Rabbit Tamm–Horsfall Urinary Glycoprotein

CHEMICAL COMPOSITION AND SUBUNIT STRUCTURE

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1. Tamm–Horsfall glycoprotein from rabbit urine has been isolated and characterized. The homogeneity of the preparation has been established by a variety of procedures including disc gel electrophoresis and ultracentrifugation in aqueous solution, sodium dodecyl sulphate and formic acid. 2. The chemical composition has been determined and a carbohydrate content of approx. 31% was obtained. The relative contents of the amino acids were shown to be very similar to those in human Tamm–Horsfall glycoprotein. A trace of lipid was also detected. 3. Leucine was identified as the only *N*-terminal amino acid. 4. The subunit structure was investigated in the presence of sodium dodecyl sulphate by gel filtration and disc gel electrophoresis. These studies indicated that the subunit possessed a molecular weight of approx. $84\,000 \pm 6000$. A similar value was obtained after reduction and *S*-alkylation of the glycoprotein indicating that the disulphide bonds were all intrachain. 5. A minimum value for the chemical molecular weight of $85\,000 \pm 6000$ was obtained from the number of *N*-terminal amino acids released by cyanogen bromide cleavage of the glycoprotein. 6. The immunological properties of the glycoprotein were studied. Cross reactivity was demonstrated between human Tamm–Horsfall glycoprotein and a guinea-pig anti-rabbit Tamm–Horsfall antiserum.

A urinary glycoprotein of high molecular weight was isolated from human urine by precipitation with 0.58M-sodium chloride (Tamm & Horsfall, 1950). Physicochemical studies have shown these preparations consist of asymmetrical macromolecules of molecular weight greater than 7×10^6 (Tamm, Bugher & Horsfall, 1955; Maxfield, 1966; Stevenson & Kent, 1970). Fluorescent labelled antibody studies have indicated that human T–H glycoprotein* is associated with the tubular epithelial cells of the kidney, although the exact region of the tubule is uncertain (Keutel, 1965; Friedmann, 1966; McKenzie & McQueen, 1969). The chemical composition of human T–H glycoprotein has recently been reinvestigated by Fletcher, Neuberger & Ratcliffe (1970b) and the subunit molecular weight has been determined in the presence of dissociating agents (Friedmann & Johnson, 1966; Stevenson & Kent, 1970; Fletcher, Neuberger & Ratcliffe, 1970c).

Rabbit T–H glycoprotein had not been characterized, although it had been isolated from rabbit urine and shown to originate from the kidney (Cornelius, Mia & Rosenfeld, 1965). As a preliminary to the development of a radioimmunoassay (Marr,

1970) and a study of the biosynthesis of the glycoprotein, it was found necessary to establish the homogeneity of the preparation and to investigate the chemical composition, subunit structure and immunological properties of rabbit T–H glycoprotein.

MATERIALS

The sources of many of the chemicals and standard proteins have been given by Fletcher *et al.* (1970b,c). In addition the following have been employed. Fucose, mannose and carboxypeptidase A were obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.), oleic acid, stearic acid, cholesteryl stearate, cholesterol, Triton X-100 and galactose were from BDH Chemicals Ltd. (Poole, Dorset, U.K.), tristearin, tripalmitin, trilaurin and cholesteryl oleate were from Eastman–Kodak Ltd. (London, U.K.), palmitic acid was from Hopkin and Williams Ltd. (Chadwell Heath, Essex, U.K.), cholesteryl palmitate was from Aldrich Chemical Co., Ralph Emanuel Ltd. (London S.E.1, U.K.), 2,5-diphenyloxazole, 1,4-bis-(5-phenyloxazol-2-yl)benzene and glucosamine hydrochloride (several times recrystallized) were from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.), chymotrypsin was from Armour Pharmaceutical Co. Ltd. (Eastbourne, U.K.), glutamate dehydrogenase was from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany), and galactosamine hydrochloride was from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Samples of

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

phosphatidylethanolamine, phosphatidylcholine and a mixture of glycolipids were kindly provided by Dr G. M. Gray (The Lister Institute of Preventive Medicine, London S.W.1, U.K.). Both complete and incomplete Freund's adjuvant were from Difco Laboratories (Detroit, Mich., U.S.A.).

METHODS

Preparation of rabbit T-H glycoprotein. An average daily volume of approx. 500 ml of pooled urine was collected from four New Zealand White rabbits (approx. 3 kg each) over a period of 9 weeks. NaN_3 was placed in the collecting bottles and 0.02% NaN_3 was included as a preservative throughout the purification. The normal pH of rabbit urine was between 8 and 8.8 and was adjusted to 5 with 3M-HCl to dissolve insoluble salts. After centrifugation at 2000 rev./min for 5 min to remove traces of remaining insoluble material, the supernatant was diluted to 2 l with water. The rabbit T-H glycoprotein was then prepared by the method described by Fletcher *et al.* (1970b) for the isolation of human T-H glycoprotein. The final aqueous solution of the glycoprotein was tested for bacterial contamination which was shown to be insignificant. The glycoprotein was recovered by freeze drying.

