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# An improved radioimmunoassay for urinary Tamm–Horsfall glycoprotein

## Investigation and resolution of factors affecting its quantification

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A rapid, specific radioimmunoassay has been used to measure Tamm–Horsfall glycoprotein (TH glycoprotein) in urine. The apparent concentration increased with increasing dilution of urine in water, reaching a plateau at 1 in 20. This increase was greater the higher the osmolality and TH glycoprotein concentration and the lower the pH of the original sample. A dilution of 1 in 100 was chosen for routine assay. Whole urine was centrifuged and the dissolved precipitate and supernatant assayed to quantify the proportion of TH glycoprotein initially present in highly aggregated form. This correlated positively and significantly with increasing osmolality, decreasing pH and increasing TH glycoprotein concentration. When the urine was diluted 1 in 100 in water, no TH glycoprotein was precipitated by centrifugation and the measured concentrations were unaffected by alterations of urine pH or calcium concentration or by addition of sodium dodecyl sulphate. Parallelism was demonstrated between the diluted samples and the disaggregated standard preparation. Recovery of added standard to diluted urine varied between 96 and 114%. The apparent concentration of TH glycoprotein in neat or diluted urine was not affected by freezing or by storage at 4°C or room temperature for at least 2 days. A physiological range for the urinary excretion rate was established as 22–56 mg/24 h, based on samples from 29 individuals with normal renal function, as defined by their creatinine clearance. There was no significant correlation between serum concentrations of TH glycoprotein and its urinary excretion rate, nor between urinary excretion rate and creatinine clearance.

TH glycoprotein is produced only by the kidney and, although found in the serum (Avis, 1977; Dawnay *et al.*, 1980; Dawnay & Cattell, 1981), is predominantly excreted in the urine as aggregates. It has been implicated in the pathogenesis of some forms of acute renal failure (Patel *et al.*, 1964) and in urinary stone formation (Hallson & Rose, 1979). Although the physiological role of TH glycoprotein is unknown, its localization in the cells lining the ascending limb of the loop of Henle and the distal convoluted tubule has led to suggestions that it may be involved in the urine-diluting mechanism of the nephron (Hoyer *et al.*, 1979; Sikri *et al.*, 1979, 1981). This precise location also suggests that its measurement may be a useful marker for renal damage at these sites.

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

A small study (Grant *et al.*, 1973) has shown that urinary TH glycoprotein output is decreased in chronic renal failure but detailed assessment of the value of its measurement has been hampered by the laborious and relatively insensitive immunoassays that have been used to date. Such methods have included varying combinations of sample treatment before assay, e.g., dialysis, gel filtration, ultrafiltration, freezing, incubation with detergents and adjustment of pH (Bichler *et al.*, 1973; Grant & Neuberger, 1973; Mazzuchi *et al.*, 1974; Wieslander *et al.*, 1977; Akesson *et al.*, 1978; Samuell, 1978; Goodall & Marshall, 1980) in attempts to achieve a uniform state of disaggregation and, by implication, a degree of immunoreactivity dependent solely on concentration.

Aggregation is promoted by high ionic strength (in particular, cation concentration), low pH and high concentrations of TH glycoprotein (Curtain,



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1953; McQueen & Engel, 1966; Stevenson *et al.*, 1971; Robinson & Puett, 1973). Simply by using a sufficiently sensitive method to allow a 1 in 100 or greater dilution of sample in water before analysis, we have developed an assay that is unaffected by these factors. We describe here the validation of this method and the excretion rates in a healthy population.

## Experimental and results

### *Materials*

Bovine albumin powder was obtained from Armour Pharmaceutical Chemical Co., Eastbourne, Sussex, U.K. Other reagents were from BDH Chemicals, Poole, Dorset, U.K.

