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The Development of a Radioimmunoassay for Tamm–Horsfall Glycoprotein in Serum

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The Tamm–Horsfall glycoprotein prepared by salt precipitation from urine was found to comprise a heterogeneous collection of aggregates. These could be disaggregated with 8 M-urea, following which chromatography on a column of Bio-Gel A.15m yielded a homogeneous glycoprotein of mol.wt. 73000 together with several unidentified impurities. Gel filtration of normal plasma showed the glycoprotein to exist predominantly in a form that is eluted identically with the purified preparation. In one case, material of higher molecular weight was also detected. The purified glycoprotein was used to develop a rapid specific radioimmunoassay for its measurement in human serum or plasma by the use of the Tamm–Horsfall glycoprotein, labelled with ^{125}I by the chloramine-T method as the tracer, an antiserum raised in rabbits, and separation of the bound and free fractions by a second antibody covalently linked to magnetizable particles. Parallelism was demonstrated between the standard preparation and samples. Recovery of added standard to serum varied between 99 and 109%. Total assay time was less than 4h with an intra-assay and inter-assay coefficient of variation of less than 10%. There were no significant differences in the ranges covered with regard to either age or sex, and no circadian rhythm was observed in normal subjects. A physiological range of 70–540ng/ml was established based on serum samples from 95 subjects with normal renal function, as defined by their serum creatinine and urea concentrations. No Tamm–Horsfall glycoprotein was detected in the serum of six anephric patients.

Tamm & Horsfall (1950) demonstrated the presence of a urinary glycoprotein which inhibited haemagglutination caused by myxoviruses. Great interest was stimulated by the finding that this glycoprotein appeared to be exclusively of renal origin (Keutel *et al.*, 1964). Salt precipitation of TH glycoprotein from urine has been shown to yield high-molecular-weight aggregates of up to 28×10^6 (Maxfield, 1961) of the basic glycoprotein, the molecular weight of which has been reported to lie in the range 75000–100000 (Fletcher *et al.*, 1970*b*; Stevenson & Kent, 1970; Hamlin & Fish, 1977). These aggregates can be dissociated by a variety of reagents without affecting their immunological properties (Burnet, 1952; Stevenson & Kent, 1970) and the size of the aggregates has been shown to depend on pH_2 ionic strength and concentration (Curtain, 1953; Maxfield, 1961; Maxfield & Davis, 1963; McQueen & Engel, 1966; Stevenson *et al.*, 1971).

TH glycoprotein has been identified in the urine produced by isolated perfused kidneys of rabbits (Cornelius *et al.*, 1965). Most immunofluorescent studies have shown it to be present in the cytoplasm and, in some cases, along the luminal surface of the tubular epithelial cells of the ascending limb of the loop of Henle, the macula densa and the distal convoluted tubule (McKenzie & McQueen, 1969; Pollak & Arbel, 1969; Schenk *et al.*, 1971; Schwartz *et al.*, 1972). However, Keutel (1965) gave its location as the proximal tubule, and Tsantoulas *et al.* (1974) located a material in liver cell membranes with similar immunological activity. Various methods including radial immunodiffusion (Bichler *et al.*, 1973; Mazzuchi *et al.*, 1974), electroimmunoassay (Bichler *et al.*, 1973; Wieslander *et al.*, 1977; Samuell, 1978) and radioimmunoassay (Grant & Neuberger, 1973) have been used to measure TH glycoprotein excretion in urine, and normal values have been reported as lying between 39 and 78mg/24h. However, all encountered technical difficulties in trying to obtain the glycoprotein in a uniform state of disaggregation.

Abbreviations used: TH glycoprotein, Tamm–Horsfall glycoprotein; SDS, sodium dodecyl sulphate.

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The question as to whether or not TH glycoprotein is present in the circulation is of importance, since it has been postulated that only with renal damage does it enter the interstitium of the kidney and hence the blood (Hodson *et al.*, 1975) and that this, in turn, leads to antibody production (Hanson *et al.*, 1976). TH glycoprotein has been reported to be absent from the normal circulation, as determined by immunodiffusion (Grant, 1959; Vaerman & Heremans, 1959; Keutel & King, 1963; Schwartz & Pallavicini, 1967). However, Avis (1977) has described an immunoradiometric assay for TH glycoprotein with which he detected it in the serum of the small number of subjects studied.

