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ISOLATION AND CHARACTERISATION OF GLYCOPEPTIDES FROM DIGESTS OF HUMAN TAMM–HORSFALL GLYCOPROTEIN

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ABSTRACT

Glycopeptides were isolated from pronase digests of human Tamm–Horsfall glycoprotein and its asialo derivative. The carbohydrate moiety of the major glycopeptide preparation isolated from the former digests had an apparent molecular weight of 4300, and those of two glycopeptides isolated from the latter digests had molecular weights of 3600 and 2300. These data, together with the compositions of the glycopeptides, indicate that the Tamm–Horsfall glycoprotein has at least five asparagine residues substituted by complex carbohydrate moieties, three being of one type, relatively rich in galactose, and two containing more sialic acid but less galactose. A small amount of a mannose-rich glycopeptide was also recovered from the digests of the Tamm–Horsfall glycoprotein.

INTRODUCTION

The urinary glycoprotein, which was discovered by Tamm and Horsfall[1,2], is produced by the kidney[3]. There may well be differences between species as to the precise parts of the tubule which produce the glycoprotein. In the Syrian hamster, the glycoprotein is produced by the cells of the ascending limb of the loop of Henle and of the distal convoluted tubule as far as its junction with the initial collecting duct[4], whereas in the rat kidney, cells of the ascending limb of the loop of Henle (which extend variable, short distances beyond the macula densa[5,6]) appear to be the only cells that produce the glycoprotein[7]. Although the function of Tamm–

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Horsfall glycoprotein is poorly understood, it has been suggested, both from a consideration of its location on the cell membranes in question[4,7] and from its gel-forming properties[8], that it may create the relatively water-impermeable barrier which must be present in the ascending limb of the loop of Henle for generation of the essential hypertonicity of the renal medulla[9]. Progress in testing this hypothesis depends in part on an understanding of the structure of the glycoprotein. We now report on the isolation of glycopeptides from proteolytic digests of human Tamm–Horsfall glycoprotein and of its asialo derivative, and some of the properties thereof.

METHODS

Materials. — Tamm–Horsfall glycoprotein was isolated from pooled, male urine by the procedure described essentially by Tamm and Horsfall[1,2]. Asialo-Tamm–Horsfall glycoprotein was obtained by heating a solution of Tamm–Horsfall glycoprotein (180 mg/15 ml) in 0.05M H_2SO_4 at 80° for 1 h. The solution, after cooling, was exhaustively dialysed against water.

SDS-gel electrophoresis was performed by the procedure of Marshall and Zamecnik[10,11].

Pronase (Sigma) was purified by acetone precipitation[12]. The product was devoid of carbohydrate, as assessed by the procedure of François *et al.*[13].

Proteolytic digestion of Tamm–Horsfall glycoprotein and of its asialo derivative.— Solutions, at pH 8.5, of each of the glycoproteins (180 mg/15 ml) in 15mM calcium acetate were digested with pronase (5.4 mg) at 37° for 24 h. The pH was kept at 8.5, and the extent of digestion was followed[14] by use of the ninhydrin reagent[15] with leucine as the standard.

Each digest was centrifuged (2000*g*; 10 min; 4°) to remove a small amount of insoluble material and the supernatant solution was freeze-dried. Each residue was taken up in 1 ml of 0.1M acetic acid, and a small amount of insoluble material was removed by centrifugation (6000*g*; 10 min; 4°) and discarded. Each supernatant solution was subjected to gel filtration at 4° on a column (46 × 1 cm) of Sephadex G-25 (fine grade) in 0.1M acetic acid, at an elution rate of 6 ml/h (2-ml fractions). The carbohydrate-containing fractions[13], with mannose as standard in the assay, were combined and freeze-dried.

Each residue was dissolved in 0.1M acetic acid (1 ml), and the solutions were chromatographed on a column (46 × 1 cm) of Sephadex G-100 at 4° and a rate of 9 ml/h with the same solvent (3-ml fractions). The carbohydrate-containing fractions were combined and freeze-dried.