All the methods that have not been given in detail were described by Fletcher *et al.* (1970b,c). Correction factors were obtained for the destruction of serine and threonine and the incomplete release of valine and isoleucine during hydrolysis of human T-H glycoprotein for 21 h at 110°C in constant-boiling HCl (Fletcher *et al.* 1970b). These correction factors were also applied to all 21 h hydrolysates of rabbit T-H glycoprotein.

Carbohydrate analysis. Qualitative examination of the component sugars was made by chromatographic comparison of an acid hydrolysate of the glycoprotein (0.5M-HCl, 18 h, 100°C) with standard sugars. Chromatography was performed on Whatman no. 4 paper in butan-1-ol-ethanol-water (20:1:2, by vol.) for 3 days and alkaline AgNO_3 was used as the location reagent.

Fucose was determined by the method of Dische & Shettles (1948) as modified by Gibbons (1955). Total hexose was determined by the phenol- H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers & Smith (1956). The orcinol- H_2SO_4 method of Winzler (1955) was also used with one-fifth of all the recommended volumes. Galactose was used as a standard in both the methods. Amino sugars were released by overnight hydrolysis of the glycoprotein (2.5 mg) in 3M-HCl at 100°C in a sealed ampoule. The component amino sugars were then separated and determined by Dr A. S. R. Donald (The Lister Institute of Preventive Medicine) by the method of Donald (1968). Sialic acid was determined by the method of Warren (1959) after hydrolysis of the glycoprotein with 50 mM- H_2SO_4 at 80°C for increasing periods of time up to 8 h.

Lipid composition. Lipid was extracted from rabbit T-H glycoprotein by two procedures. An aqueous solution of the glycoprotein (20 mg in 2 ml) was extracted with 7.5 ml of chloroform-methanol (2:1, v/v). The second procedure involved extraction of T-H glycoprotein (20 mg) with 7 ml of ethanol-ether (3:1, v/v) at 4°C.

The lipid components were separated by t.l.c. on silica gel G and compared with pure chromatographic

standards and a serum extract. Neutral lipids were separated in solvent A, hexane-ether-acetic acid (80:20:1, by vol.), and phospholipids were resolved in solvent B, chloroform-methanol-water-acetic acid (65:25:4:1, by vol.). Lipids were detected with iodine vapour and by charring with H_2SO_4 . Components possessing free NH_2 groups were detected with ninhydrin (0.3% in ethanol).

Gel filtration in aqueous buffer. A column (39 cm $\times$ 2 cm diam.) of Sephadex G-200 with a layer (0.25 cm) of Sephadex G-25 on the glass filter was equilibrated and then washed with 7 mM-sodium phosphate buffer, pH 7.0, with added 43 mM-NaCl and 0.02% serum albumin. A 1.0 ml aqueous sample of ^{125}I -labelled rabbit T-H glycoprotein (see Marr, 1970) was loaded on the column and 5 ml fractions were collected. A sample (25 μl) was removed from each fraction and was dissolved in a mixture (1:2, v/v) of Triton X-100 and a scintillation fluid consisting of 5 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene. The void volume of the column was determined with Dextran Blue 2000.

Disc gel electrophoresis in sodium dodecyl sulphate. Procedure I. The method of Davis (1964) as modified by Marshall & Zamecnik (1969) was used as described by Fletcher *et al.* (1970c). The protein samples (20–40 μg) were allowed to equilibrate with sodium dodecyl sulphate overnight before electrophoresis. An increased load of the rabbit T-H glycoprotein (100–200 μg) was necessary because of its poor staining properties. Protein bands were located with a freshly prepared solution of Eriochrome Black T or Amido Black 10B as described by Fletcher *et al.* (1970c). The gels were calibrated with the protein standards of Fletcher *et al.* (1970c) and also carboxypeptidase A (mol.wt. 34300) (Bargetzi, Sampath Kumar, Cox, Walsh & Neurath, 1963).

Procedure II. This method was based on that described by Weber & Osborn (1969). Gels of 10% (w/v) acrylamide containing either 2.7 or 1.35% cross-linker (relative to acrylamide) were used. Pretreatment of the protein samples involved incubation for at least 2 h at 37°C in 10 mM-sodium phosphate buffer, pH 7.0, containing (a) 1% sodium dodecyl sulphate, (b) 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol or (c) 1% sodium dodecyl sulphate, 1% 2-mercaptoethanol and 8M-urea. Pretreatment (a) was followed by overnight dialysis against 0.1% sodium dodecyl sulphate in the pH 7.0 buffer and pretreatments (b) and (c) were each followed by overnight dialysis against the pH 7.0 buffer containing 0.1% sodium dodecyl sulphate and 0.1% 2-mercaptoethanol. In addition to the protein standards described by Fletcher *et al.* (1970c), the following proteins (10 μg) were treated in the same way as the glycoproteins except for the omission of 8M-urea in pretreatment (c): chymotrypsin (two chains, mol.wt. 11000 and 13000) [calculated from amino acid sequence given in Dayhoff & Eck (1967–1968)]; bovine β -lactoglobulin (mol.wt. 18400) (Tanford, Kawahara & Lapanje, 1967); ox liver glutamate dehydrogenase (mol.wt. 40000) (Ullmann, Goldberg, Perrin & Monod, 1968).

