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Affinity-purified antibodies of defined specificity for use in a solid-phase microplate radioimmunoassay of human Tamm–Horsfall glycoprotein in urine

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Rabbit antibodies to human Tamm–Horsfall glycoprotein (prepared by salt precipitation from normal urine) were purified by affinity chromatography using columns containing Tamm–Horsfall glycoprotein linked to CNBr-activated Sepharose 4B. The specificity of these antibodies was determined by analysis of their binding characteristics on Western blots of Tamm–Horsfall protein from sodium dodecyl sulphate/polyacrylamide gradient gels and comparison with the reactivity of monoclonal antibodies to this glycoprotein. Optimal conditions of adsorption to poly(vinyl chloride) microtitre plates were established such that these purified antibodies could be used in a solid-phase radioimmunoassay for the determination of urinary Tamm–Horsfall-glycoprotein concentration. The specificity of the immunoassay was confirmed by competitive inhibition of the urinary Tamm–Horsfall glycoprotein by purified freeze-dried material in solution. A standard curve obtained with this material showed the radioimmunoassay to have a sensitivity of at least 5ng/ml, with linearity between 30 and 600ng/ml. The mean coefficient of variation over the linear section of the curve was $11.3 \pm 2.2\%$ ($n = 13$). The effects of dialysis and freezing of urine samples before determination of Tamm–Horsfall-glycoprotein concentrations were investigated and the mean 24h urinary excretion rate in 60 normal donors was shown to be 84.9 ± 44.1 mg.

Urine contains a glycoprotein, Tamm–Horsfall protein (THP) (Tamm & Horsfall, 1950, 1952), which exists as a polymer with a subunit M_r of approx. 100000 (Fletcher *et al.*, 1970*a*; Stevenson & Kent, 1970; Hunt *et al.*, 1980). It is produced by a discrete part of the distal tubule (Hoyer *et al.*, 1979; Sikri *et al.*, 1979) and is the main component of urinary casts (McQueen, 1962; Fletcher *et al.*, 1970*b*). THP may be present in serum, but radioimmunoassays directed at establishing this (Avis, 1977; Dawnay *et al.*, 1980) have suffered from the potential problem of antibodies to THP cross-reacting with other material in serum (Lynn & Marshall, 1981).

The glycoprotein's physiological role is unclear, but it has been implicated in renal disease (see Hoyer & Seiler, 1979; Lynn, 1982), and a specific immunoassay would be useful in evaluating pathogenetic processes in the kidney.

Abbreviations used: SDS, sodium dodecyl sulphate; THP, Tamm–Horsfall proteins; PVC, poly(vinyl chloride).

Methods used for the determination of THP in human urine include salt precipitation (McKenzie *et al.*, 1964), radial immunodiffusion (Mazzuchi *et al.*, 1974), electroimmunoassay (Bichler *et al.*, 1973; Wieslander *et al.*, 1977; Samuel, 1978) and radioimmunoassay (Grant & Neuberger, 1973; Goodall & Marshall, 1978, 1980; Lynn & Marshall, 1981; Dawnay *et al.*, 1982). Most of these methods have required a dissociating agent, usually SDS.

We have established a sensitive and specific solid-phase radioimmunoassay for THP using affinity-purified antibodies whose specificity has been compared with that of monoclonal antibodies to human THP. We report data defining the specificity of these antibodies, their application in the development of a radioimmunoassay for THP and the measurement of urinary THP excretion in normal subjects.

Materials and methods

Suppliers of materials

CNBr-activated Sepharose 4B, Sephadex G-50 and G-200 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; complete Freund's adjuvant from Grand Island Biological Co., NY, U.S.A.; $Na^{125}I$ (IMS-30) from Amersham International, Amersham, Bucks., U.K., polystyrene and flexible PVC Cooke microtitre plate with U-shaped wells from Dynatech Laboratories, Alexandria, VA, U.S.A.; bovine and human serum albumins (fraction V), 3,3'-diaminobenzidine tetrahydrochloride and polyoxyethylene sorbitan monododecanoate (Tween-20) from Sigma Chemical Co., St. Louis, MO, U.S.A.; affinity-purified goat anti-mouse antibodies (fluorescein isothiocyanate and horseradish peroxidase conjugates) from Tago, Burlingame, CA, U.S.A.; nitrocellulose membranes from Bio-Rad, Richmond, CA, U.S.A. All chemicals used were of laboratory-reagent grade or better.

