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Tamm–Horsfall Urinary Glycoprotein

THE CHEMICAL COMPOSITION

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1. A revised amino acid and carbohydrate composition of human Tamm–Horsfall glycoprotein is presented. 2. No significant differences were obtained in the amino acid composition of Tamm–Horsfall glycoprotein isolated from patients with cystic fibrosis. 3. The glycoprotein was shown to possess a high half-cystine content of 1 per 11–12 amino acid residues, which has been confirmed by performic acid oxidation and *S*-alkylation with iodoacetate and iodoacetamide. No thiol groups were detected in the glycoprotein. 4. Treatment of the glycoprotein with 0.5 M-sodium hydroxide at 4°C for 2 days did not release heterosaccharide material, which suggests that the predominant carbohydrate–protein linkages present are not of the *O*-glycosidic type. 5. No *N*-terminal amino acid was detected in the glycoprotein.

A glycoprotein of high molecular weight was prepared from human urine by Tamm & Horsfall (1950, 1952) by adding sodium chloride to increase the salt concentration of urine by 0.58 M. Evidence suggests that the T–H glycoprotein* originates in the kidney, and labelled-antibody techniques (Friedmann, 1966; Keutel, 1965; McKenzie & McQueen, 1969) have indicated that T–H glycoprotein is associated with the epithelial cells of the kidney tubule. T–H glycoprotein has been identified by both chemical and immunological methods as a major component of urinary casts occurring in the nephrotic syndrome (McQueen, 1962; Fletcher, McLaughlin, Ratcliffe & Woods, 1970*a*). Sedimentation studies by Tamm, Bugher & Horsfall (1955) and Maxfield (1961) have suggested that the glycoprotein has a molecular weight of at least 7.0×10^6, and from viscosity data a mean axial ratio of over 100 was calculated, indicating that the macromolecule is highly asymmetrical. Electron microscopy confirmed that the glycoprotein possessed an unbranched fibrillar structure, possibly consisting of a linear arrangement of subunits (Porter & Tamm, 1955; Bayer, 1964; Fletcher *et al.* 1970*a*). Maxfield & Wolins (1962) reported that T–H glycoprotein from the urine of patients with cystic fibrosis had an abnormally high molecular weight and possessed a greater tendency to aggregate compared with the T–H glycoprotein from normal urine. They suggested that these abnormalities were a direct expression of the genetic defect in cystic fibrosis.

* Abbreviation: T–H glycoprotein, Tamm–Horsfall glycoprotein.

The amino acid analyses of T–H glycoprotein by Maxfield & Stefanye (1962) and Friedmann & Johnson (1966*a*,*b*) show discrepancies that are beyond the usual limits of experimental error. Amino acid analysis has been included in the present investigation in an attempt to account for these differences. It was thought possible that careful amino acid analysis of T–H glycoprotein in cystic fibrosis might reveal significant differences in this disease. Carbohydrate analyses on T–H glycoprotein by Gottschalk (1952), Odin (1952), Rozenfel'd & Yusipova (1967) and Schwartz & Pallavicini (1967) have agreed that the glycoprotein contains about 25% carbohydrate, although the amounts of the individual sugars varied considerably. Preparations of high-molecular-weight urinary glycoproteins by King, Fielden & Boyce (1961) have been shown to contain small amounts of lipid, and this has been confirmed by Fletcher *et al.* (1970*a*) in both soluble T–H glycoprotein and T–H glycoprotein isolated from urinary casts.

MATERIALS

Ethyleneimine and serotonin creatinine sulphate were obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.), sialic acid, 5,5′-dithiobis-(2-nitrobenzoic acid) and crystalline insulin (bovine pancreas) were from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.), *S*-carboxymethylcysteine, *S*-2-aminoethylcysteine and DNS chloride were from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and 'polyamide layers' were from the Cheng-Chin Trading Co. Ltd. (no. 75 Section 1, Hankow Street, Taipei, Taiwan). Mannose, fucose, glucosamine

hydrochloride, sodium *p*-chloromercuribenzoate, iodoacetic acid and iodoacetamide had been recrystallized before use. Ovomucoid was obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Ovalbumin was prepared by Dr R. D. Marshall. Guanidine hydrochloride was prepared from guanidine carbonate (BDH Chemicals Ltd.) and recrystallized from aqueous ethanol after treatment with charcoal.