Disc gel electrophoresis in urea. The system used was essentially that of procedure I omitting sodium dodecyl sulphate but including urea. The gels contained 7.5% (w/v) acrylamide and 6M-urea. The glycoprotein samples

were equilibrated overnight in the electrode buffer (25 mM-tris-glycine, pH 8.4) which contained 8 M-urea. The rabbit T-H glycoprotein was also dissolved in 6 M-guanidinium hydrochloride and dialysed overnight against the above buffer containing 8 M-urea. Serum albumin, γ -globulin and human T-H glycoprotein were also run in this system.

A second procedure closely followed that of Takayama, MacLennan, Tzagoloff & Stoner (1966), except that the acrylamide content of the gel was decreased to 5% and the protein samples were dissolved in phenol-urea-acetic acid-water (10:6:5:5, w/w/v/v; Baum, Silman, Rieske & Lipton, 1967). It was found necessary to heat the gels at 50–60°C for 20–30 min to increase the rate of polymerization. The gels were then pre-run for 30 min before the glycoprotein (200 and 400 μ g) and protein (60 μ g) samples were loaded. Ovalbumin, serum albumin, γ -globulin and human T-H glycoprotein were also run in this system.

Ultracentrifugation studies. Rabbit T-H glycoprotein (4.8 mg/ml in 0.1 M-glycine-NaOH buffer, pH 8.0) was allowed to stand overnight after vigorous shaking. Insoluble material was removed by centrifuging twice at 3000 rev./min for 20 min, the supernatant was removed and the glycoprotein concentration was determined by the Lowry reaction (Lowry, Rosebrough, Farr & Randall, 1951). Dr J. M. Creeth (The Lister Institute of Preventive Medicine) kindly performed these ultracentrifugation studies on the soluble fraction with a Spinco model E analytical ultracentrifuge. The sedimentation rate of rabbit T-H glycoprotein in 0.1 M-glycine-NaOH buffer, pH 8.0, was examined at two concentrations, 4.2 mg/ml (44770 rev./min) and 1.05 mg/ml (50740 rev./min), at 25°C.

The sedimentation rates of native and iodoacetamide-alkylated rabbit T-H glycoprotein were compared with that of albumin in 1% sodium dodecyl sulphate in 0.1 M-tris-HCl buffer, pH 8.0, and 70% (w/v) formic acid respectively. These experiments, A and B, were performed at 60000 rev./min and at temperatures of 23 and 22°C respectively.

Immunology. Antibodies were raised to human T-H glycoprotein in rabbits by twice-weekly intramuscular injections of 1 ml of an emulsion containing equal volumes of the glycoprotein (1 mg/ml) and complete Freund's adjuvant, and antibodies were first detected after 4 weeks. If additional samples of antisera were required, single booster injections were given and the animals were bled 8–10 days later. Similarly antibodies were raised to rabbit T-H glycoprotein in guinea pigs with incomplete Freund's adjuvant. Ouchterlony plates for double-diffusion studies were prepared with 1% agar in 0.1 M-sodium barbitone buffer, pH 8.6. Human T-H glycoprotein (1–4 mg/ml) and rabbit T-H glycoprotein (1–8 mg/ml) were dissolved in 25 mM-tris-glycine buffer, pH 8.0, containing one of several protein dissociating agents: 0.1% sodium dodecyl sulphate, 8 M-urea, 6 M-guanidine hydrochloride, 0.1% Triton X-100, 0.1% Nonidet P-42. Immunoelectrophoresis was performed on plates of 1% agar in 50 mM-tris-HCl buffer, pH 8.63. The antisera were placed in the wells and electrophoresis was performed at a constant current of 7 mA for 1.5 h in the above tris-HCl buffer. The human and rabbit T-H glycoproteins (1 mg/ml in 25 mM-NaOH) were then placed in the troughs and allowed to diffuse against the antisera for 24 h. The

plate was then washed and stained with Eriochrome Black T.