Isolation of THP

THP was isolated from urine as previously described (Hunt & McGiven, 1978). SDS/polyacrylamide-gel electrophoresis of these preparations (Hunt *et al.*, 1980) showed one major protein band with an M_r of 100000 (±10%).

Collection of urine samples for analysis

Urine samples (24h) were collected from patients and healthy laboratory staff over NaN_3 (final concn. 0.2g/litre), the volume measured, and 20ml portions stored at −20°C. Samples to be assayed were thawed in a water bath at 37°C, mixed well and 1ml portions were dialysed overnight with stirring at 4°C in distilled water containing 0.02% NaN_3.

Preparation of THP–Sepharose 4B for affinity chromatography

THP (obtained as above) was coupled to CNBr-activated Sepharose 4B according to the standard method outlined by Pharmacia. THP (15mg) was solubilized in 2ml of 0.1M-sodium borate/NaOH buffer, pH9, added to 1g of CNBr-activated Sepharose 4B and mixed for 2h at 22°C. After blocking any remaining active groups with 1M-ethanolamine, pH8, the gel was extensively washed and stored at 4°C in phosphate-buffered saline (57mM-Na_2HPO_4/18mM-KH_2PO_4/77mM-NaCl, pH7.4), containing 0.02% NaN_3.

Affinity purification of rabbit anti-(human THP) antiserum

Rabbit anti-(human THP) antiserum was produced in New Zealand White rabbits after subcutaneous injections at multiple sites with a total of 1mg of THP emulsified in complete Freund's adjuvant (2mg of THP in 1ml of alkaline phosphate-buffered saline, pH9, plus 1ml of complete Freund's adjuvant). Animals were similarly boosted after 1 month and bled from an ear vein 1 week later. Immunodiffusion of this antiserum in 1% (w/v) agar (Ouchterlony, 1958) against a solution of freeze-dried THP (1mg/ml) showed one precipitin arc. Antiserum (2.5ml) was diluted 1:1 with 0.2M-Tris/HCl buffer, pH8, and specific anti-human antibodies were prepared by using an affinity column of Sepharose 4B–THP (Hunt & McGiven, 1980). After dialysis against phosphate-buffered saline and concentration to about 1mg/ml with an Amicon stirred cell, eluted specific antibodies were divided into portions (250μg) and stored in silicone-treated glass tubes at −70°C.

Iodination of proteins

Proteins were labelled with $Na^{125}I$ by the chloramine-T method (Hunter, 1978). THP (2mg/ml) was dissolved in 0.5M-NH_4HCO_3 buffer, pH9, adjusted to pH8 with 0.5M-phosphate buffer, pH7.4, and 100μl portions were iodinated as described by Hunt & McGiven (1980) to give specific radioactivities of 0.1–0.2μCi/μg. Affinity-purified rabbit anti-(human THP) antibody (250μg) in phosphate-buffered saline was labelled in a similar manner to give specific radioactivities of 1–2μCi/μg.

Radioimmunoassay procedure

The radioimmunoassay was based on the solid-phase procedure described by Kalmakoff *et al.* (1977) with flexible PVC microtitre plates with U-shaped wells coated with affinity-purified rabbit anti-(human THP) antibody (100μl of 0.5–1μg/ml in phosphate-buffered saline) by incubation for 2h at 37°C. After washing the wells three times by filling and emptying with cold phosphate-buffered saline, non-specific binding was blocked with human serum albumin (1%, v/v, in phosphate-buffered saline at 200μl/well) by incubation for 1h at room temperature. After three further washes as described above, dialysed urine samples (100μl), diluted 1:200 with phosphate-buffered saline containing 0.05% Tween-20, were incubated in triplicate in the antibody-coated wells for 1h at 37°C. After three washes with cold phosphate-buffered saline, affinity-purified ^{125}I-labelled rabbit anti-(human THP) antibody (50μl of a 0.25μg/ml solution) was added and incubated for 1h at 37°C. After three washes with phosphate-buffered saline the wells were drained, and cut out and placed in plastic tubes for radioactivity counting in a γ-radiation spectrometer (Beckman Biogamma). Standard curves were constructed with freeze-

