Alanine content has been arbitrarily equated to 8. 0, on a molar ratio basis.

[**] Cystine + cysteine content estimated as cysteic acid after oxydation by performic acid.

whereas the total amount of the protein moiety is recovered within the dialysis bag, as demonstrated by ninhydrine and total nitrogen analysises. The molar ratios of the amino acids of the resulting protein (Table I) were in agreement with the molar ratios of the native protein, with the exception of threonine and serine, the amount of which decreases respectively by 78 % and 60 %. When lysozyme was treated under the same conditions and analyzed, decrease of serine and threonine amounted to less than 15 %.

An additional experiment performed with alkali-treated OSM consisted in an α-β addition of a nucleophilic substituent on the double bond of the unsaturated amino acids formed by β-elimination of diosides. Sulfite 0.1 M was used and the reaction took place at room temperature, 24 h., pH 9.0; the resulting protein was dialyzed, air dried and its amino acid content analyzed (Table I). Cysteic acid found accounted on a molar basis for 55 % of the serine lost. This result clearly demonstrates that the loss of serine during alkaline treatment of OSM is at least partially due to the formation of unsaturated amino acids through a β-elimination mechanism. Similar results have been recently obtained by Strumeyer et al. (1963) with the catalytically active serine residue in chymotrypsin. No sulfonic derivative of threonine was detected, which may be due either to steric hindrance or to an inductive effect of the vicinal $-CH_3$ group on the double bond formed by alkaline treatment on the threonine residues. This interpretation is confirmed by recent experiments of Sokolowsky (1964) concerning α-β-unsaturated amino acids.

Thus it appears that conclusive evidence is presently accumulating concerning the glycosidic nature of the link between sugars and amino acids in ovine submaxillary mucoprotein. Contradiction between these results and the data previously published (Gottschalk and Murphy, 1961; Graham et al., 1963) should be however appreciated with caution: additional ester linkages between sugar -OH groups and ω -COOH groups of dicarboxylic amino acids could be present in mucoproteins since these linkages would be extremely sensitive to alkaline treatment and could also be cleaved by pronase digestion as shown by control experiments with esterified proteins.

In addition, the present experiments suggest that glycosidic links between sugars and hydroxylated amino acids in glycoproteins may be demonstrated by β-substitution when the amino acid concerned is serine.

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