RESULTS AND DISCUSSION

Amino acid composition. The amino acid composition of rabbit T-H glycoprotein (Table 1) is compared with that of human T-H glycoprotein. The amino acid content of rabbit T-H glycoprotein (534 mol of residues/100 000 g) accounted for 58.6% of the weight of the glycoprotein as anhydro-amino acid residues, whereas the amino acid content of human T-H glycoprotein (619 mol of residues/100 000 g) accounted for 68% of its weight (Fletcher *et al.* 1970b). Although small differences exist in the relative proportions of the individual amino acids in the two glycoproteins, in general there is a marked similarity. Thus they both possess similar

Table 1. *Amino acid composition of rabbit T-H glycoprotein*

The standard deviations were calculated from the analysis of six hydrolysates of rabbit T-H glycoprotein. The *S*-carboxymethylcysteine content is the average of two analyses of iodoacetamide-alkylated rabbit T-H glycoprotein.

	Amino acid composition		
	(mol of residue/ 100 000 g of glycoprotein)	(residues/100 residues in the glycoprotein)	
		Native rabbit T-H glyco- protein	Native human T-H glyco- protein (Fletcher <i>et al.</i> 1970b)
Asp	54.3 $\pm$ 0.9	10.17	10.90
Thr	47.8 $\pm$ 1.6	8.96	7.63
Ser	43.4 $\pm$ 1.8	8.13	7.86
Glu	52.3 $\pm$ 1.2	9.79	8.44
Pro	21.3 $\pm$ 1.5	3.99	4.26
Gly	45.7 $\pm$ 1.2	8.56	8.41
Ala	26.1 $\pm$ 1.0	4.89	6.79
Val	37.5 $\pm$ 0.9	7.03	6.40
Met	8.2 $\pm$ 0.6	1.54	2.05
Ile	16.3 $\pm$ 0.4	3.05	2.46
Leu	42.5 $\pm$ 1.5	7.96	7.58
Tyr	18.1 $\pm$ 0.5	3.39	3.83
Phe	18.6 $\pm$ 1.0	3.49	3.14
Lys	11.0 $\pm$ 0.4	2.06	2.65
His	14.7 $\pm$ 0.6	2.75	2.67
Arg	20.2 $\pm$ 0.9	3.78	4.49
Trp*	7.3 $\pm$ 0.5	1.37	1.68
Cys	48.4†	9.07†	8.41‡
Total	533.7	99.98	100.00

* Average of two spectrophotometric methods (Table 3).

† Determined as *S*-carboxymethylcysteine.

‡ Determined as cysteic acid.

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

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

Method	Amino acid content (mol of residue/100000 g of glycoprotein)	
	Tyrosine	Tryptophan
Amino acid analysis	18.1 ± 0.5 (6)	Absent
Edelhoch (1967)	16.8 ± 0.5 (5)	7.1 ± 0.4 (5)
Beaven & Holiday (1952)	17.5 ± 1.2 (10)	7.4 ± 0.6 (10)

high half-cystine contents, similar tyrosine/tryptophan ratios and similar excess of acidic over basic residues. The half-cystine content of rabbit T-H glycoprotein was determined as *S*-carboxymethylcysteine (48.4 mol of residues/100000 g) after *S*-alkylation with iodoacetamide, and as cysteic acid (45.8 mol of residues/100000 g) after performic acid oxidation. No galactosamine was detected in a 21 h hydrolysate of rabbit T-H glycoprotein.

Aqueous solutions of freeze-dried rabbit T-H glycoprotein (0.46 mg/ml) gave considerable light-scattering at 277 nm equivalent to approx. 36% of the total extinction at this wavelength. At similar protein concentrations in 6M-guanidine hydrochloride the light scattering was decreased to approx. 9% of the extinction at 277 nm. A value of 6.7 was obtained for $E_{1\text{cm}}^{1\%}$ at 277 nm both in water and in 6M-guanidine hydrochloride, after correcting for light-scattering by the method described by Beaven & Holiday (1952).

The tyrosine content obtained by amino acid analysis after acid hydrolysis compared favourably with those given by the two spectrophotometric methods. Further the tryptophan contents obtained in the two spectrophotometric methods were in close agreement (Table 2).

Amide nitrogen content. Rabbit T-H glycoprotein was hydrolysed for 1 h in 50 mM-sulphuric acid at 80°C to release sialic acid; this treatment also liberated 3 mol of ammonia/100000 g resulting from the hydrolysis of amide groups. Hydrolysis of the sialic acid-free glycoprotein (2M-hydrochloric acid, 3 h, 100°C) released a further 52 mol of ammonia/100000 g yielding a total amide content of 55 mol of residues/100000 g. Amino acid analysis revealed an excess of 61 mol of acidic over basic residues, 55 of which are amidated or involved in carbohydrate-protein linkages of the *N*-acylglycosylamine type.

It was later found that the hydrolysis conditions used to liberate sialic acid in the first stage of the

Table 3. *Carbohydrate composition of rabbit T-H glycoprotein*

The standard deviations were calculated from the number of different determinations given in parentheses. For details of determination of total hexose and sialic acid see the text.