dried THP (2mg/ml in 0.01M-NH_4HCO_3/NaOH, pH9) diluted in phosphate-buffered saline/Tween to give concentrations from 5 to 10000ng/ml. Each concentration was assayed in triplicate as for the urine samples.

Immunoblotting technique

Antiserum, affinity-purified antibodies and monoclonal anti-THP antibodies (Hunt *et al.*, 1985) were allowed to react with Western blots (Towbin *et al.*, 1979) formed after transfer to nitrocellulose of human and rat THP and human serum samples from SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) in 5–20%-(w/v)-polyacrylamide-gradient slab gels. Autoradiography was carried out using affinity-purified ^{125}I-F(ab')$_2$ fragments of rabbit anti-mouse IgG or sheep anti-rabbit IgG (Dalchau & Fabre, 1979).

Results

Specificity of antibodies

Western blots (Fig. 1) showed that both the antiserum (lane 3) and affinity-purified antibodies (lane 7) reacted with human THP. Our THP preparations appeared primarily as a single band of M_r 100000. Higher-M_r bands were faintly visible on silver staining (lane 2). There was also some weakly stained material (M_r 60000–100000), which was less noticeable with rat THP (lane 1). The antiserum showed some cross-reactivity with rat THP (lane 4), most of which was removed by affinity purification (lane 8). The reactivity of the affinity-purified antibodies was identical with that of our monoclonal antibody THP/C4 (lane 5), which showed strong reactivity with the 100000-M_r band and faint binding with a higher-M_r (about 185000–190000) band, probably representing a dimer of THP. Neither of these antibodies, nor five other anti-THP monoclonal antibodies, showed any cross-reactivity on normal human heart, liver or spleen when tested histologically by immunofluorescence. These monoclonal and affinity-purified antibodies bound to tubular casts and distal-tubular cells in normal human kidney on screening by immunofluorescence and were clearly demonstrated by an immunoperoxidase technique (K. L. Lynn, P. Ellingsen & R. Chambers, unpublished work).

Adsorption of ^{125}I-labelled rabbit anti-(human THP) antibody to plastic plates

Preliminary experiments showed that labelled antibody bound to both PVC and polystyrene plates throughout the pH range (7.5–9.0) tested, that binding increased with time, and was slightly better at low concentrations of antibody. After 2h incubation at 37°C, approx. 55% of the labelled antibody (0.5μg/ml in phosphate-buffered saline) was adsorbed and this remained bound to the PVC after coating with bovine serum albumin and washing with phosphate-buffered saline.