### *Assay of TH glycoprotein in serum and urine*

Concentrations of TH glycoprotein were determined by a rapid (2h) radioimmunoassay with a sensitivity of 23ng/ml. Samples with lower values were re-assayed by a more sensitive procedure capable of detecting 1.6ng/ml (Dawnay *et al.*, 1980). Intra- and inter-assay coefficients of variation were less than 10% at all concentrations. Urine samples were diluted before assay in either distilled water or in assay diluent [0.052M-$Na_2HPO_4$/0.013M-$NaH_2PO_4$ containing 0.01M-EDTA and 2% (w/v) bovine serum albumin, pH7.4] as specified in the text. The concentrations quoted for TH glycoprotein in urine samples have been corrected for dilution.

### *Statistics*

Non-parametric statistics were used to assess correlation coefficients and their significance as described by Siegel (1956).

### *Evaluation of factors affecting the aggregation of TH glycoprotein in urine*

The proportion of TH glycoprotein in a highly aggregated form was ascertained as follows. Portions (1ml) of 20 samples of fresh urine of known osmolality and pH were centrifuged at 1500*g* ($r_{av.} = 26$cm) for 10min. The supernatant was carefully removed and the precipitate dissolved in 1ml of water. Both the supernatant and the dissolved precipitate were diluted 1 in 100 in water before assay and the ratio of the amount of TH glycoprotein precipitated to that remaining in the supernatant was calculated. Increases in this ratio correlated positively and significantly with increasing osmolality, decreasing pH and increasing concentrations of TH glycoprotein in the original urine sample (Fig. 1).

When whole urine was diluted 1 in 100 in water and assayed for TH glycoprotein before and after centrifugation as described above, no TH glycoprotein was precipitated.

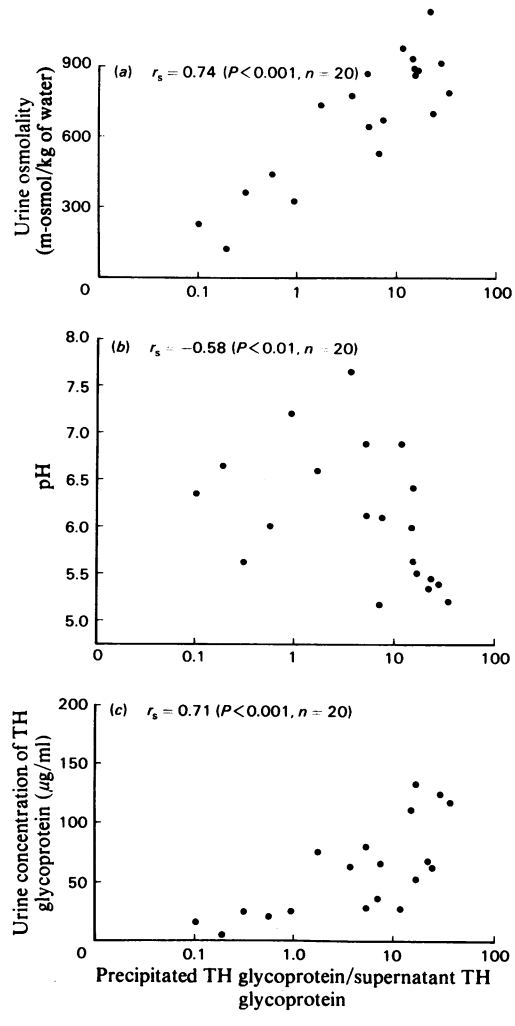


Fig. 1. *Evaluation of factors affecting the aggregation of TH glycoprotein in urine*

Samples of urine were centrifuged and the supernatant and dissolved precipitate assayed for TH glycoprotein as described in the text. The ratio of the amount precipitated to that remaining in the supernatant was calculated and compared with urine osmolality (*a*), urine pH (*b*) and the concentration of TH glycoprotein (*c*) in the original sample.

### *Investigation of sample treatment before assay*

*Effect of diluent.* Eleven samples of urine of various osmolalities (121–1145 m-osmol/kg of water) and pH (5.17–7.20) were either diluted 1 in 100 in water or 1 in 100 in assay diluent or in various proportions of the two such that the final dilution was 1 in 100.