Each residue was dissolved in 0.1M acetic acid (1 ml), and the solutions were placed on a column (68 × 1 cm) of DEAE-Sephadex A-25 equilibrated with the same solvent at 4°. Elution of the column with 0.1M acetic acid (200 ml; flow rate, 9 ml/h) was followed by elution with 0.5M NaCl in 0.1M acetic acid. The carbohydrate-containing fractions were combined appropriately and freeze-dried.

Each residue was dissolved in water (1–2 ml), and each solution was subjected

to gel filtration on Sephadex G-25 (fine grade; 46 × 1 cm) with water as the eluting agent. The carbohydrate-containing fractions were combined and freeze-dried. These materials were used for determinations of molecular weight by ultracentrifugation, for analyses of their compositions, and for high-voltage paper electrophoresis.

Sugar analysis was performed by g.l.c. of the trimethylsilyl derivatives of the methyl glycosides[16], and amino acid analysis was performed on hydrolysates prepared in 3M toluene-*p*-sulphonic acid[17].

High-voltage paper electrophoresis was performed on Whatman 3MM paper, in sodium acetate buffer (0.1M in acetate) at pH 5.0, for 30 min at 80 V/cm. Ninhydrin in acetone containing 2% of pyridine was used for detection.

Molecular weights of glycopeptides were measured at 25° in a Beckman model E ultracentrifuge by the short-column, Schlieren method of Yphantis[18]. To suppress charge effects as much as possible, the buffer solutions used were of high salt strength (0.5M NaCl, 0.1M sodium phosphate, pH 6.0 for GP-TH-II and GP-AsTH-II; 0.2M NaCl, 0.1M sodium phosphate, pH 6.0 for GP-AsTH-I). The glycopeptides were at low concentrations, which were measured refractometrically. Photographs were recorded at 32-min intervals once sufficient time had elapsed to allow for the attainment of equilibrium. For GP-TH-II, a second, higher speed was used. The apparent molecular weight (M) at the centre of each (0.8 mm) column was calculated from the relation: $M = RT(dc/dr)/\omega^2 rc(1 - \bar{v}\rho)$, where c and dc/dr are the concentration and concentration gradient, respectively, at the centre of the column, r is the distance from the axis of rotation, ω the angular velocity, and ρ the density of the solvent, and the other symbols have their usual significance. The concentration is practically equal to the initial value, but small corrections were applied. As it was not practicable to measure the partial specific volume ($\bar{v}$), it was calculated by taking a value of 0.66 for the carbohydrate, estimating it for the peptide moiety from the contributions of the individual amino-acid residues, and assuming additivity[19]. Overall values did not differ significantly from 0.66, which was therefore used in all the calculations of molecular weight.

RESULTS

Polyacrylamide-gel electrophoresis[10] of human Tamm–Horsfall glycoprotein revealed the presence of one band staining with Coomassie Brilliant Blue. Treatment of the glycoprotein with 0.05M H_2SO_4 at 80° for 1 h (to release sialic acid residues[20,21])

Fig. 1. Polyacrylamide-gel electrophoresis in the presence of SDS of Tamm–Horsfall glycoprotein, which had been treated with 0.05M H_2SO_4 at 80° for 1 h.

TABLE I

RECOVERIES OF NEUTRAL SUGARS AT VARIOUS STAGES OF PURIFICATION OF GLYCOPEPTIDES FROM PRONASE DIGESTS OF TAMM–HORSFALL GLYCOPROTEIN AND OF ASIALO-TAMM–HORSFALL GLYCOPROTEIN

	Tamm–Horsfall glycoprotein			*Asialo-Tamm–Horsfall glycoprotein*		
		Amount of neutral sugar (mg)	*Recovery (%)*		*Amount of neutral sugar (mg)*	*Recovery (%)*
Original glycoprotein		19.8	100		18.7	100
G-25 in 0.1M acetic acid		20.0	101		16.8	90
G-100 in 0.1M acetic acid		18.5	93		16.6	89
DEAE-Sephadex A-25	TH-A[a]	0.5	2.5	AsTH-A[c]	9.4	50
	TH-B[a]	14.7	74	AsTH-B[a]	4.8	26
A-peaks on G-25 in water	GP-TH-I[b]	0.3	1.5	GP-AsTH-I[b]	9.2	49
B-peaks on G-25 in water	GP-TH-II[b]	13.9	70	GP-AsTH-II[b]	4.5	24

[a]See Fig. 4. [b]See Fig. 5.