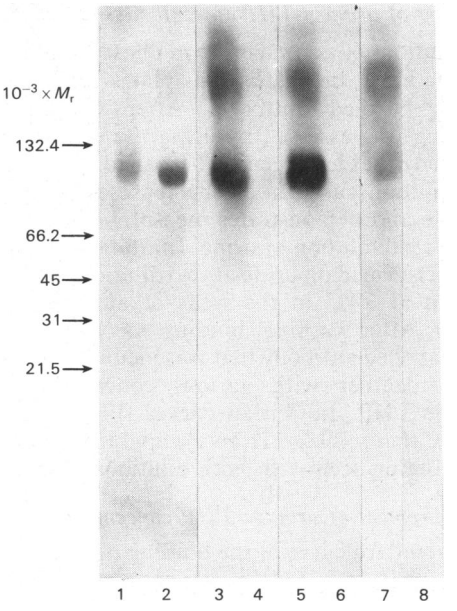

Fig. 1. *Electrophoretic and Western-blotting profiles of THP*

Samples (20μg) of human and rat THP were run on Laemmli SDS/5–20%-(w/v)-polyacrylamide-gradient slab gels and blotted overnight on to nitrocellulose paper. Blots were developed by using ^{125}I-F(ab')$_2$ rabbit anti-mouse IgG or sheep anti-rabbit IgG and autoradiographs exposed for 24h at −70°C. Lanes 2, 3, 5 and 7, human THP; lanes 1, 4, 6 and 8, rat THP; lanes 1 and 2, silver-stained gel containing 50μg of rat THP (lane 1) and 20μg of human THP (lane 2); lanes 3 and 4, blots incubated with rabbit anti-human THP antiserum (1:500, ~2–4μg antibody/ml); lanes 5 and 6, blots incubated with THP/C4 ascitic fluid (2.5μg of monoclonal antibody/ml; lanes 7 and 8, blots incubated with affinity-purified antibody (0.5μg/ml). Arrows represent positions of standards, which were, from the bottom: soya-bean trypsin inhibitor, carbonic anhydrase, ovalbumin, and bovine serum albumin monomer and dimer.

Binding of soluble THP to antibody-coated plates

THP binding increased with increasing antibody concentrations, but reached a plateau with plates incubated with antibody at 2μg/ml. Plates coated with antibody at this concentration had the capacity to bind approx. 50% of labelled THP. Subsequent adsorption of antibody to plastic plates was performed after incubation at 37°C for 2h with antibody at 1μg/ml.

Binding of urinary THP to antibody-coated plates

Dilutions of normal urine in phosphate-buffered saline were incubated on plates coated with affinity-purified antibody. After washing, the binding was assessed by using the same purified antibody that had been ^{125}I-labelled. Fig. 2 shows the dilution curve from one representative urine sample and demonstrates measurable binding up to a 1:500 dilution of urine. Inhibition of binding was performed on urine at two dilutions incubated for 1 h at 37°C in the wells of antibody-coated plates. After washing, binding was assessed with ^{125}I-labelled antibody that was incubated for 1 h at 37°C together with various concentrations of soluble THP. Inhibition curves (Fig. 3) demonstrated that soluble THP was capable of inhibiting the binding seen with both dilutions of urine.

Measurement of urinary THP concentration

A standard curve of the binding of soluble THP to antibody-coated plates was constructed by using dilutions of a stock solution of THP (2mg/ml in 0.01 M-NH_4HCO_3, pH9). Portions were thawed, diluted 1:200 in phosphate-buffered saline/Tween and subsequently serially diluted to give THP concentrations from 10000 to 5ng/ml. The standard curve (Fig. 4) showed linearity over a THP concentration range of 30–600ng/ml. An initial dilution of 1:200 for urine samples was found to be most convenient. A standard curve and a control urine sample were included on each plate of urine samples assayed. The plate-to-plate coefficient of variation amongst the different concentrations of the curve ranged from a minimum of 8.8% at 78ng/ml to a maximum of 20% at 20ng/ml ($n = 13$), with a mean coefficient of variation for the five concentrations on the linear section of the curve of $11.3 \pm 2.2\%$.

Effects of dialysis and freezing on apparent urinary THP concentration

Four urine samples were collected from laboratory staff and assayed after a variety of treatments. Urinary THP concentrations were apparently decreased if undialysed urine was assayed and elevated if measured after dialysis and freezing