METHODS

Preparation. The glycoprotein was precipitated from pooled samples of urine from male medical students by the addition of sufficient NaCl to increase the concentration by 0.58 M. The precipitate was collected by centrifugation, washed at least once with 0.58 M-NaCl and then exhaustively dialysed against water. After two further precipitations with 0.58 M-NaCl the final aqueous solution of the glycoprotein was centrifuged at 13000*g* for 30 min and the supernatant was freeze-dried. The T–H glycoprotein was also prepared from a 5-litre pooled sample of urine from two fibrocystic children that had been collected in the presence of toluene.

Amino acid analysis. Dry weights of the glycoproteins were obtained after drying *in vacuo* at 100°C for 24 h. The glycoprotein was hydrolysed *in vacuo* by constant-boiling HCl at 110°C for 21 h. Analysis was carried out on a Technicon amino acid analyser with the standard buffer gradient. In certain analyses norleucine was added before hydrolysis as a standard. Half-cystine was determined as cysteic acid after performic acid oxidation of the glycoprotein for 4 h at 0°C (Hirs, 1956). Glucosamine and galactosamine were resolved when the starting buffer was changed from pH 2.875 to 2.80. The glycoprotein was hydrolysed for 14, 21, 48 and 70 h and correction factors were obtained by extrapolation for the destruction or incomplete hydrolysis of certain amino acids in a 21 h hydrolysis. The ash content of the glycoprotein was obtained after heating of the dry glycoprotein at 400°C.

Tryptophan and tyrosine were measured spectroscopically by the method of Beaven & Holiday (1952) on the basis of the u.v. absorption in 0.1 M-NaOH, and values were calculated from the formula of Goodwin & Morton (1946). A second method, based on absorption measurements in 6 M-guanidine hydrochloride at neutral pH, was also used (Edelhoch, 1967). Corrections were made for absorption not due to tryptophan and tyrosine by taking extinction measurements at 320 and 360 nm and projecting these as far as 280 nm, thus forming a base line for absorption measurements, as suggested by Beaven & Holiday (1952).

Amide nitrogen content. Sialic acid is degraded during acid hydrolysis of amide groups, and releases NH_3. The amide nitrogen content was therefore determined by a modification of the two-step hydrolysis method recommended by Marshall & Gottschalk (1966) in which sialic acid is removed before amide hydrolysis. All NH_3 measurements were related to a known weight of glycoprotein that had been dried overnight at 90°C *in vacuo* over P_2O_5. Sialic acid was released by hydrolysis in 50 mM-H_2SO_4 at 80°C for 1 h. As a control, glycoprotein was allowed to stand in 50 mM-H_2SO_4 at room temperature for 1 h. A portion of the hydrolysate was added to the outer well of a Conway unit, and the NH_3 was liberated by the addition of 0.5 ml of saturated K_2CO_3 and distilled to the centre well containing 1 ml of 10 mM-H_2SO_4 during 18 h at 5°C. The contents of the centre well were made up to a known volume, and the NH_3 was determined with a ninhydrin reagent (Rosen, 1957). The sialic acid-free protein was extensively dialysed against water and recovered by freeze-drying. Amide NH_3 was released in 2 M-HCl at 100°C for 3 h in evacuated sealed ampoules, and the hydrolysates were evaporated to dryness *in vacuo* over P_2O_5 and NaOH pellets. Samples of protein dissolved in 2 M-HCl at room temperature for 3 h served as controls for the amide hydrolysis. The dried hydrolysates were washed into the outer well of a Conway unit with about 1 ml of water and the NH_3 was determined as described above.