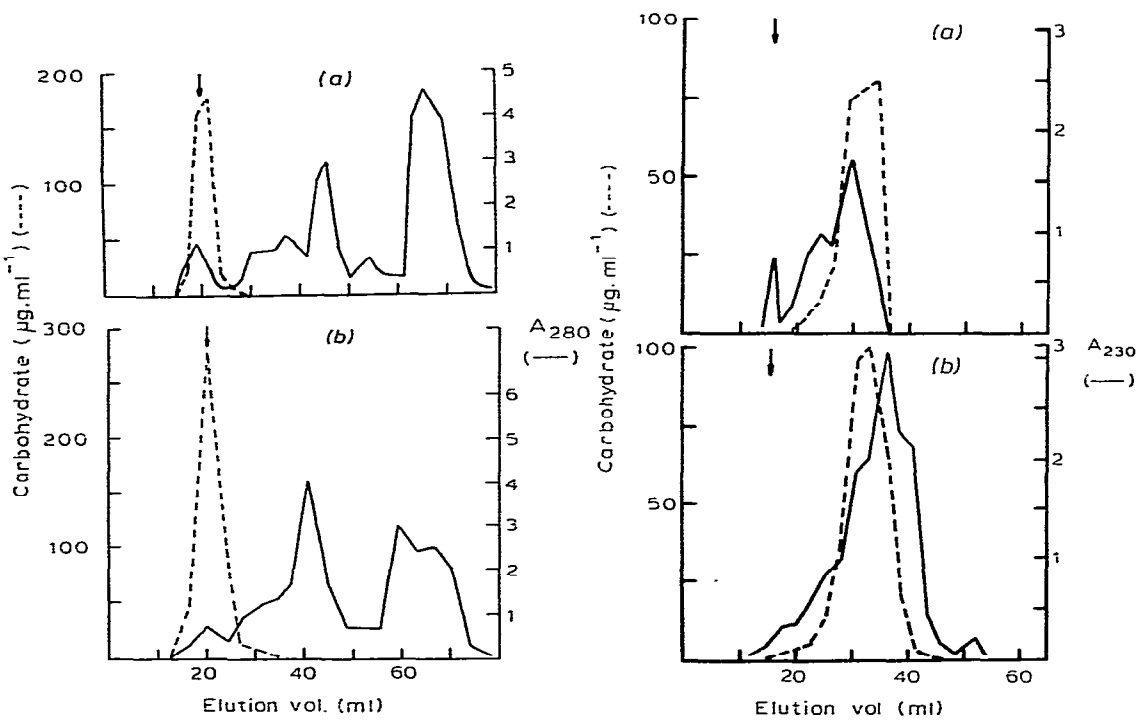


Fig. 2. Gel filtration on Sephadex G-25 in 0.1M acetic acid of pronase digests of (*a*) human Tamm–Horsfall glycoprotein and (*b*) human asialo-Tamm–Horsfall glycoprotein (see text; the void volume is indicated by an arrow).

Fig. 3. Gel filtration on Sephadex G-100 in 0.1M acetic acid of (*a*) the carbohydrate-containing fractions from Tamm–Horsfall glycoprotein (Fig. 2*a*) and (*b*) the carbohydrate-containing fractions from asialo-Tamm–Horsfall glycoprotein (Fig. 2*b*) (see text; the void volume of the column is indicated by an arrow).