Sugar	Composition (% dry wt.)	Method
Fucose	<0.5	Gibbons (1955)
Total hexose	16.3 ± 0.2 (3)	Dubois <i>et al.</i> (1956)
	12.4 ± 0.2 (3)	Winzler (1955)
<i>N</i> -Acetylglucosamine	13.3 ± 0.1 (4)	Donald (1968)
<i>N</i> -Acetylgalactosamine	0.2 ± 0.1 (4)	Donald (1968)
Sialic acid	4.6 ± 0.1 (3)	Warren (1959)
Ash	8.5*	

* An average of two determinations made by heating for 4 days at 450°C.

amide analysis were possibly inadequate (see below). The sialic acid content of rabbit T-H glycoprotein that had been hydrolysed for only 1 h (50 mM-sulphuric acid at 80°C) was hydrolysed for a further 5 h and found to contain 0.7% residual sialic acid, which would not be expected seriously to affect the amide value obtained for rabbit T-H glycoprotein.

Lipid composition. Trace amounts of lipid had been found to be associated with both human urinary T-H glycoprotein (Fletcher *et al.* 1970b) and human T-H glycoprotein from urinary casts occurring in the nephrotic syndrome (Fletcher, McLaughlin, Ratcliffe & Woods, 1970a). Trace amounts of lipid have now also been found in rabbit T-H glycoprotein. Approx. 4 mg of glycoprotein yielded sufficient lipid extract for a single chromatographic separation. Chromatography in solvent A yielded components that were chromatographically similar to cholesterol, cholesteryl esters, triglycerides and free fatty acids. After chromatography in solvent B traces of several ninhydrin-positive components were detected. The main one had a mobility slightly less than that of phosphatidylethanolamine. A spot was also detected that was chromatographically identical with phosphatidylcholine.

Carbohydrate analysis. Examination of the acid hydrolysate of the glycoprotein showed that only sugars chromatographically identical with mannose, galactose and glucosamine hydrochloride were present. In addition trace amounts of fucose and galactosamine were detected by quantitative methods (Table 3). It was notable that galactosamine was virtually absent from the rabbit T-H glycoprotein as this is present in measurable quantities in the human T-H glycoprotein (Fletcher *et al.* 1970b).

Fig. 1. Rate of release of sialic acid from rabbit T-H glycoprotein. The rabbit T-H glycoprotein (1.36 mg/ml) was hydrolysed in 50 mM-H₂SO₄ at 80°C. Duplicate samples (0.2 ml) were removed after 1, 3, 5, 7 and 8 h hydrolysis and free sialic acid was determined.

The two methods of hexose determination yielded different values due to the different colour yields given by galactose and mannose. In the phenol-sulphuric acid procedure the colour yield ratio of galactose/mannose is 1.0:1.5, whereas in the orcinol-sulphuric acid method it is 1.0:0.9. In both methods galactose was used as the standard, and total hexose contents of 16.3 and 12.4% respectively were obtained. From simultaneous equations derived from these results the contents of galactose and mannose were both calculated to be 6.5% giving a total hexose content of 13.0%.

The usual hydrolysis conditions (50 mM-sulphuric acid, 1 h, 80°C) were found to be inadequate for the complete release of sialic acid from rabbit T-H glycoprotein (Fig. 1). Between 96 and 100% of the maximum amount obtained (4.6%) was liberated after 5 h hydrolysis, whereas the usual hydrolysis time of 1 h is sufficient for the complete release of sialic acid from human T-H glycoprotein. It is clear that the value obtained will be a minimum value as no correction has been made for destruction under these hydrolysis conditions (Gibbons, 1963). A slow release of sialic acid has been reported for two other glycoproteins (Gibbons, 1963).

From Table 3 the total carbohydrate content of the glycoprotein is 31.0% if the total hexose content is taken as 12.4%. Therefore 98.1% by weight of the glycoprotein was accounted for as carbohydrate, protein and ash. It was noted that the carbohydrate content of rabbit T-H glycoprotein relative to the protein content was greater than that of human T-H glycoprotein (Fletcher *et al.* 1970b). Although the isoelectric point has not been directly measured, the sialic acid content and the slight excess of acidic amino acid residues over basic residues suggests that rabbit T-H glycoprotein, like human T-H glycoprotein, has an acidic isoelectric point.

The high ash content of 8.5% may therefore be due in part to the binding of metal cations not removed by extensive dialysis of the glycoprotein during its preparation.

N-Terminal analysis. The cyanate method of Stark (1967), after correction for a blank determination, yielded 0.84 mol of leucine/100 000 g of glycoprotein and trace quantities of aspartic acid, glutamic acid and histidine (0.05, 0.03 and 0.09 mol/100 000 g of glycoprotein respectively). The presence of N-terminal leucine was also shown by the dansyl chloride method as described by Fletcher *et al.* (1970b) and rechromatography after inclusion of DNS-leucine confirmed this result. Thus leucine was identified as the only N-terminal residue of rabbit T-H glycoprotein, whereas no N-terminal residue was detected in human T-H glycoprotein by the same two methods (Fletcher *et al.* 1970b).