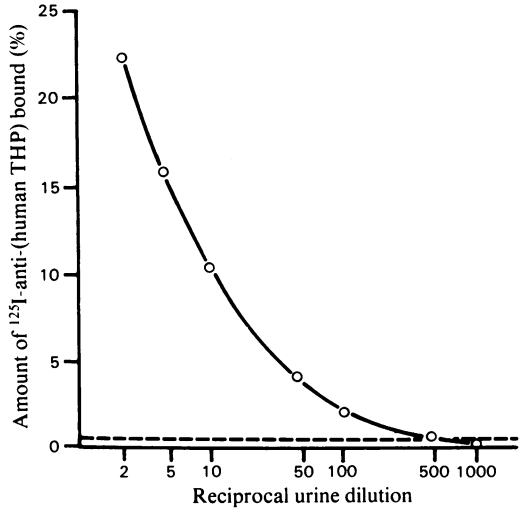

Fig. 2. *Urine dilution curve on PVC plate coated with affinity-purified rabbit anti-(human THP) antibody*
Dialysed urine diluted in phosphate-buffered saline/Tween was incubated in triplicate for 1 h at 37°C on a microtitre plate coated with purified antibody (1 μg/ml) for 1 h at 37°C, followed by 1% bovine serum albumin for 1 h at room temperature. After washing three times with phosphate-buffered saline, binding was measured by using ^{125}I-labelled purified rabbit anti-(human THP) antibody (0.5 μg/ml) in phosphate-buffered saline/Tween and incubation for 1 h at 37°C. Each point is the mean of triplicate determinations. The broken line represents a phosphate-buffered-saline blank control.

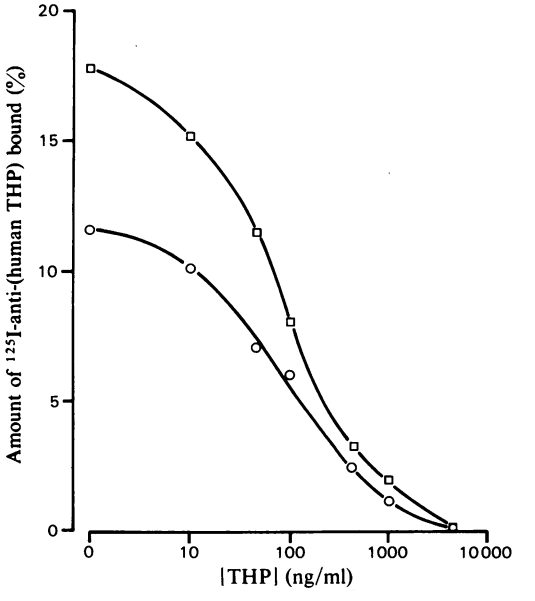

Fig. 3. *Inhibition of urine binding by soluble THP*
Dialysed urine at two dilutions in phosphate-buffered saline/Tween was incubated for 1 h at 37°C on PVC microtitre plates coated with affinity-purified rabbit anti-(human THP) antibody. After washing three times, binding was assessed by using ^{125}I-rabbit anti-(human THP) antibody, which was incubated in the wells (1 h at 37°C) together with increasing concentrations of a solution made from freeze-dried THP in phosphate-buffered saline. Each point is the mean of triplicate determinations. □, Urine diluted 1:5; ○, urine diluted 1:50.

(Table 1). The apparent concentration of THP in dialysed urine appeared considerably more elevated after snap-freezing to −70°C in solid CO_2/ethanol than after slow freezing by placing in a deep-freeze at −20°C. If fresh urine was dialysed and assayed, concentrations very similar to those reported by Goodall & Marshall (1978, 1980) were observed.

Further experiments where fresh urine samples, or urine samples thawed after storage at −20°C, were dialysed at 4°C for various times from 15 min to 48 h showed that the apparent THP concentration in urine increased with dialysis up to 1–2 h and then reached a plateau (results not shown) in both fresh and frozen stored urine.

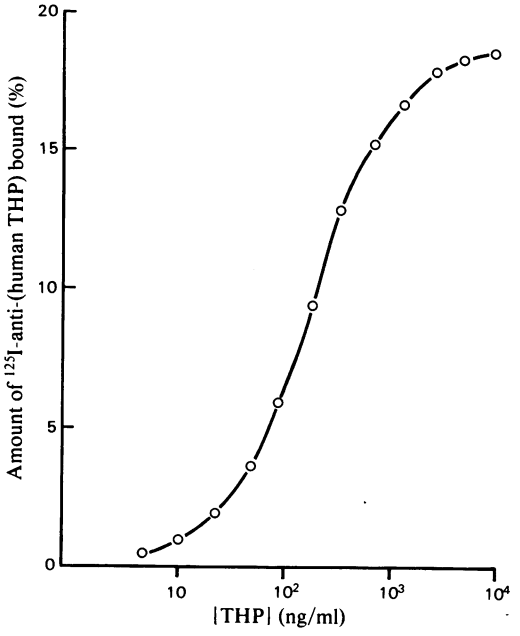

Fig. 4. *Standard curve of THP concentration*
A stock solution of THP at 2 mg/ml was diluted in phosphate-buffered saline/Tween and each concentration measured in triplicate on PVC microtitre plates coated with affinity-purified rabbit anti-(human THP) antibody as described in the Materials and methods section. Each point is the mean of 13 determinations.