The apparent concentration of TH glycoprotein in urine increased with increasing dilution of sample in water and reached a plateau at a dilution of 1 in 20 (Fig. 2). The maximum increase in the apparent concentration correlated positively and significantly with increasing osmolality ($r_s = 0.773$, $P < 0.01$, $n = 11$), decreasing pH ($r_s = 0.682$, $P < 0.05$, $n = 11$) and increasing TH glycoprotein concentration ($r_s = 0.755$, $P < 0.01$, $n = 11$) of the urine samples. A dilution of 1 in 100 in water was chosen for routine use and results were independent of incubation time at this dilution.

*Effect of pH.* (*a*) The pH of five urine samples was adjusted into the range pH 5 to pH 12 by the addition of 5 M-NaOH or 5 M-HCl such that there was negligible change in sample volume. After overnight incubation, portions (1 ml) of urine were centrifuged as described above, the supernatant removed and the precipitate dissolved in water. All samples were diluted 1 in 100 in assay diluent before the measurement of TH glycoprotein.

As the pH of whole urine was increased, the amount of TH glycoprotein precipitated by centrifugation decreased and greater amounts remained in the supernatant. At pH 11.0–11.5, no TH glycoprotein was detectable in the precipitate and the apparent concentration in the supernatant reached a maximum. Thus the mean concentration of TH glycoprotein in the original sample was 7.0 μg/ml (range 4.0–8.8), which increased to 38 μg/ml (range 20–74) at pH 11.0–11.5. Any further increase in pH resulted in a decrease in the concentration of TH glycoprotein.

(*b*) Five urine samples were initially diluted 1 in 100 in water and the pH then adjusted into the same range as above. No variation in the apparent concentration of TH glycoprotein was found. Increasing pH to greater than pH 11.0–11.5 again resulted in a decrease in TH glycoprotein concentration.

*Effect of $Ca^{2+}$ concentration.* To four urine samples containing $Ca^{2+}$ in the range 5.5–9.2 mmol/litre was added an equal volume of water or of $CaCl_2$ at concentrations of 10, 20 or 40 mmol/litre in water. Samples were stored at 4°C overnight and diluted 1 in 100 in either water or assay diluent before assay.

As expected from the previous experiments, the apparent concentration of TH glycoprotein was much greater in the water-diluted samples than in those diluted in assay diluent. However, no difference was found in its concentration in relation to the amount of $Ca^{2+}$ added.

*Effect of SDS.* Four samples of urine were diluted 1 in 20 or greater in various concentrations of SDS up to 0.01% (w/v) in water. They were incubated for 1 h at 37°C before assay. Such treatment had no effect on the measured concentration of TH glycoprotein.

### *Stability of TH glycoprotein*

Portions (1 ml) of six samples of neat urine and urine after a 1 in 100 dilution in water were frozen either at −20°C or in a mixture of methanol and $CO_2$ in a wide diameter (1.4 cm) tube. Samples were also stored at room temperature and at 4°C for 2 days. The concentration of TH glycoprotein after any such modes of storage did not differ from that measured in fresh samples (mean 40 μg/ml, range 9–69).

### *Validation of the assay for urinary TH glycoprotein*

Urine samples were routinely diluted 1 in 100 in water and then doubly diluted in assay diluent to give three further dilutions. Parallelism was demonstrated between the standard preparation and urine samples at these four dilutions. Occasionally, and especially in concentrated urine samples, a deviation was seen at the lowest dilution but not in the remaining three.

The recovery of various amounts (94–1500 ng/ml) of standard TH glycoprotein added to urine samples that contained 292 and 200 ng/ml after a 1 in 100 dilution in water varied in the range 96 to 114% (mean 106%).