Evidence to date leaves uncertain the question of whether TH glycoprotein is present in the normal circulation and, if so, in what form. Attempts at its measurement have, so far, been complicated by the range of molecular aggregates shown to exist when it is isolated from urine by simple salt precipitation. For these reasons it was decided to purify TH glycoprotein from urine and develop a radioimmunoassay for its measurement in human serum.

Experimental

Materials

Bio-Gel A.15m, agarose and SDS were from Bio-Rad Laboratories, Watford, Herts., U.K. Sephadex G-200 and Sepharose 6B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide, *NNN'N'*-tetramethylethylenediamine, *NN'*-methylenebisacrylamide, ninhydrin and Tween 20 were from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Standard amino acid solution (A grade) and lactoperoxidase were from Calbiochem, Bishop's Stortford, Herts., U.K. Phosphorylase *a*, catalase and CNBr were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Alcohol dehydrogenase and carbonic anhydrase were from Miles Laboratories, Slough, Berks., U.K. β-Galactosidase was from Cambrian Chemicals, Croydon, Surrey, U.K. Bovine albumin powder was from Armour Pharmaceutical Chemical Co., Eastbourne, Sussex, U.K. Magnetizable particles coupled to cellulose as the solid phase and sheep anti-(rabbit immunoglobulin G) were from Technia Diagnostics, London, E.C.1, U.K. Freund's complete adjuvant was from Difco Laboratories, Detroit, MI, U.S.A. Carrier-free $Na^{125}I$ (100mCi/ml) was from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were obtained from BDH Chemicals, Poole, Dorset, U.K.

Preparation of TH glycoprotein by salt precipitation

Human TH glycoprotein was precipitated from pooled samples of urine (26 litres), obtained from laboratory staff, by the addition of solid NaCl sufficient to increase the concentration by 0.58M (Tamm & Horsfall, 1952). After being left overnight at 4°C, the precipitate was collected by centrifugation at 1500*g* ($r_{av.}$ 26cm) for 1h and the supernatant discarded. The precipitate was dissolved in water (4.2 litres) overnight at 4°C, and any insoluble material was removed by centrifugation at 1500*g* ($r_{av.}$ 26cm) for 1h. The glycoprotein was reprecipitated and collected by centrifugation as above, dissolved in water (1.0 litre) and clarified by centrifugation at 1500*g* ($r_{av.}$ 26cm). This procedure was repeated twice at an increased centrifuge speed of 10000*g* ($r_{av.}$ 6.2cm), resulting in an aqueous solution of 800ml, which was dialysed against three changes of 2.5 litres of water for 4 days and freeze-dried.

Purification of TH glycoprotein

Some (36mg) of this preparation was dissolved in 5ml of 0.014M-Na_2HPO_4/0.016M-NaH_2PO_4, pH6.8, containing 8M-urea and left overnight at room temperature. The solution was chromatographed on a column (2.5cm × 114cm) of Bio-Gel A.15m in the same buffer containing 2M-urea, at a flow rate of 6ml/h. The absorbance of fractions was monitored at 280nm and the peak fractions were pooled and dialysed against water before being freeze-dried.

Characterization

Analytical chromatography. Gel filtration of the two preparations of TH glycoprotein and of two plasma samples was carried out on a column (1cm × 94cm) of Sepharose 6B in 0.052M-Na_2HPO_4/0.013M-NaH_2PO_4, pH7.4. The flow rate was 1.8ml/h and the fractions collected were assayed for TH glycoprotein as described below.

Polyacrylamide-gel electrophoresis. Procedure 1. Polyacrylamide-gel electrophoresis in the presence of urea and SDS was carried out by the method of Guenther *et al.* (1977), with a final acrylamide concentration of 7.5% (w/v). Samples (20μg) were dissolved in the electrode buffer containing 8M-urea and 2% (w/v) SDS both in the presence and absence of 4% (v/v) β-mercaptoethanol and heated in a boiling-water bath for 5min. Gels of diameter 5mm were prerun for 30min at 1mA/gel. Electrophoresis was performed at a constant current of 2mA/gel until the tracking dye, Bromophenol Blue, had reached the bottom of the gel. The gels were stained for 2h with 0.25% (w/v) Coomassie Blue in a mixture of 45% (v/v) methanol and 7% (v/v) acetic acid in water and then destained by repeated washings in the same solution without added Coomassie Blue.