Reduction and alkylation. The method used was similar to that described by Press, Piggot & Porter (1966) for the complete reduction and alkylation of γ-globulin, in the presence of 6 M-guanidine hydrochloride. The reduced glycoprotein was alkylated with a 1.2 M excess of iodoacetamide relative to 2-mercaptoethanol, or a 1.5 M excess of iodoacetic acid, for approx. 1 h at 0°C or until the reaction solution gave a negative nitroprusside reaction. The pH was maintained at 8.0 by the addition of M-NaOH. Alkylation with a five- to ten-fold excess of ethyleneimine (Raftery & Cole, 1966) at 0°C took about 30 min and the pH was maintained at 8.0 by the addition of M-HCl. The reaction solutions were desalted on either a column (60 cm × 2.5 cm diam.) of Sephadex G-25 in 0.2 M-acetic acid or by dialysis and the protein was recovered by freeze-drying. *S*-Carboxymethylcysteine and *S*-2-aminoethylcysteine were used as reference standards in amino acid analysis.

Determination of free thiol groups. (1) Alkylation with iodoacetamide without prior reduction. The glycoprotein was dissolved in 6 M-guanidine hydrochloride in 0.5 M-tris–HCl buffer, pH 8.2, and allowed to react at 0°C for 30 min with a twofold molar excess of iodoacetamide relative to the known half-cystine content of the glycoprotein. The pH was adjusted to 5.0 with acetic acid and the solution was dialysed against water at 4°C. The *S*-carboxymethylcysteine content of the hydrolysed glycoprotein was determined by amino acid analysis.

(2) Mercaptide formation with *p*-chloromercuribenzoate. The reaction of *p*-chloromercuribenzoate with ovalbumin and T–H glycoprotein was followed spectrophotometrically in 50 mM-sodium phosphate buffer, pH 7.4, by the method of Isles & Jocelyn (1963).

(3) Reaction with 5,5′-dithiobis-(2-nitrobenzoic acid). A modification of the conditions of Janatova, Fuller & Hunter (1968) was used to measure the reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) with ovalbumin and T–H glycoprotein in 6 M-guanidine hydrochloride in 10 mM-sodium phosphate buffer, pH 6.9.

Alkali treatment of the glycoprotein. The glycoprotein (50 mg) was dissolved in 10 ml of 0.5 M-NaOH and dialysed against 0.5 M-NaOH at 4°C for 2 days, followed by several changes of water, and then recovered by freeze-drying. Subsequent neutral sugar and amino sugar analysis, and amino acid analysis, were related to the dry weight of the alkali-treated glycoprotein.

Carbohydrate analysis. All sugar analyses were related to the dry weight of the glycoprotein. Neutral sugars were determined by the orcinol–H_2SO_4 method, modified by Johansen, Marshall & Neuberger (1960) and François,

Marshall & Neuberger (1962). Hexosamines were released by hydrolysis of the glycoprotein in 4M-HCl at 100°C for 4h in evacuated sealed ampoules. The procedure of Kraan & Muir (1957) was used to measure hexosamines, with recrystallized glucosamine hydrochloride as standard. The method of Gibbons (1955) was used for analysis of fucose. Sialic acid was determined by the method of Warren (1959*a*) after hydrolysis in 50mM-H_2SO_4 at 80°C for 1h.

High-voltage paper electrophoresis. The glycoprotein (2mg) was subjected to high-voltage electrophoresis at pH2.0 in 1.5M-formic acid–2M-acetic acid buffer for 20min at 70V/cm. Amino acids were detected by a ninhydrin spray reagent.

N-*Terminal analysis.* (1) The cyanate method for the identification of *N*-terminal residues described by Stark (1967) was applied to both the native and the iodoacetamide-alkylated glycoprotein, and also to crystalline insulin. Ion-free 8M-urea was included in the carbamoylation reaction. The cyclization step was carried out in the presence of serotonin creatinine sulphate to prevent destruction of tryptophan. A separate blank determination was performed with cyanate omitted from the carbamoylation step to correct for small quantities of amino acids not derived from *N*-termini. Hydantoin fractions A and B were obtained as described by Stark (1967) from a Dowex 50 (X2) column. Fractions C_1 and C_2 were combined to give a fraction C before they were hydrolysed for amino acid analysis. (2) The DNS chloride *N*-terminal method described by Gray (1967) was applied to both the native and the iodoacetamide-alkylated glycoprotein and also to insulin and ovomucoid. The dansylation of ovomucoid and T–H glycoprotein was carried out in 8M-urea. Insulin was dansylated in 0.5M-$NaHCO_3$. DNS-amino acids were identified after high-voltage electrophoresis at pH4.4 and 1.9 (Gray, 1967) and by two-dimensional 'polyamide-layer' chromatography as described by Woods & Wang (1967) with their solvents 1 and 2. In both methods ethyl acetate extracts of the hydrolysate gave much clearer separations (Gray, 1967).