Gel filtration in aqueous buffer. The rabbit ¹²⁵I-labelled T-H glycoprotein was eluted from a column of Sephadex G-200 as a symmetrical peak with the void volume. This suggests that the molecular weight of the iodinated glycoprotein is greater than approx. 500 000, the nominal exclusion limit of the gel.

Ultracentrifugation studies. The solubility of human T-H glycoprotein is known to be very dependent on ionic strength and pH (Curtain, 1953; McQueen & Engel, 1966). The ionic strength of the buffer chosen for ultracentrifugation studies and the concentration of rabbit T-H glycoprotein were both limited by insolubility of the glycoprotein in buffers of high salt concentration. The following three buffers were investigated: 0.1 M-sodium phosphate, pH 8.0, 0.1 M-sodium barbitone, pH 8.5, and 0.1 M-glycine-sodium hydroxide, pH 8.0. The rabbit T-H glycoprotein was most soluble in the glycine-sodium hydroxide buffer as 88% of the added glycoprotein remained in the supernatant after centrifugation (4.20 mg/ml). Sedimentation-velocity studies were carried out on the soluble fraction in this buffer at two glycoprotein concentrations.

The glycoprotein sedimented in a single symmetrical boundary (see Fig. 2) indicating that by far the greater fraction of the glycoprotein was substantially homogeneous. Sedimentation coefficients of $s_{25,w}$ 27.0S (1.05 mg/ml) and $s_{25,w}$ 15.9S (4.20 mg/ml) were obtained, demonstrating the concentration-dependence of the sedimentation coefficient. This behaviour has also been observed with human T-H glycoprotein (Tamm *et al.* 1955).

Disc gel electrophoresis in the presence of urea. Rabbit T-H glycoprotein behaved as a single diffuse band on disc gel electrophoresis in 6 M-urea. The protein band had a mobility of 0.25–0.38 relative to Bromophenol Blue. A similar less diffuse band was obtained for human T-H glycoprotein in

Fig. 2. Ultracentrifugation of rabbit T-H glycoprotein (4.2 mg/ml in 0.1 M-glycine-NaOH buffer, pH 8.0). Exposure was made after 45 min at 44 770 rev./min, 25°C, phase-plate angle 65°.

this system. γ -Globulin also entered the gel under these conditions and serum albumin was clearly resolved into several components. Pretreatment of the glycoprotein with 6 M-guanidine hydrochloride did not affect its mobility.

The method of Takayama *et al.* (1966) was used originally to solubilize, dissociate and separate the hydrophobic proteins of the mitochondrial electron-transfer chain. T-H glycoprotein and the standard proteins were dissolved in a phenol-urea-acetic acid-water mixture and the electrophoresis was performed in gels containing 5 M-urea and 35% acetic acid. In this system all the proteins examined migrated towards the cathode. The rabbit T-H glycoprotein and its iodoacetamide-alkylated derivative migrated a short distance as a single diffuse band. Electrophoresis of the standard proteins in this system gave in each case several well-resolved bands. The absence of fast-running components in the rabbit T-H glycoprotein in this highly resolving system is good evidence of the homogeneity of the preparation.

Molecular-weight determination by gel filtration in sodium dodecyl sulphate. The molecular weight of rabbit T-H glycoprotein was determined by gel filtration in the presence of 0.5% sodium dodecyl sulphate with a column of Sephadex G-200 calibrated as described by Fletcher *et al.* (1970c). The elution volumes of human and rabbit T-H glycoproteins yielded molecular weights of 76 000 and 110 000 respectively. Rabbit T-H glycoprotein was eluted as a broader peak than human T-H glycoprotein (Fig. 3). Extensive dialysis of either human or

Fig. 3. Comparison of the elution profiles of human and rabbit T-H glycoprotein from a column of Sephadex G-200 in 0.5% sodium dodecyl sulphate. A, Human T-H glycoprotein (3 mg in 1 ml) applied directly to the column. B, Rabbit T-H glycoprotein (4.5 mg in 1 ml) allowed to stand overnight in buffer before application to the column. Column: Sephadex G-200 in 0.5% sodium dodecyl sulphate in 0.05 M-tris-acetic acid buffer, pH 8.0 (76 cm $\times$ 4 cm²). Flow rate, 6 ml/h, fraction size, 2.0 ml. Void volume, 114 ml.

Table 4. *Molecular-weight determination by disc gel electrophoresis in sodium dodecyl sulphate*

For details of procedure I and procedure II see the Methods section. In procedure II, the inclusion of 8 M-urea (pretreatment c) gave similar results to those obtained by pretreatment (b). The standard deviations were calculated from the number of values obtained in different experiments, these being indicated in parentheses. Abbreviation: SDS, sodium dodecyl sulphate.