Procedure 2. Polyacrylamide-gel electrophoresis was performed as described in a commercial instruction manual (Shandon Southern Instruments,

Runcorn, Cheshire, U.K.) in the absence of urea and SDS and at a final acrylamide concentration of 7.5% (w/v). Gels of diameter 5mm were routinely prerun for 1h at 4mA/gel in 0.0375M-Tris/0.06M-HCl, pH8.8. Samples (20 and 100μg) were dissolved in the electrode buffer (0.05M-Tris/0.385M-glycine, pH8.5) and electrophoresis was carried out at a constant current of 4mA/gel until the Bromophenol Blue tracking dye had reached the bottom of the gel. Gels were stained and destained as in Procedure 1.

Amino acid analysis. Freeze-dried samples for amino acid analysis were hydrolysed *in vacuo* by 6M-HCl in the presence of a crystal of phenol at 110°C for 24h. The preparation of TH glycoprotein after urea disaggregation was also hydrolysed for 48 and 72h to enable extrapolation to zero time of hydrolysis for serine and threonine. Analysis was carried out on a JLC-6AH automatic amino acid analyser (Japan Electron Optics Laboratory, Tokyo, Japan). Peak areas were computed by a digital integrator and compared with results obtained from analysis of standard solutions of amino acids.

Molecular-weight determination. The molecular weights of TH glycoprotein and its aggregates were determined in the presence of SDS by a method based on that of Laemmli (1970) using a discontinuous buffer system. The 10% (w/v) polyacrylamide slab gels (2mm thick, 10.5cm long and 15cm wide) were used with a 5% stacking gel 1cm high. Before electrophoresis the samples were boiled for 5min in sample buffer containing 1% β-mercaptoethanol. After electrophoresis the gels were stained for 1h in a solution of 0.05% Coomassie Brilliant Blue in a mixture of 40% (v/v) methanol and 10% (v/v) acetic acid in water, and then destained overnight in the same solution omitting the Coomassie Blue. The following proteins of known molecular weight were chosen to standardize the method: β-galactosidase (mol.wt. 130000); phosphorylase *a* (mol.wt. 94000); lactoperoxidase (mol.wt. 80000); catalase (mol.wt. 60000);' alcohol dehydrogenase (mol. wt. 41000); carbonic anhydrase (mol.wt. 29000). The unknown molecular weights were calculated by plotting the distance migrated by each standard protein against the logarithm of their molecular weights.

Radioimmunoassay

Preparation of antisera. Antisera to both preparations of human TH glycoprotein were prepared by immunizing five rabbits each with 1ml of an emulsified mixture of 0.9ml of Freund's complete adjuvant and 250μg of glycoprotein dissolved in 0.1ml of distilled water. The dose was administered subcutaneously at three separate sites. The rabbits were given further injections of 100μg of glycoprotein in Freund's complete adjuvant at monthly intervals for 4 months, and were bled 10 days after the final injection. The serum was separated and 1ml portions were stored at −20°C.

Immunodiffusion. Ouchterlony plates for double-diffusion studies were prepared with 1.2% (w/v) agarose in 0.025M-Tris/0.01M-HCl, pH8.3. Samples of freeze-dried TH glycoprotein and its aggregates were dissolved in distilled water to a concentration of 2mg/ml and brought to 1mg/ml by the addition of 0.065M-sodium phosphate buffer, pH7.4. Each antigen was diffused against each antiserum.

Labelling of TH glycoprotein. TH glycoprotein was labelled with $Na^{125}I$ by the chloramine-T method of Hunter & Greenwood (1962). All solutions were made up in 0.065M-sodium phosphate buffer, pH7.4. $Na^{125}I$ (1mCi, 10μl) was allowed to react with 10μg of chloramine-T (20μl) for 5s before being mixed with 10μg of TH glycoprotein (20μl). The reaction was stopped after 30s by the addition of 25μg of sodium metabisulphite (50μl). After a further 30s, 2mg of KI (100μl) was added. ^{125}I-labelled TH glycoprotein was separated from free radioactive iodide on a column (1cm × 36cm) of Sephadex G-200 in 0.065M-sodium phosphate buffer, pH7.4, containing 0.5% (w/v) bovine serum albumin. The column was previously equilibrated with 60ml of the same buffer. The peak fractions with highest binding when assayed in the presence of anti-(TH glycoprotein) serum were pooled, divided into portions and stored at −20°C.