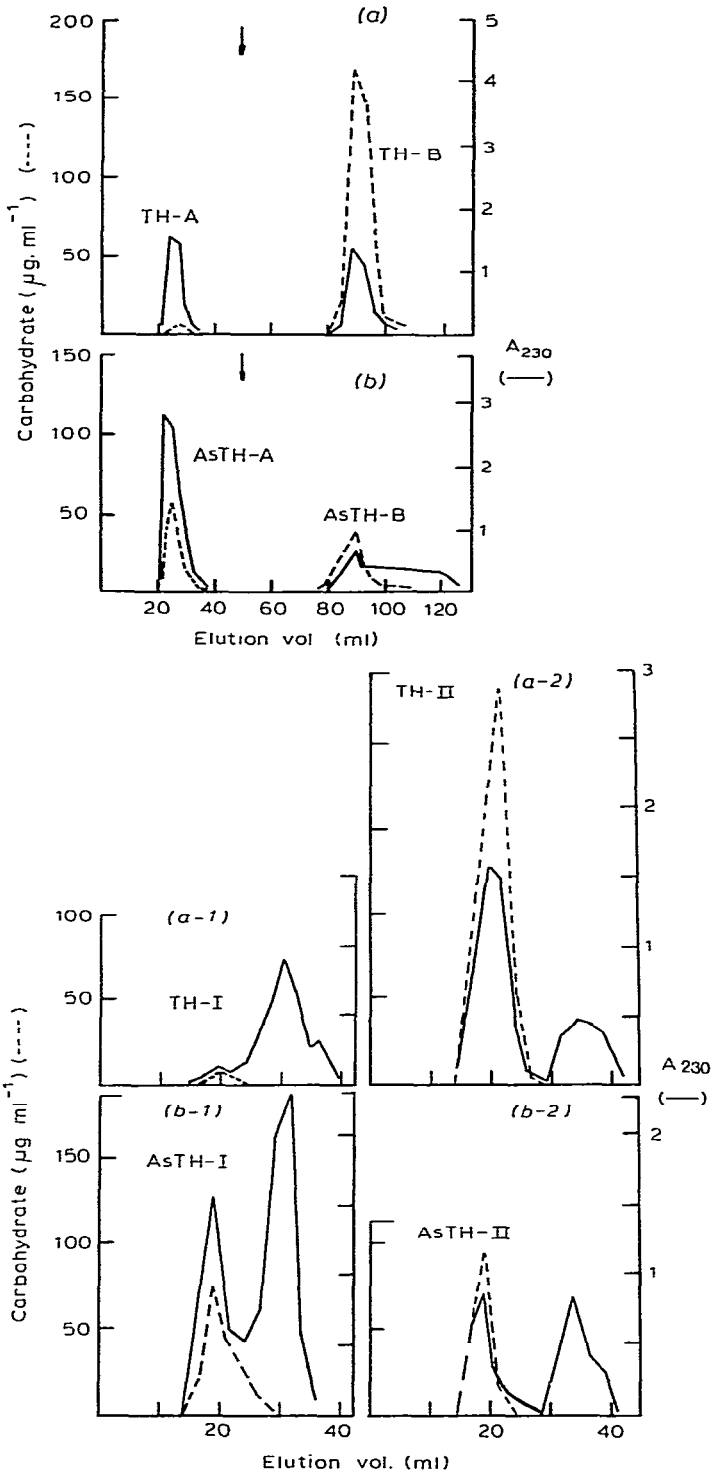


Fig. 4. Fractionation (see text) of glycopeptide mixtures on DEAE-Sephadex A-25: (*a*) chromatography of the carbohydrate-containing fractions from Fig. 3*a* (glycopeptides from Tamm–Horsfall glycoprotein) and (*b*) chromatography of carbohydrate-containing fractions from Fig. 3*b* (glycopeptides from asialo-Tamm–Horsfall glycoprotein). The column was eluted first with 0.1M acetic acid and then, as indicated by the arrow, with 0.1M NaCl in 0.1M acetic acid.