### *Physiological range*

A blood sample and a 24 h urine collection were obtained from 15 female and 14 male volunteers

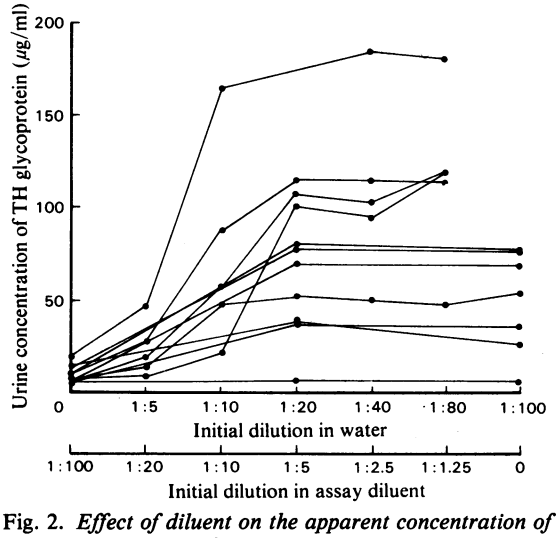


Fig. 2. *Effect of diluent on the apparent concentration of TH glycoprotein in urine*

Samples were diluted 1 in 100 in water or 1 in 100 in assay diluent or in various proportions of the two such that the final dilution was 1 in 100.

with creatinine clearances within the normal range (70 to 130 ml/min, uncorrected for body-surface area). The blood sample was drawn at 09:00 h as the concentration of TH glycoprotein at this time has been shown to be representative of the 24 h mean (Dawnay, 1981). Creatinine was estimated by a standard continuous-flow Jaffe reaction. Creatinine clearances were calculated from the 24 h urinary output of creatinine and the serum creatinine result on the above blood sample, which was drawn on the morning of completion of the urine collection.

The urinary excretion of TH glycoprotein ranged from 22 to 53 mg/24 h (mean 41) in males and from 26 to 56 mg/24 h (mean 40) in females; concentrations in serum were within the normal range (70–540 ng/ml) (Dawnay & Cattell, 1981). There was no significant correlation between serum concentrations of TH glycoprotein and urinary excretion rate ($r_s = 0.332$, $0.05 < P < 0.1$, $n = 29$), nor between urinary excretion rate and creatinine clearance ($r_s = 0.035$, $P > 0.2$, $n = 29$), even when the latter had been corrected for body-surface area ($r_s = 0.154$, $P > 0.2$, $n = 29$).

## Discussion

We have described a simple, rapid and reliable method for the assay of urinary TH glycoprotein in which the sample behaves in an immunologically identical manner with the disaggregated standard preparation. The results are unaffected by variations in the concentration and pH of the sample, provided that it is sufficiently diluted in water before assay. This suggests that a uniform state of disaggregation has been attained. The increasing proportion of TH glycoprotein precipitated in urines of high osmolality, low pH or high concentrations of TH glycoprotein is the first direct measurement of the effect of these factors on the glycoprotein in whole urine. Although the contribution of these factors has been assessed separately it must be remembered that they may be acting either together or competitively and that the relative importance of each is not known.

The aggregated form of TH glycoprotein may be isolated from the urine by salt precipitation (Bichler *et al.*, 1973). McKenzie and co-workers (1964) demonstrated that increasing dilution of urine decreased the proportion of TH glycoprotein that could be salt-precipitated, suggesting that disaggregation had occurred on dilution. Although no details of urine concentration and pH were given, they found that at concentrations of TH glycoprotein greater than 25 μg/ml, most of it was precipitated by the addition of 0.58 M-NaCl. This was similar to the concentration of TH glycoprotein above which we found large increases in the proportion precipitated after centrifugation only (Fig. 1).

The normal range in urine pH and concentration may result in considerable aggregation of TH glycoprotein. Since aggregation may mask antibody binding sites, leading to falsely low results, complicated assays were developed (see the Introduction). The increase in apparent TH glycoprotein concentration with increasing dilution in water appears to confirm this theory. It is also supported by the observation that a greater increase occurred on dilution when the original urine sample was of high osmolality, low pH or high concentration of TH glycoprotein. The fact that such increases were not observed after dilution in assay diluent only is presumably due to its high concentration of $Na^+$ (117 mmol/litre) preventing disaggregation (Stevenson *et al.*, 1971). These results parallel those of Tamm & Horsfall (1950), who measured the ability of urine to inhibit haemagglutination by various viruses. The apparent concentration of inhibitor (subsequently known as TH glycoprotein) increased when the sample osmolality was decreased and this increase was greater the higher the initial osmolality. They also found that large amounts of their inhibitor could be precipitated by centrifugation at moderate speeds (7000 *g*).