Preparation of magnetizable solid-phase second antibody. A sample (1g) of the magnetizable solid phase was washed several times with water until the supernatant was clear. After several further washings with 0.05M-Na_2HPO_4/0.002M-NaOH, pH11.5, the particles were suspended in 10ml of the buffer and transferred to a fume cupboard where CNBr (1g) dissolved in 15ml of the same buffer was added and stirred for 12min. The pH was maintained at 11.5 throughout by the addition of 2M-NaOH. The solid phase was then washed four times with ice-cold water followed by two washings with 0.1M-$NaHCO_3$, adjusted to pH8.6 with NaOH. In all washings the supernatant was separated from the solid phase by a magnet. The activated solid phase was finally suspended in 20ml of 0.1M-$NaHCO_3$. The coupling of antibody to solid phase was based on the method described by Porath *et al.* (1967). Sheep anti-(rabbit immunoglobulin G) (2ml) was added to the solid phase (1g) and the mixture rotated at 4°C for 72h. The solid phase was washed twice with 0.1M-$NaHCO_3$ pH8.6, followed by one wash with the same solution containing 0.05M-ethanolamine. The solid phase was resuspended in the ethanolamine/bicarbonate solution and mixed for 30min to block any remaining active sites. One wash with 0.018M-sodium acetate/0.082M-acetic acid,

pH 4.0, was followed by resuspension in the same buffer with mixing for 30 min. This step was repeated with mixing for 4 h. The solid phase was finally washed three times with 0.065 M-sodium phosphate buffer, pH 7.4, containing 0.25% (v/v) Tween 20 and 0.02% (w/v) NaN_3. The amount of solid phase in the final solution was accurately determined by acetone-washing and oven-drying portions of known volume in preweighed bottles.

Assay procedure. All dilutions were prepared in an assay buffer comprising 0.065 M-sodium phosphate, pH 7.4, 2% (w/v) bovine serum albumin, 0.01 M-EDTA and 0.02% (w/v) NaN_3. Freeze-dried TH glycoprotein was weighed and diluted to a concentration of 2500 ng/ml and stored at −70°C. For each assay the stock solution was doubly diluted over the range 1250–19.5 ng/ml. To each tube was added 200 μl of standard, or a 1 in 4 dilution of sample, in assay buffer, ^{125}I-labelled TH glycoprotein (50 μl, 1.6 ng) and, finally, rabbit anti-(TH-glycoprotein) serum (50 μl, 1:30000 dilution). The tubes were mixed and incubated for 2 h at room temperature. Magnetizable solid-phase second antibody (100 μl, 0.5 mg) in assay buffer containing 0.25% (v/v) Tween 20 was added, and the tubes were mixed and incubated for 5 min, and then mixed and incubated for a further 10 min at room temperature. The bound fraction was separated by centrifugation at 1500 *g* ($r_{av.}$ 26 cm) for 10 min, and the radioactivity in the pellet counted. A range of incubation times from 30 min to 24 h and a variety of other separation techniques were examined. The measurement of TH glycoprotein in fractions after chromatography of serum samples required a more sensitive assay. For this purpose the assay differed as follows: the standards ranged from 1.6 to 51 ng/ml; a greater dilution of antiserum (50 μl, 1:150000) was employed and the tubes were incubated overnight at room temperature. Late addition of label (50 μl, 0.8 ng) was then followed by a further incubation period overnight at room temperature before separation.

Validation. A standard curve was constructed in serum from an anephric patient and compared with one constructed in assay buffer. Serum or plasma from five volunteers was doubly diluted in assay buffer to give four different dilutions and compared with the standard curve on a logit/log plot (Midgley *et al.*, 1969). The recovery of various amounts (78–625 ng/ml) of standard TH glycoprotein added to normal human serum was also assessed.

Criteria for sample collection. The stability, effect of haemolysis, type of specimen, time of day and day-to-day samples were all investigated for possible effects on the concentration of TH glycoprotein.

(*a*) Heparinized blood from five volunteers was collected into four separate tubes of which one was centrifuged, separated and frozen immediately. The rest were left at room temperature for up to 3 days, one sample from each person being spun, separated and frozen each day.

(*b*) Three samples of haemolysed and unhaemolysed plasma were compared. Haemolysis was effected by vigorous mixing.

(*c*) Blood from six volunteers was collected into both a lithium–heparin (LH 10; Searle) and a plain glass tube and separated after 1 h.

(*d*) Three volunteers had blood taken half-hourly or hourly over a period of 24 h.

(*e*) Six volunteers had blood taken at the same time every day for 5 days.

Physiological range. A normal range for TH glycoprotein was determined on 48 male and 47 female volunteers with serum creatinine and blood urea concentrations within their normal ranges of $<100\mu$M and 2.5–6.7 mM respectively. In addition, the concentrations of TH glycoprotein in six anephric patients on between one and nineteen separate occasions were measured.