Lipid. The glycoprotein was extracted with chloroform-methanol (2:1, v/v) at room temperature. The extract was back-washed with 0.2vol. of 0.1% KCl and the chloroform layer was evaporated to dryness under N_2. Samples of the glycoprotein, the lipid extracts and the lipid-extracted glycoprotein were analysed for phosphorus (Bartlett, 1959). Total cholesterol was determined by the procedure of Henly (1957).

RESULTS AND DISCUSSION

T–H glycoprotein is quantitatively an important constituent of normal urine, and is readily prepared by repeated salt precipitations, which yield a material relatively constant in its physical properties and chemical composition. In the following paper (Fletcher, Neuberger & Ratcliffe, 1970*b*) the subunit structure of the protein is investigated by disc gel electrophoresis and gel chromatography on Sephadex G-200 in the presence of the dissociating agent sodium dodecyl sulphate. By both techniques the subunit of the protein appeared to be a single polypeptide chain of molecular weight approx. 80000, and there was no evidence to suggest the presence of contaminating proteins in the preparation. The restricted number of *N*-terminal groups released by specific cyanogen bromide cleavage of the glycoprotein is further evidence of its homogeneity. Friedmann & Johnson (1966*b*) have shown that T–H glycoprotein on high-voltage electrophoresis in formic acid at pH2.1 produced nine ninhydrin-positive spots, which they attributed to free amino acid adsorbed on the glycoprotein. We were unable to demonstrate the presence of amino acids adsorbed to our preparation by high-voltage electrophoresis with loads of up to 2 mg of glycoprotein.

Amino acid composition. The serine and threonine values obtained by amino acid analysis after increasing times of hydrolysis were extrapolated to zero time, indicating that 10% and 6% respectively were destroyed after 21h of hydrolysis. Similarly extrapolation to infinite time of hydrolysis of the valine and isoleucine values showed that after 21h 6 and 11% respectively were incompletely released. These correction factors have been applied to all our amino acid analyses. All other amino acids, except for half-cystine, appeared to be stable for up to 70h of hydrolysis. T–H glycoprotein contains all the amino acids normally present in proteins and no unidentified peaks were obtained. In Table 1 the amino acid content of T–H glycoprotein is expressed as mol of residues/100000g of dry protein, which represents a quantitative recovery of anhydro amino acid residues of 68%. These values have also been expressed in Table 1 as residues/100 residues to compare the relative proportions of the amino acids present with those found by earlier workers. The greatest differences between our results and those of Maxfield & Stefanye (1962) and Friedmann & Johnson (1966*b*) are in the values obtained for half-cystine, tryptophan and tyrosine. The same amino acid composition, within the limits shown for the native glycoprotein (Table 1), was obtained for the various alkylated modifications of this preparation and also other preparations of T–H glycoprotein.

The *S*-carboxymethylcysteine contents of the iodoacetate- and iodoacetamide-alkylated protein were 8.8 and 8.7% respectively. These values are in good agreement with the cysteic acid content of the glycoprotein and confirm the high half-cystine content. Alkylation with ethyleneimine was incomplete, however, and converted only 77% of the cystine into *S*-aminoethylcysteine, the remainder not being recovered on amino acid analysis. Stevenson & Kent (1970) obtained a half-cystine content, determined as cysteic acid, of 50.0mol of residues/100000g, in good agreement with our values.

Table 1. *Amino acid composition of T–H glycoprotein*

The standard deviations were calculated from the analysis of six and four hydrolysates of T–H glycoprotein from normal and fibrocystic subjects respectively.