Fig. 5. Gel filtration of glycopeptides obtained from DEAE-Sephadex A-25 chromatography on columns of Sephadex G-25 by elution with water: (*a-1*) TH-A from Fig. 4*a*, (*a-2*) TH-B from Fig. 4*a*, (*b-1*) AsTH-A from Fig. 4*b*, and (*b-2*) AsTH-B from Fig. 4*b*. This step led, in each case, to the separation of peptide from the glycopeptides, separations which had not resulted from gel filtration

led to formation of a product that gave one major band and two minor bands after SDS-gel electrophoresis (Fig. 1).

Extensive degradation of the Tamm–Horsfall glycoprotein occurred on digestion with pronase, resulting in cleavage of ~60% of the peptide bonds and a quantitative recovery of neutral sugar.

Gel filtration of the digest on a Sephadex G-25 column in 0.1M acetic acid led to quantitative recovery of the carbohydrate (Table I) close to the void volume of the column (Fig. 2*a*). Further gel filtration of the glycopeptide-containing material on Sephadex G-100 in 0.1M acetic acid led to a good recovery of the carbohydrate-containing material (Table I) with a V/V_0 value of 2.1 (Fig. 3*a*).

The glycopeptide material was fractionated by chromatography on DEAE-Sephadex A-25 in 0.1M acetic acid, to yield Peak TH-A which was not retarded by the column, and peak TH-B which was the dominant one (Fig. 4*a*) and required salt for elution from the column. Peak TH-A was relatively richer in peptide material, as judged by absorption at 230 nm, compared with carbohydrate than was TH-B, but in each case, non-carbohydrate-containing peptide could be separated from the glycopeptides by gel filtration on Sephadex G-25 with water as eluting agent (Fig. 5*a*). The recoveries of carbohydrate in glycopeptide fractions GP-TH-I and GP-TH-II were 1.5 and 70%, respectively (an overall recovery of ~72%).

The pronase digest of asialo-Tamm–Horsfall glycoprotein, upon fractionation in the same way (Figs. 2*b* to 5*b*), yielded glycopeptide fractions GP-AsTH-I and GP-AsTH-II with recoveries of carbohydrate of 49% and 24%, respectively [an overall yield of 73% (Table I)].

Three of the four glycopeptide fractions (GP-TH-I, GP-AsTH-I, and GP-AsTH-II) gave predominantly one compact spot each when subjected to high-voltage electrophoresis at pH 5.0 and visualised with ninhydrin reagent (Table II). The mobility, as assessed from that of mannose, was the same as that due to electroendosmosis. One of the fractions, which exhibited extensive mobility towards the anode, was GP-TH-II. It gave an elongated spot (Table II) which suggests the possibility of

TABLE II

MOBILITIES OF GLYCOPEPTIDE FRACTIONS UPON HIGH-VOLTAGE ELECTROPHORESIS AT pH 5.0

Glycopeptides	*Mobilities*[a] *(cm)*	
	Anodic	*Cathodic*
GP-TH-I		1.1–2.5 (intense)
GP-TH-II	2.7–7.7 (intense)	1.3–2.1 (very weak)[b]
GP-AsTH-I	0.2–1.6 (very weak)	0.8–2.0 (intense)
GP-AsTH-II		0.8–2.1 (intense)

[a]The degree of staining with ninhydrin is indicated in parentheses. [b]Staining with ninhydrin in acetone, containing pyridine, gave a yellow–brown colour.

TABLE III

APPARENT MOLECULAR WEIGHTS OF GLYCOPEPTIDES DERIVED FROM HUMAN TAMM–HORSFALL GLYCOPROTEIN

Glycopeptide	*Conc. (mg/ml)*	*Speed (r.p.m.)*	*No. of measurements*	*M*
GP-TH-II	3.5	29,500	3	4870
GP-TH-II	3.0	29,500	3	4950
GP-TH-II	2.5	29,500	3	4980
GP-TH-II	2.0	29,500	3	4990
GP-TH-II	3.5	44,770	5	4540
GP-TH-II	3.0	44,770	5	4710
GP-TH-II	2.5	44,770	5	4600
GP-TH-II	2.0	44,770	5	4870
GP-AsTH-I	2.0	44,770	10	4530
GP-AsTH-II	1.5	44,770	10	2150