Increasing the pH of urine samples resulted in an apparent increase in the concentration of TH glycoprotein when samples were subsequently diluted in assay diluent but not if they had already been diluted in water. At a pH greater than approx. 11.5 the concentration decreased, presumably due to denaturation. Similar results were reported by Mazzuchi and co-workers (1974) by increasing the NaOH concentration in a single urine sample, although they did not measure the pH. These observations would again support the theory that increasing disaggregation results in the exposure of previously hidden antibody binding sites. The absence of any pH effect after the sample had been diluted 1 in 100 in water shows that dilution is at least as effective in irreversibly exposing such binding sites.

The lack of effect of $Ca^{2+}$, which was expected to decrease the apparent concentration of TH glycoprotein, may be due to the fact that the endogenous concentrations (5.5–9.2 mmol/litre) were high enough to cause aggregation, as only 6–7 mol/litre has been shown to suffice (Stevenson *et al.*, 1971). The presence of EDTA in the assay diluent would also help to reduce the effect of $Ca^{2+}$ by chelation.

Incubation with SDS had no effect on the apparent concentration of TH glycoprotein. It has been shown that SDS concentrations of 0.25% are sufficient to completely disaggregate solutions of the glycoprotein of 1 mg/ml in water (Bichler *et al.*, 1973). Thus the concentration used here (0.01%) should be adequate for a solution of 40 μg/ml, a concentration much higher than was found in any

samples after dilution in water. Thus either the TH glycoprotein was already disaggregated by dilution alone or any subsequent disaggregation due to SDS had no effect on quantification.

Quantification of TH glycoprotein was found to be unaffected by storage of urine samples at room temperature or at 4°C for several days or after freezing at −20°C or in methanol/$CO_2$. Other workers, using different methods, have found no change (Akesson *et al.*, 1978; Samuell, 1978), small increases (Grant & Neuberger, 1973) or decreases (Wieslander *et al.*, 1977) in the concentration of TH glycoprotein. It has also been reported that only by freezing urine in methanol/$CO_2$ after dialysis against water (i.e. virtually salt-free) can the 'true' concentration of TH glycoprotein be estimated (Goodall & Marshall, 1978, 1980) and that omission of this procedure results in values of approx. 10% of the 'true' value. However, our results on urine samples that were diluted 1 in 100 in water, and were thus virtually salt-free, were unaffected by this method of freezing. It may be that rendering urine salt-free by dialysis only, although reducing the osmolality and the $H^+$ concentration, still results in a solution containing a high concentration of TH glycoprotein. This may result in it remaining aggregated or may cause it to re-aggregate when subsequently diluted in phosphate-buffered saline that contains a high concentration of NaCl (154 mmol/litre) and only 0.0011% SDS, as in the method of Goodall & Marshall (1980). This latter aggregation might be prevented if freezing altered the structure.

The physiological range established by our method (22–56 mg/24 h) is in good agreement with most other estimates (Grant *et al.*, 1973; Bichler *et al.*, 1976; Mazzuchi *et al.*, 1974; Haugen *et al.*, 1978; Samuell, 1978). However, we could find no significant correlation between urinary TH glycoprotein excretion and creatinine clearance as reported elsewhere (Grant *et al.*, 1973; $r = 0.71$, $P < 0.0025$, $n = 15$) even when the creatinine clearance was corrected for body-surface area. Such discrepancies may reflect small sample numbers or variable results in the assays for TH glycoprotein.

This paper is the first to compare the urinary excretion rate and serum concentration of TH glycoprotein. It should be noted that the amount excreted per day is considerably greater than the amount present in serum. The poor correlation between the two suggests that they do not depend simply on the amount of functioning renal mass. Determination of the factors governing the excretion and secretion of TH glycoprotein may help to elucidate its physiological role.

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