	Amino acid composition				
	(mol of residues/100000g of protein)		(residues/100 residues in the protein)		
			Native T–H glycoprotein		
	Native T–H glycoprotein	Fibrocystic T–H glycoprotein	Present work	Maxfield & Stefanye (1962)*	Friedmann & Johnson (1966b)*
Asp	67.4 ± 0.9	65.1 ± 1.3	10.90	11.83	11.75
Thr	47.2 ± 0.9	47.4 ± 1.7	7.63	8.22	7.67
Ser	48.6 ± 1.2	51.0 ± 1.8	7.86	8.55	8.00
Glu	52.2 ± 2.0	50.9 ± 1.0	8.44	8.55	9.22
Pro	28.6 ± 1.8	29.4 ± 3.0	4.26	5.61	4.73
Gly	52.0 ± 1.5	53.4 ± 3.4	8.41	8.88	8.28
Ala	42.0 ± 1.8	41.6 ± 1.8	6.79	6.72	7.89
Val	39.6 ± 1.3	38.7 ± 1.2	6.40	6.89	5.91
Cys†	52.0 ± 0.5	50.3 ± 1.4	8.41	2.17	7.12
Met	12.7 ± 0.9	12.0 ± 0.4	2.05	2.22	2.05
Ile	15.2 ± 0.6	14.6 ± 0.7	2.46	2.67	2.55
Leu	46.9 ± 1.4	45.7 ± 0.6	7.58	8.27	7.64
Tyr	23.7 ± 0.8	21.1 ± 0.5	3.83	2.78	3.15
Phe	19.4 ± 0.8	19.0 ± 1.0	3.14	3.44	3.35
Lys	16.4 ± 0.7	15.0 ± 0.6	2.65	2.78	3.10
His	16.5 ± 0.9	14.8 ± 0.5	2.67	2.78	2.54
Arg	27.8 ± 0.9	26.4 ± 1.0	4.49	4.72	3.92
Trp	10.4 ± 0.3‡	(10.4 ± 0.3§)	1.68	2.94	1.11
Total	618.6	606.9	100.00	100.02	99.98

* Recalculated from the published data.
† Determined as cysteic acid.
‡ Average of two spectrophotometric methods.
§ Assumed from native T–H glycoprotein.

No free thiol groups were detected in the glycoprotein in phosphate buffer with *p*-chloromercuribenzoate, or in 6M-guanidine hydrochloride with either 5,5′-dithiobis-(2-nitrobenzoic acid) or iodoacetamide. Alkylation with iodoacetamide yielded less than 1 mol of residue of *S*-carboxymethylcysteine/100000g. In control experiments with ovalbumin, of the four cysteine residues known to be present (Fothergill & Fothergill, 1967) 3.8 and 3.7 residues reacted with *p*-chloromercuribenzoate and 5,5′-dithiobis-(2-nitrobenzoic acid) respectively. The absence of free thiol groups from T–H glycoprotein implies that it has an unusually high cystine content, about 1 per 11–12 amino acid residues being half-cystine. Since the reduced and alkylated glycoprotein and the native glycoprotein both had similar molecular weights in the presence of the denaturing agent sodium dodecyl sulphate (see Fletcher *et al.* 1970*b*), these disulphide bonds must be intrachain and involved only in the folding of the subunit polypeptide chain. Non-covalent bonds must therefore be involved in the aggregation of the protein subunits in the native macromolecule.

Amino acid analysis of T–H glycoprotein from fibrocystic subjects revealed that its amino acid composition was very similar to that of the glycoprotein from normal pooled urine. Friedmann & Johnson (1966*b*) also claim to have found no significant difference in the amino acid composition of T–H glycoprotein, from normal and fibrocystic subjects, although they obtained considerable variations in certain residues. Amino acid analysis cannot be expected to detect small changes in the primary structure of the protein should they exist. A reinvestigation of the viscosity of T–H glycoprotein in cystic fibrosis by Stevenson (1969) has failed to reveal a difference in $[\eta]$ of T–H glycoprotein from normal and fibrocystic subjects. This, together with the similarity of carbohydrate composition and immunological properties found by Schwartz & Pallavicini (1967), have so far failed to account for the tendency of T–H glycoprotein from fibrocystic subjects to exist as an aggregate of higher molecular weight as claimed by Maxfield & Wolins (1962).

The measurement of tyrosine by amino acid

Table 2. *Comparison of the tyrosine and tryptophan contents of T–H glycoprotein measured spectrophotometrically and by amino acid analysis*

The standard deviations were calculated from the numbers of determinations given in parentheses.