	Mol.wt.
Procedure I	
Native rabbit T-H glycoprotein	129 000 $\pm$ 5000 (5)
Procedure II	
Pretreatment (a) 1% SDS	
Native rabbit T-H glycoprotein	103 000 $\pm$ 7000 (3)
Iodoacetamide-alkylated T-H glycoprotein	113 000 $\pm$ 8000 (3)
Pretreatment (b) 1% SDS and 1% 2-mercaptoethanol	
Native rabbit T-H glycoprotein	84 000 $\pm$ 6000 (10)
Iodoacetamide-alkylated rabbit T-H glycoprotein	89 000 $\pm$ 8000 (4)

rabbit T-H glycoprotein against 0.5% sodium dodecyl sulphate before their application to the column had no effect on the resulting elution volume or the elution profile.

Molecular-weight determination by disc gel electrophoresis in sodium dodecyl sulphate. In procedure I a single standard protein was applied per gel whereas two standard proteins per gel were run in procedure II. A linear relation between the

electrophoretic mobility of the standard proteins and the logarithm of the molecular weight was obtained by both procedures. A calibration curve was constructed with every determination from a series of standard proteins run simultaneously with the glycoproteins. The results of the subunit molecular-weight determinations are summarized in Table 4.

In procedure I a single diffuse band was obtained for rabbit T-H glycoprotein which behaved as a broader band of higher molecular weight (129 000) than the human T-H glycoprotein (mol.wt. 104 000) (Fletcher *et al.* 1970c). This difference in the behaviour of the glycoproteins is consistent with that observed on gel filtration on Sephadex G-200 in 0.5% sodium dodecyl sulphate (Fig. 3). When the disc gel electrophoresis was repeated at an increased sodium dodecyl sulphate concentration (0.5%) in an attempt to further disaggregate the glycoprotein, the molecular weight was unchanged. Similarly dialysis against 0.5% sodium dodecyl sulphate for 3 days also failed to disaggregate the glycoprotein further. It was found necessary to increase the protein loads applied to the gels as the resulting protein band was more diffuse and thus stained less strongly at the higher sodium dodecyl sulphate concentration.

In procedure II (see the Methods section) a slightly lower estimate of the molecular weight was obtained when the rabbit T-H glycoprotein was pretreated with 1% sodium dodecyl sulphate (see Table 4). However, treatment of the glycoprotein with 2-mercaptoethanol (pretreatment *b*) further decreased the molecular weight of the glycoprotein to 84 000. Inclusion of 8M-urea in the samples (pretreatment *c*) made no difference to the molecular weight obtained. Since the lowest estimate of the subunit molecular weight of human T-H glycoprotein (79 000) was also obtained by this procedure, after pretreatment with 2-mercaptoethanol, the subunit molecular weight of rabbit T-H glycoprotein was taken to be $84\,000 \pm 6000$. Work by Tanford and co-workers has confirmed our interpretation of these results (Reynolds & Tanford, 1970*a,b*; Fish, Reynolds & Tanford, 1970). It was demonstrated that under certain conditions a given weight of protein, after reduction of the disulphide bonds, bound a constant weight of sodium dodecyl sulphate. The empirical relationship that exists between electrophoretic mobility and the molecular weight on gel electrophoresis of protein-sodium dodecyl sulphate complexes was explained in terms of the uniform charge densities of the complexes. Further, they proposed that the hydrodynamic properties of the complexes were a function of the length of the polypeptide chain only. It was suggested that the failure of a non-reduced protein to bind the maximum amount of sodium

Fig. 4. Disc gel electrophoresis of rabbit T-H glycoprotein and its iodoacetamide-alkylated derivative in sodium dodecyl sulphate by the method of Weber & Osborn (1969). A, Rabbit T-H glycoprotein; B, rabbit T-H glycoprotein; C, iodoacetamide-alkylated T-H glycoprotein. Sample B was pretreated with 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol and samples A and C were pretreated with 1% sodium dodecyl sulphate.

dodecyl sulphate might lead to a decreased charge density producing an artificially high estimate of the molecular weight. This explanation would agree very well with our findings. It is noteworthy that the glycoproteins ovomucoid and α_1 -acid glycoprotein containing approx. 25 and 40% carbohydrate respectively have also been shown to give good estimates of their molecular weights by this method (Fletcher *et al.* 1970c; Li & Li, 1970). Therefore the subunit molecular weight of rabbit T-H glycoprotein by this method was taken to be $84\,000 \pm 6000$.

Only traces of one or two components of lower molecular weight than the main band of the rabbit T-H glycoprotein were occasionally detected after pretreatment with 2-mercaptoethanol, which indicated that the preparation was free from significant contamination by lower-molecular-weight material. Although the leading edge of the rabbit T-H glycoprotein band was always well defined, a considerable proportion of material of higher molecular weight was always present (Fig. 4). This was assumed to be undissociated glycoprotein.