TABLE IV

COMPOSITION (RESIDUES/MOLE) OF GLYCOPEPTIDES AND OF TAMM–HORSFALL GLYCOPROTEIN[16]

	Glycoprotein	*GP-TH-I*	*GP-TH-II*	*GP-AsTH-I*	*GP-AsTH-II*
Fucose	4.3	0.16	1.0	1.2	1.1
Galactose	36	0.73	5.0	6.9	4.0
Mannose	27	5.9	3.2	3.6	3.3
N-Acetylglucosamine	38	1.9	7.0	8.6	3.7
N-Acetylgalactosamine	11[a]	0.15	0.92	0	0.87
Sialic acid	19	0	4.5	0	0
Aspartic acid		1.0	1.5	2.9	1.0
Threonine		0.70	0.50	1.0	0.31
Serine		0.73	1.0	1.3	0.44
Glutamic acid		0.47	0.23	0.76	0.19
Proline		0	0.29	0 70	0.16
Glycine		0.63	0.23	0.80	0.14
Alanine		0.33	0.47	0.70	0.26
Half-cystine		0	0.29	0.43	0.15
Leucine		0.11	0.10	0.36	0.16
Valine		0.16	0	0	0
Mol. wt. of glycopeptide or glycoprotein (determined)	79,000	n.d.[b]	4810	4530	2150
Average mol. wt. of carbohydrate moiety (calc.)		1514[b]	4354[c]	3640[c]	2320[d]

[a]Reported, variously, from 3.6[23] to 15.5[21] residues per mole. The amount present in a given preparation may depend upon the proportion of blood-group Sd[a]-positive protein present[25]. [b]Not determined because of paucity of material. The calc. value is based on 1 residue of aspartic acid; there must be at least one asparagine-linked carbohydrate moiety in the glycopeptide. [c]Based on the composition, and measured molecular weight, of the glycopeptide. [d]Based on one residue of aspartic acid.

variations in the sialic acid content of the carbohydrate moieties. GP-TH-II contained, as well as the major anodic glycopeptide, traces of a neutral substance at pH 5.0. In view of the yellow–brown colour given with the ninhydrin reagent, it is likely to be an oligosaccharide moiety linked to a single asparagine residue[22] and probably lacking sialic acid.

The molecular weights of the glycopeptides determined in the ultracentrifuge, apart from GP-TH-I for which too little was available, are indicated in Table III. There was no trend with time in any set of measurements, confirming that an adequate interval had been allowed for equilibration. The apparent molecular weight of GP-TH-II, calculated from the equilibrium data, was larger at lower concentrations of the glycopeptide solutions on which measurements were made, at both speeds (Table III). These observations serve to emphasise the accuracy of the technique. The values found at 44,770 r.p.m. are ~5% smaller than those at 29,500 r.p.m., which is suggestive of a degree of polydispersity. Because of these uncertainties, mean values are noted in Table IV, which also includes the molar amounts of the sugars and the amino acids present in the glycopeptides.

DISCUSSION

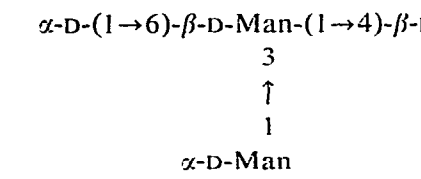
Treatment of human Tamm–Horsfall glycoprotein with 0.05M H_2SO_4 at 80° for 1 h led to partial degradation of the polypeptide chain (Fig. 1)[26]. Similar partial fragmentation of the glycoprotein isolated from the urine of hamsters[27] and of cattle[28] occurs under similar conditions. The digest was fractionated to give glycopeptides GP-AsTH-I and GP-AsTH-II, the latter being the smaller (Table III).

GP-AsTH-II contained 8.4 neutral sugar residues (Table IV) per mole and was recovered in 24% yield. Since the whole glycoprotein contains 67 neutral sugar residues[16], the former must contain at least two carbohydrate moieties of the type found in GP-AsTH-II, on the reasonable assumption that there was no substantial removal of any type of sugar other than sialic acid during the acid treatment of the glycoprotein.