	Amino acid content (mol of residues/100000g of protein)	
Method	Tyrosine	Tryptophan
Amino acid analysis	23.7 ± 0.8 (6)	Absent
Edelhoch (1967)	23.4 ± 2.0 (3)	10.3 ± 0.3 (3)
Beaven & Holiday (1952)	23.1 ± 1.2 (5)	10.5 ± 0.3 (5)

analysis can occasionally give low values owing to chlorination during acid hydrolysis, but we found tyrosine to be stable for prolonged periods of acid hydrolysis. The value obtained by amino acid analysis compared favourably with those obtained from both spectrophotometric methods (Table 2). The values for tryptophan obtained were also in good agreement, but were considerably lower than the values obtained by Maxfield & Stefanye (1962). Solutions of the freeze-dried glycoprotein in water (0.5mg/ml) produced considerable light-scattering, and base-line corrections were up to 48% of the total extinction at 277nm. The base-line correction was considerably less if the preparation of glycoprotein had not been freeze-dried. In this case the correction was 28% of the total extinction at 277nm and was proportional to the glycoprotein concentrations in the range examined (0.2–0.8mg/ml). The disaggregating effect of the solvent 6M-guanidine hydrochloride decreased the correction required to 10% of the total extinction of a solution of 0.5 mg/ml. However, after these corrections had been made, the extinction coefficients of the protein in water and guanidine were found to be very similar. $E_{1\,cm}^{1\%}$ at 277nm in 6M-guanidine hydrochloride was found to be 9.5 and that in water 9.4. Maxfield (1961) obtained a higher value (10.8) for the extinction coefficient of T–H glycoprotein in water. Such a value could be obtained by failure to correct for light-scattering and non-specific absorption.

Maxfield & Stefanye (1962) calculated a minimum molecular weight of 28100 for T–H glycoprotein from its amino acid composition. More recent evidence, however, has shown that the subunit molecular weight of T–H glycoprotein is in the region of 80000–100000 (Friedmann & Johnson, 1966*a*; Stevenson & Kent, 1970; Fletcher *et al.* 1970*b*), and it would therefore be unwise to attempt to calculate the minimum molecular weight of such a large polypeptide chain from its amino acid composition.

Amide nitrogen content. Sialic acid is known to be quantitatively released in 50mM-sulphuric acid at 80°C for 1h (Warren, 1959*b*) and the ammonia released under these conditions is derived from amide groups in the protein. Under these conditions 4.9mol of ammonia was liberated/100000g. Hydrolysis of the sialic acid-free protein by 2M-hydrochloric acid at 100°C for 3h released a further 48.8mol of ammonia/100000g. The amide content is therefore the sum of the ammonia released in both hydrolytic steps, namely 54mol of residues/100000 g. This value represents the total amide content of the glycoprotein, and includes the *N*-acylglycosylamine carbohydrate-protein linkages. Amino acid analysis revealed an excess of 59mol of acidic over basic residues/100000g of protein, 54 of which are amidated or involved in carbohydrate–protein linkages. The low isoelectric point of the glycoprotein of approx. pH 3.0 (Tamm & Horsfall, 1950) is therefore due to the presence of 14.6mol of sialic acid residues/100000g and an excess of five acidic amino acids. Maxfield & Stefanye (1962) estimated the amide content to be 38mol of residues/100000g after hydrolysis in constant-boiling hydrochloric acid at 110°C. An amide value obtained by these hydrolysis conditions would be a serious overestimate, since it would include ammonia derived from the degradation of sialic acid, amino sugars and certain amino acids. It is somewhat surprising therefore that their value is considerably lower than the value we have obtained.

Table 3. *Carbohydrate and protein composition of T–H glycoprotein*

The carbohydrate compositions are expressed as free monosaccharides. For details of the alkali treatment see the Methods section. N.D., Not determined.

	Composition (% of dry wt.)	
	T–H glycoprotein	Alkali-treated T–H glycoprotein
Hexose	11.7 ± 0.3	11.5 ± 0.2
Fucose	0.8 ± 0.2	N.D.
N-Acetylhexosamine	11.2 ± 0.2	11.0 ± 0.2
Sialic acid	4.4 ± 0.2	N.D.
Ash	2.3	N.D.
Protein	67.6	N.D.