Results

Preparation, purification and characterization of TH glycoprotein

Salt precipitation of 26 litres of urine yielded 330 mg of a TH glycoprotein preparation which, on polyacrylamide-gel electrophoresis in the presence of urea and SDS (procedure 1), appeared as a single band. In the absence of either (procedure 2), it did not migrate into the gel. Results of amino acid analysis are shown in Table 1. The molecular weight of the reduced glycoprotein in the presence of SDS was calculated as 92000. On gel filtration (Fig. 1*a*) this preparation appeared as a heterogeneous mixture, the elution of which began immediately after the void volume and continued throughout most of the run.

After disaggregation in 8 M-urea, gel filtration revealed one main peak (TH glycoprotein) and two minor ones (Fig. 2), which were termed A, B and C depending on their elution position. After freeze-drying, their relative recoveries by weight were 55, 13 and 32% respectively. On polyacrylamide-gel electrophoresis, both in the presence and absence of urea and SDS, TH glycoprotein appeared as a single band. Results of amino acid analysis of peaks A and B are shown in Table 1. Peak C contained no amino acids. The molecular weight of TH glycoprotein (peak A) after reduction and in the presence of SDS was calculated as 73000. When peaks B and C were assayed for TH glycoprotein, peak B was found to exhibit 1% cross-reactivity (w/w) and peak C showed no reactivity. In order to determine whether the former was due to cross-reaction of a similar molecule or to contamination of peak B with a small amount of TH glycoprotein carried over during gel

Table 1. *Amino acid analysis*

The amino acid compositions of TH glycoprotein from salt precipitation of urine, and peaks A and B in Fig. 2 are expressed as residues per 608 residues in order to facilitate comparison with those published by Fletcher *et al.* (1970*a*) for TH glycoprotein prepared by salt precipitation alone. The high values obtained for tyrosine are due to amino sugars eluted with this amino acid after acid hydrolysis.

	Fletcher *et al.* (1970*a*)	TH glycoprotein from the salt precipitation of urine	Peak A (TH glycoprotein)	Peak B
Asp	67.4	72.9	76.8	74.5
Thr	47.2*	42.3	38.7*	53.8
Ser	48.6*	42.2	44.3*	48.1
Glu	52.2	48.5	48.4	55.8
Pro	28.6	30.1	30.3	35.6
Gly	52.0	52.0	54.4	43.7
Ala	42.0	42.0	45.5	31.8
½Cys	52.0§	43.9	49.3	26.0
Val	39.6‡	36.9	40.3†	38.0
Met	12.7	13.9	9.4	9.2
Ile	15.1‡	13.6	15.2†	13.0
Leu	46.9	46.5	42.2	52.4
Tyr	23.7	47.9	43.2	38.5
Phe	19.4	18.2	12.9	33.2
Lys	16.4	15.5	15.2	14.4
His	16.5	14.7	15.6	13.0
Arg	27.8	27.5	26.0	26.5
	608.2	608.6	607.7	607.5

* Values are corrected to zero time.
† Values of 72 h hydrolysis.
‡ Values corrected to infinite time.
§ Determined as cysteic acid.

filtration on the urea column (Fig. 2), a sample from peak B was further chromatographed on a column of Sepharose 6B (Fig. 1*c*). TH glycoprotein and human serum albumin were similarly examined (Figs. 1*b* and 1*d* respectively). TH glycoprotein was eluted as one peak slightly in advance of the albumin. The cross-reacting component of peak B was eluted in the same position as TH glycoprotein and, of the 125μg loaded on to the column, only 1μg was detected in the assay.

Immunodiffusion of both preparations of TH glycoprotein against rabbit antiserum raised against either of them gave a single precipitin line. The two antisera showed a clear reaction of identity when diffused against either antigen.

Radioimmunoassay

Iodination. About 65–75% of the ^{125}I was incorporated into TH glycoprotein during iodination, resulting in specific radioactivities of 65–75μCi/μg. After storage for 2 months at −20°C there was a 50% decrease in the binding of ^{125}I-labelled TH glycoprotein to antibody in the absence of unlabelled TH glycoprotein. The final dilution of antiserum (1:180000) chosen for use in the assay bound 60–65% of ^{125}I-labelled TH glycoprotein in the absence of unlabelled TH glycoprotein. In the absence of antibody 3–4% was precipitated.

Assay conditions. The effect of varying incubation times from 30 min to 24 h was investigated. As brevity of assay was considered of prime importance, a 2 h incubation period was chosen. Although this resulted in a non-equilibrium assay, quality controls indicated that if addition of the separating reagent was completed within 10 min, no assay drift was detectable.