The lack of sialic acid residues in this glycopeptide would explain its behaviour upon electrophoresis at pH 5.0, although it would seem that the interaction with DEAE-Sephadex A-25 in 0.1M acetic acid is likely to involve forces other than charge effects. This glycopeptide, like all the others, is heterogeneous with respect to the peptide moiety, because of incomplete enzymic splitting of the peptide bonds around a carbohydrate moiety, and the reasons for this have been discussed[29]. The polydispersity seen in the ultracentrifuge is probably due to this fact, as well as to the known heterogeneity of the carbohydrate moieties of glycoproteins.

The other glycopeptide obtained from asialo-Tamm–Horsfall glycoprotein, GP-AsTH-I, carries a carbohydrate moiety which is richer in galactose and in 2-acetamido-2-deoxyglucose (*N*-acetylglucosamine) than is GP-AsTH-II. In view of the mannose content of the glycopeptide, it seems likely that there is only one asparagine-linked carbohydrate moiety in the glycopeptide. This is because the "core"

region of carbohydrate prosthetic groups linked in this way almost invariably have the structure (see ref. 30):



so that the mannose content of GP-AsTH-I is largely accounted for.

GP-AsTH-I was predominantly neutrally charged at pH 5.0, so that the aspartic acid residues present in acid hydrolysates of the glycopeptide must have been present either as asparagine or as glycosylated asparagine residues. The glycopeptide contains 11.7 neutral sugar residues and was recovered in 49% yield. Thus, on average, there are at least three carbohydrate moieties of the type present in GP-AsTH-I in the molecule of the asialo-glycoprotein.

An interesting glycopeptide, GP-TH-I, was isolated from digests of Tamm–Horsfall glycoprotein itself. The recovery of neutral sugar in this fraction was <2%. This glycopeptide was richer in mannose than any of the other glycopeptides, containing, in addition to the three mannose residues expected in the "core" region of the carbohydrate moiety, three further mannose residues: it lacked sialic acid. It might represent a carbohydrate incompletely "processed" during synthesis[31].

The major glycopeptide fraction derived from Tamm–Horsfall glycoprotein, GP-TH-II, was obtained in a yield of 70%, and its content of sialic acid would explain why it was negatively charged at pH 5.0. It is likely to be a mixture of sialylated analogues of GP-AsTH-I and GP-AsTH-II, each of which must have a carbohydrate moiety with a molecular weight close to 4300. From a consideration of the molecular weights of GP-AsTH-I and GP-AsTH-II, the sialylated form of the former would contain 2 or 3 sialic acid residues and that of the latter 5 or 6 residues. A mixture of sialylated forms of GP-AsTH-I and GP-AsTH-II in the ratio of 3:2, as might be expected in the whole glycoprotein (see above), may be calculated to contain 1.2 fucose, 5.7 galactose, 3.5 mannose, and 6.7 *N*-acetylglucosamine residues, which are of the order found for GP-TH-II.

The mode of attachment of the *N*-acetylgalactosamine residues is unknown. It is reported that alkaline treatment of human[32] or of calf[28] Tamm–Horsfall glycoprotein does not cause significant loss of serine or threonine residues, but not all glycosylated amino acids of these types readily undergo β-elimination under alkaline conditions[33,34].

Tamm–Horsfall glycoprotein has at least five asparagine residues substituted by complex carbohydrate moieties, three being of one type, relatively rich in galactose, and two of another having more sialic acid but less galactose. Each has a molecular weight of ~4300. Their distribution along the polypeptide chain is unknown. It has been suggested[35] that all of the asparagine-linked carbohydrate moieties of the glycoprotein are restricted to a relatively short segment of the protein chain, largely

on the assignment of a molecular weight of 18,000 to glycopeptides derived from Tamm–Horsfall glycoprotein. This value was calculated from the results of gel-filtration experiments performed in 0.1M acetic acid, and it seems likely to be an overestimate.

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