Carbohydrate composition. The sum of carbohydrate values (Table 3) represents a total carbohydrate content of 28%, if it is assumed that the hexosamine is *N*-acetylated. A total hexosamine content of 9.1% was obtained by a modification of the Elson–Morgan reaction after acid hydrolysis. Graham & Neuberger (1968), by an isotope-dilution

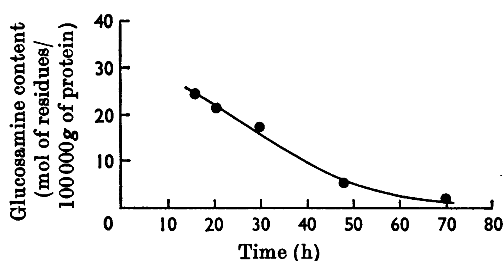

Fig. 1. Destruction of glucosamine in T–H glycoprotein by hydrolysis in constant-boiling HCl at 110°C *in vacuo*.

technique, found values of 9.8 and 1.6% respectively for the glucosamine and galactosamine contents of this preparation of T–H glycoprotein. From this glucosamine : galactosamine ratio the glucosamine content calculated from our total hexosamine value is 7.8% or 43.6 mol of residues/100000g. Both glucosamine and galactosamine were resolved on amino acid analysis, but the galactosamine peak was usually too small to be accurately measured. The recovery of glucosamine decreased considerably with increasing times of hydrolysis for amino acid analysis (Fig. 1), and in view of the high rate of destruction it seems unwise to extrapolate the curve to zero time of hydrolysis. The glucosamine content of a 21h hydrolysate was 22 mol of residues/100000g of protein, which is approx. 50% of the value obtained after hydrolysis in 4M-hydrochloric acid at 100°C for 4h. A similar value for the destruction of glucosamine in constant-boiling hydrochloric acid at 110°C for 20h was obtained by Nolan & Smith (1962). The rate of destruction of glucosamine is probably dependent on the concentration of other hydrolysis products of the glycoprotein, especially cysteine and tryptophan. The *N*-acetylhexosamine values obtained by g.l.c. by Stevenson & Kent (1970) are very close to our value of 11.2%. Some preliminary work in this laboratory by Dr J. G. Ratcliffe on the g.l.c. of the trimethylsilyl derivatives of the neutral sugars of the glycoprotein by the method of Bolton, Clamp & Hough (1965) gave somewhat higher values for the hexose content. However, the galactose : mannose ratio was found to be 1.0 : 1.0, in agreement with the values given by Stevenson & Kent (1970), and not 2.0 : 1.0 as stated by Gottschalk (1952). The presence of glucose in the glycoprotein could not be demonstrated by this technique, contrary to the findings of Rozenfel'd & Yusipova (1967).

The values in the literature for the sialic acid content of T–H glycoprotein are particularly variable, ranging from 0 to over 10%. The sialic acid values we obtained were consistently in the region of 4% for different preparations assayed at different times. It is possible that some of the higher values for sialic acid that have been reported were due to the use of less specific methods of analysis. We have observed that bacterial contamination occurring during the preparation resulted in a low value of sialic acid (1%), although the composition of the other sugars was unchanged.

Carbohydrate analysis of T–H glycoprotein by g.l.c. gave a carbohydrate content of 30.8% (Stevenson & Kent, 1970) and the percentage compositions of the sugars present expressed as ratios were as follows: hexose : *N*-acetylhexosamine : sialic acid : fucose (0.42 : 0.34 : 0.20 : 0.04). These compare well with our values (0.41 : 0.40 : 0.16 : 0.03). The sugar proportions reported by earlier workers have been highly variable and may be partly due to the difficulties involved in determining the various carbohydrate components present (Neuberger & Marshall, 1966). It is also possible that differences in carbohydrate composition exist between preparations as a result of heterogeneity in the carbohydrate prosthetic groups, as has been found in so many glycoproteins (Marshall & Neuberger, 1970).