Methods of separation found to give satisfactory results were the use of liquid-phase second antibody either alone or in conjunction with poly(ethylene glycol) or $(NH_4)_2SO_4$, and second antibody covalently linked to magnetizable particles. Second antibody alone required overnight incubation to effect maximum precipitation of the bound fraction. All of the other methods required only 20–30 min to achieve complete precipitation, but the solid-phase approach was adopted because it could be added in one step and needed only 10 min centrifuge time.

Validation. Standard curves constructed in assay buffer or in the serum from an anephric patient were essentially superimposable (Fig. 3). Parallelism between standard TH glycoprotein and serum or

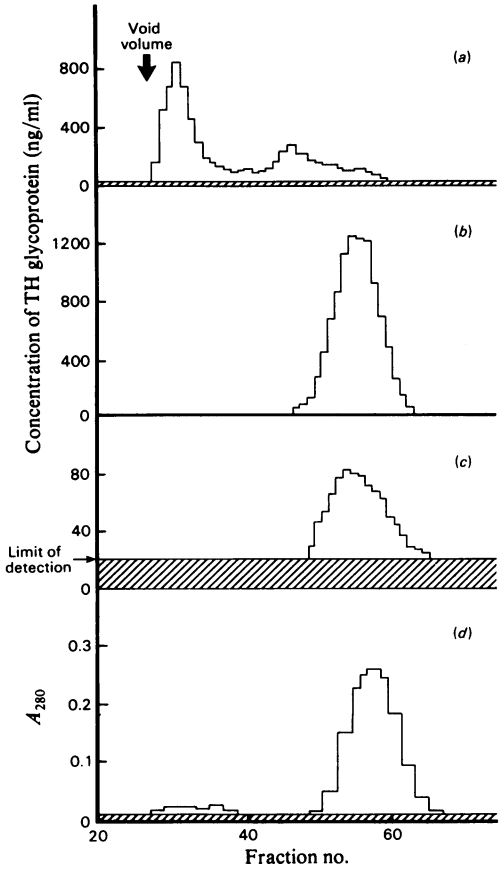

Fig. 1. *Analytical chromatography of TH glycoprotein before and after urea disaggregation*

Gel filtration on a column (1 cm × 94 cm) of Sepharose 6B in 0.065 M-sodium phosphate buffer, pH 7.4, of the following samples: (*a*) 20 μg of TH glycoprotein prepared by salt precipitation from the urine; (*b*) 10 μg of TH glycoprotein after treatment with 8 M-urea (peak A in Fig. 2); (*c*) 125 μg of peak B in Fig. 2; (*d*) 5 mg of human serum albumin. The flow rate was 1.8 ml/h and fractions of volume 1 ml were collected. Fractions in (*a*)–(*c*) were assayed for TH glycoprotein. The fractions in (*d*) were monitored at 280 nm.

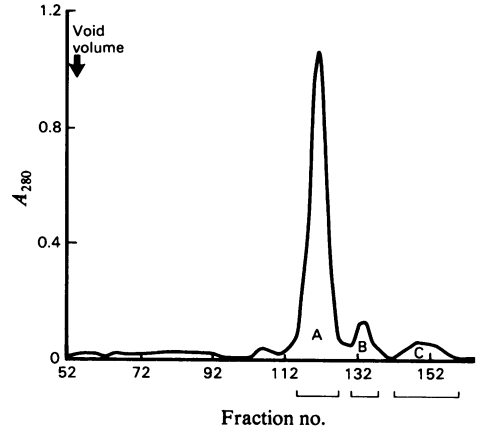

Fig. 2. *Gel filtration of TH glycoprotein after disaggregation in* 8 M-*urea*

TH glycoprotein from the salt precipitation of urine (approx. 36 mg) was dissolved in 0.03 M-sodium phosphate buffer, pH 6.8, containing 8 M-urea and chromatographed on a column (2.5 cm × 114 cm) of Bio-Gel A.15 m in the same buffer containing 2 M-urea. The flow rate was 6 ml/h and fractions of volume 3.4 ml were collected. Fractions underlined were pooled separately, dialysed against water and freeze-dried.