Ultracentrifugation studies in sodium dodecyl sulphate and in formic acid. Rabbit T-H glyco-

Table 5. *Comparative ultracentrifugation studies on rabbit T-H glycoprotein and serum albumin in sodium dodecyl sulphate and formic acid*

The sedimentation coefficients have not been corrected to water at 20°C, nor have corrections been applied for the amount of sodium dodecyl sulphate bound by the proteins. Abbreviation: SDS, sodium dodecyl sulphate.

Expt.	Protein	Concn. of protein (mg/ml)	Solvent	SDS/protein ratio (w/w)	$s_{\text{obs.}}$ (S)
A	Rabbit T-H glycoprotein	0.60	1.0% SDS	1.7:1	3.3
	Serum albumin	0.63	1.0% SDS	1.6:1	2.3
B	Iodoacetamide-alkylated T-H glycoprotein	0.6	70% formic acid	—	1.0
	Serum albumin	0.6	70% formic acid	—	0.60

Fig. 5. Cross-reaction of human T-H glycoprotein with guinea-pig anti-(rabbit T-H glycoprotein) serum demonstrated by immunoelectrophoresis. The wells contained rabbit anti-(human T-H glycoprotein) serum (Anti-H) and guinea-pig anti-(rabbit T-H glycoprotein) serum (Anti-R). The troughs contained human T-H glycoprotein (H) and rabbit T-H glycoprotein (R).

protein and its *S*-alkylated modification sedimented as single components in 1.0% sodium dodecyl sulphate and 70% (v/v) formic acid respectively which provided good evidence for the homogeneity of the preparation. In both solvents studied, rabbit T-H glycoprotein (see Table 5) and human T-H glycoprotein (Fletcher *et al.* 1970c) sedimented approx. 50% faster than serum albumin at similar protein concentrations. This suggests that the subunit molecular weight of rabbit T-H glycoprotein is unlikely to be less than that of serum albumin (68 000).

Cyanogen bromide cleavage of rabbit T-H glycoprotein. Cleavage of methionyl peptide bonds with cyanogen bromide released peptides with the following *N*-terminal amino acids identified as the dansyl derivatives: arginine, aspartate, lysine, tyrosine, valine, isoleucine, leucine and phenylalanine. Trace amounts of threonine and serine were

also occasionally detected in the residue remaining after ethyl acetate extraction, but the amounts present suggested that these were probably not *N*-termini. Since leucine was shown to be the *N*-terminus of rabbit T-H glycoprotein, these results indicate that there were at least seven different methionyl peptide bonds/molecule of glycoprotein. From the methionine content of the glycoprotein (8.2 ± 0.6 mol of residues/100 000 g), a minimum molecular weight of rabbit T-H glycoprotein of $85\,000 \pm 6000$ was calculated (uncorrected for the ash content of the preparation).

Immunological properties. After the close similarity in the chemical composition and subunit molecular weight of T-H glycoprotein from humans and rabbits was shown, the immunological properties were compared. Both native human and native rabbit T-H macromolecules were unable to diffuse through agar gel due to their molecular weights

being of the order of several millions, but pretreatment of both glycoproteins with a variety of dissociating agents (see the Methods section) enabled them to penetrate the gel. Precipitin lines were obtained for each glycoprotein with its homologous antibody even after pretreatment with the following denaturing agents: 0.1% sodium dodecyl sulphate, 8M-urea, 6M-guanidine hydrochloride, 0.1% Triton X-100 and 0.1% Nonidet P-42. These precipitin lines gave reactions of identity (Marr, 1970) indicating that an antigenic determinant was preserved after these different pretreatments. It was observed in both cases, however, that complete S-alkylation of the cystine residues completely destroyed the immunological determinant. These observations have, in part, been confirmed for both native and S-alkylated human T-H glycoprotein in 6M-guanidine hydrochloride by Stevenson & Kent (1970). It seems that there is at least one immunological determinant in each glycoprotein that does not depend on hydrogen bonding but does depend on intrachain disulphide bonding to maintain its structure.

Reactions of both the glycoproteins with their heterologous antisera were studied by immunoelectrophoresis. Both glycoproteins gave precipitin lines with their homologous antisera (Fig. 5). The single precipitin line observed with human T-H glycoprotein and the guinea-pig anti-(rabbit T-H glycoprotein) serum suggested the presence of a common determinant. However, no precipitin lines were observed between the rabbit T-H glycoprotein and the rabbit anti-(human T-H glycoprotein) serum. This may be explained by immune tolerance. The antibodies raised in rabbits to human T-H glycoprotein may only be directed against immunological determinants present specifically on the human but not the rabbit glycoprotein. This is supported by the fact that no detectable circulating antibodies were produced in six New Zealand White rabbits after repeated injection of rabbit T-H glycoprotein with complete Freund's adjuvant over a period of 9 weeks.

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