The glycoprotein was treated under mildly alkaline conditions (see the Methods section) to determine whether alkali-labile *O*-glycosidic linkages were present. Amino acid analysis of the alkali-treated glycoprotein revealed a very small and probably insignificant decrease in both serine and threonine. The serine and threonine contents of the alkali-treated protein were 7.8 and 7.5% respectively. Cystine was almost completely absent, but *meso*-lanthionine and lanthionine resulting from the alkaline degradation of cystine emerged from the column between alanine and valine (Williams & Woodhouse, 1967). The neutral sugar and amino sugar contents of the glycoprotein, related to its dry weight, were not significantly altered by the alkali treatment (Table 3). The predominant type of carbohydrate–protein linkage in T–H glycoprotein is therefore stable in the alkaline conditions known to hydrolyse the *O*-glycosidic linkages, present in certain glycoproteins, that involve the hydroxyl groups of serine and threonine (Anderson *et al.* 1964). This indirect evidence therefore suggests that the linkages may be of the *N*-acylglycosylamine type.

Lipid composition. Fletcher *et al.* (1970*a*) have shown that the small quantity of lipid associated with T–H glycoprotein consisted of triglycerides, free fatty acids, free and esterified cholesterol and phospholipids. Phosphorus determinations on T–H glycoprotein were in the range 0.7–1.25 μg/mg, only part of which could be removed by lipid extraction. Non-extractable phosphorus corresponded to 2.07 mol/100000g and could possibly be present in the glycoprotein as phosphate covalently linked to

serine. The average phospholipid content of the preparation was found to be 0.26% and the total cholesterol 0.15%. The presence of lipid was found to be a constant feature of all our preparations and, although we have not determined all the lipid components quantitatively these values suggest that the lipid content of the glycoprotein is very low and possibly approx. 1%. Fletcher *et al.* (1970*a*) showed that lipid was also associated with T–H glycoprotein extracted from urinary casts and discussed its possible significance.

N-Terminal analysis. Attempts were made to identify the *N*-terminal residues of T–H glycoprotein by dinitrophenylation (Fraenkel-Conrat, Harris & Levy, 1956) and by the Edman method of Eriksson & Sjöquist (1960), but these methods gave unsatisfactory results. The cyanate method described by Stark (1967) should both identify and give a quantitative measure of the *N*-terminal residue. The results obtained on both the native and the iodoacetamide-alkylated T–H glycoprotein by this method are summarized in Table 4. No amino acids other than those mentioned were found in significant amounts. The small quantities of aspartic acid, threonine and arginine are probably artifacts, which are frequently obtained by this method (Stark, 1967). In a control experiment the *N*-terminal amino acids of insulin, glycine and phenylalanine, were obtained in 84 and 73% yields respectively.

The dansyl method was also found to be satisfactory when applied to the glycoprotein. Two-dimensional chromatography on polyamide sheets was performed on the hydrolysate of the dansylated glycoprotein. Fluorescent areas corresponding to 1-dimethylaminonaphthalene-5-sulphonic acid, *O*-DNS-tyrosine and 1-dimethylaminonaphthalene-5-sulphonamide were obtained. The ethyl acetate extract of the hydrolysate contained *O*-DNS-tyrosine and 1-dimethylaminonaphthalene-5-sulphonamide and a small number of unidentified fluorescent areas, not corresponding in intensity to that expected of an *N*-terminal residue. *N*-Terminal amino acids of insulin and ovomucoid were readily identified in control experiments, and their intensity suggested at least 50% dansylation of the terminal residues. High-voltage electrophoresis at pH 4.4 and 1.8 of the hydrolysate of dansylated T–H glycoprotein also failed to reveal an *N*-terminal. Friedmann & Johnson (1966*b*) have claimed to have identified tyrosine at the *N*-terminal of T–H glycoprotein by the dansyl method. In the electrophoresis system they used, however, bis-DNS-tyrosine remains at the origin and could be confused with other fluorescent dansyl products.

Table 4. *Results of* N-*terminal determinations by the method of Stark* (1967)

The values given are the average of determinations on both the native and the reduced and alkylated T–H glycoprotein, and have been corrected for blank determinations and for destruction during hydrolysis (Stark, 1967).

Amino acid	*N*-Terminal residue (mol/100000 g of protein)
Asp	0.033
Thr	0.062
Arg	0.052

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