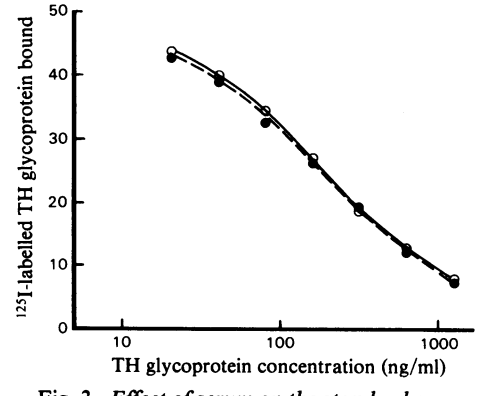

Fig. 3. *Effect of serum on the standard curve*

Standard curves were constructed in assay buffer (O) and in the serum of an anephric patient (●) by the addition of known amounts of TH glycoprotein. These were assayed as described for samples in the text.

plasma samples was demonstrated on a logit/log plot. The amount of standard TH glycoprotein recovered after addition to human serum varied between 99 and 109%.

Criteria for sample collection. No variation was found in TH glycoprotein concentrations between serum and plasma, haemolysed and unhaemolysed plasma, or whole blood after 3 days at room temperature. Concentrations throughout 24 h varied slightly, but there was no apparent circadian rhythm. There was no significant day-to-day variation over the 5 days studied.

Physiological range. The physiological range of TH glycoprotein in the 95 normal subjects tested

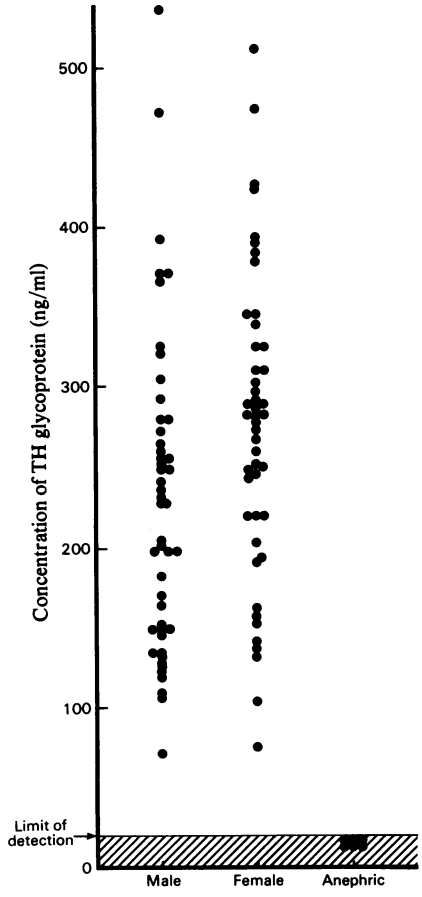

Fig. 4. *Physiological range of TH glycoprotein*
Normal concentrations of TH glycoprotein in 48 males and 47 females ranged from 71 to 538 ng/ml. Concentrations in six anephric patients were less than 20 ng/ml.

was determined as 70–540 ng/ml (Fig. 4). Age and sex made no difference to the range detected. In addition, the concentrations of TH glycoprotein in six anephric patients were always less than 20 ng/ml. Six of these samples from four of these patients were subsequently investigated in the more-sensitive assay and all had concentrations below 1.6 ng/ml.

Reproducibility. The intra-assay coefficient of variation at a mean concentration of 47 ng/ml was 9.0% ($n = 10$) and at 301 ng/ml was 5.1% ($n = 11$). The inter-assay coefficient of variation at a mean concentration of 54 ng/ml was 9.3% ($n = 19$) and at 290 ng/ml was 7.2% ($n = 17$).

Gel filtration of plasma

Fig. 5 shows the gel filtration of two fresh plasma samples from normal volunteers compared with the

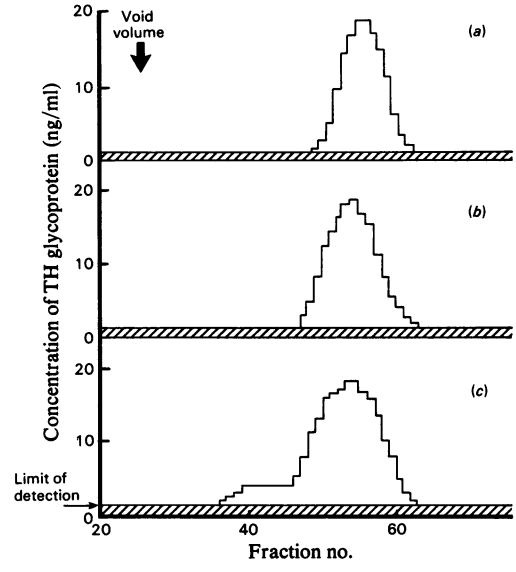

Fig. 5. *Gel filtration of plasma from normal subjects compared with a standard preparation of TH glycoprotein*
(*a*) 0.5 ml of standard TH glycoprotein (312 ng/ml); (*b*) and (*c*) 0.5 ml of fresh plasma. Samples were chromatographed on a column (1 cm × 94 cm) of Sepharose 6B in 0.065 M-sodium phosphate buffer, pH 7.4, at a flow rate of 1.8 ml/h. Fractions of volume 1 ml were collected and assayed for TH glycoprotein in the sensitive assay described in the text.

TH glycoprotein standard. Both samples show peaks in the position of the standard. However, one of them also contains material of apparently higher molecular weight, which reacts in the TH glycoprotein assay.

Discussion

Gel filtration of TH glycoprotein prepared by salt precipitation from urine showed a heterogeneous mixture of different molecular weights. After disaggregation and reduction, the calculated mol.wt. was 92000 for this preparation, which is similar to previously reported values of from 74000 to 100000 (Fletcher *et al.*, 1970*b*; Stevenson & Kent, 1970; Hamlin & Fish, 1977). The amino acid analysis of TH glycoprotein is in good agreement with other published data (Maxfield & Stefanye, 1962; Friedmann & Johnson, 1966*b*; Fletcher *et al.*, 1970*a*). On polyacrylamide-gel electrophoresis in the absence of urea and SDS, TH glycoprotein did not migrate into the gel, owing to its large molecular size. However, in their presence the preparation appeared homo-

geneous, in contrast with the chromatographic pattern obtained after disaggregation with 8 M-urea when three peaks were found. The fact that none of these had a mol.wt. greater than 73000 suggests that the original preparation of higher molecular weight was composed of molecules held together by non-covalent bonds, confirming previous work using various denaturants (Friedmann & Johnson, 1966*a*; Stevenson & Kent, 1970; Hamlin & Fish, 1977). Only 55% by weight of the original preparation was TH glycoprotein. The remainder consisted of a small amount of an immunologically dissimilar protein of lower molecular weight and a material accounting for one-third of the original preparation, which was shown to contain no amino acids. More detailed analysis of this component has yet to be undertaken, but reports that immunoglobulin G isolated from the urine is heavily contaminated with low-molecular-weight urinary carbohydrate (Bourrillon, 1972) suggest that this third peak could be composed of sugars that are non-specifically adsorbed on TH glycoprotein in the urine and are therefore also precipitated by NaCl. This explains why, after polyacrylamide-gel electrophoresis, the original preparation appeared to be homogeneous, as non-protein material does not stain with Coomassie Blue and the remaining contaminant consists of only 13% of the total by weight, which is insufficient to detect.

Both chromatography and polyacrylamide-gel electrophoresis showed that after disaggregation the TH glycoprotein was homogeneous. The differences seen in the amino acid composition of TH glycoprotein compared with the original preparation are presumably due to the material in peak B having been removed. The difference in the two molecular weights, even after reduction in the presence of SDS, can be explained by the observation made by Hamlin & Fish (1977) that TH glycoprotein behaves differently hydrodynamically from most glycopolypeptides when complexed with SDS.

The stability of ^{125}I-labelled TH glycoprotein on storage was not particularly good; binding to antibody in the absence of unlabelled TH glycoprotein was reduced by 50% after 2 months. This may be due, in part, to the phenomenon of 'decay catastrophe' (Yalow & Berson, 1968), which occurs when molecules contain at least two radioactive iodine atoms. The stability was improved by lowering the specific radioactivity, but this resulted in a longer counting time which was thought to create an unnecessary increase in length of assay time.

The gel filtration of the two plasma samples showed both to contain TH glycoprotein which was eluted in the same position as the standard. The material of higher molecular weight found in one of the samples could be due to either aggregation of TH glycoprotein or binding of TH glycoprotein to another plasma protein and requires further investigation.

The only other reported assay for TH glycoprotein in human serum is that of Avis (1977), which required 2 days. For the assay to be of clinical use in the possible diagnosis of renal-tubular disease or damage, especially in cases such as renal transplantation, it is particularly important for such a test to be rapid and specific. With the incubation time set at 2h, the assay described here can produce results within 4h on a batch of 50 samples. The validation of the assay, including the tests of parallelism and recovery and the assay of samples from anephric patients, suggests that one is dealing with a renal-specific material reacting in a manner identical with the standard. The physiological role of TH glycoprotein has yet to be understood, and it is hoped that the establishment of this assay will further this understanding.

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