Effects of diluent on apparent urinary THP concentration

A number of dialysed and undialysed urine samples were diluted 1:100 in distilled water, 0.1% SDS in distilled water or 0.05% Tween-20 in phosphate-buffered saline before being assayed for apparent THP concentration. All undialysed samples and dialysed samples diluted in distilled water or 0.1% SDS had very low apparent THP concentrations ($\leqslant 1\,\mu$g/ml), whereas dialysed samples diluted in phosphate-buffered saline/Tween had concentrations of 10–50 μg/ml.

Urinary THP concentrations in 60 normal subjects

Urine collections (24 h) were made by 60 healthy volunteers (29 men, 31 women) with a mean age of 29.8 ± 9.5 years. Portions (20 ml) were stored at −20°C until the day before assay. After dialysis of 1 ml of urine, 0.5 ml was snap-frozen in a solid-CO_2/ethanol mixture at −70°C and kept at this temperature for 5 min before being thawed and assayed together with the remaining 0.5 ml in the solid-phase radioimmunoassay for THP concentration. There was a considerable increase in the range of THP concentration after snap-freezing at −70°C for 5 min (Table 2). In general, the apparent concentrations increased, although many individual measurements were actually decreased

Table 1. *Effects of dialysis and freezing on urinary THP concentrations determined by solid-phase radioimmunoassay*
Binding of urine at three dilutions in phosphate-buffered saline/Tween was measured on PVC microtitre plates coated with affinity-purified rabbit anti-(human THP) antibody as described in the Materials and methods section. Samples (1 ml) were measured before (fresh) and after (dialysed) overnight dialysis against distilled water at 4°C, after dialysis and snap-freezing at −70°C in solid CO_2, after dialysis and slow freezing at −20°C in a deep-freeze, or after dialysis of fresh urine that had been stored frozen at −20°C. Concentrations are means ± S.D. of triplicate determinations at each dilution calculated from a standard curve of THP binding run on the same plate.

	Apparent THP concentration (μg/ml)				
Sample	Fresh	Dialysed	Dialysed and snap-frozen	Dialysed and slow-frozen	Frozen fresh and dialysed
1	1.5 ± 0.2	35.1 ± 2.9	143.2 ± 16.5	80.2 ± 1.0	49.1 ± 6.8
2	1.3 ± 0.6	25.4 ± 1.8	64.8 ± 14.8	40.2 ± 0.5	33.6 ± 5.4
3	14.8 ± 1.2	24.8 ± 1.0	71.8 ± 8.3	40.2 ± 0.5	31.9 ± 4.3
4	9.2 ± 1.7	20.1 ± 4.8	40.1 ± 8.7	27.3 ± 5.6	22.0 ± 3.4

Table 2. *THP concentration in urine from normal donors*
A total of 60 24h urine samples were assessed on anti-(human THP) antibody-coated PVC plates after dialysis alone or dialysis and snap-freezing as for Table 1. Concentrations were calculated from triplicate determinations after comparison with the standard curve run on each plate.

Urine	THP concentration (μg/ml)		THP excretion (mg/24h)	
	Mean ± S.D.	Range	Mean ± S.D.	Range
Dialysed	60.9 ± 47.3	8.9–252.4	84.9 ± 44.1	17.8–203
Dialysed and snap-frozen	76.7 ± 101	4.5–679.2	102.0 ± 70.2	13.3–387.7

(29/60 increased, 22/60 decreased, 9/60 unchanged).

Discussion

THP has been shown to be confined to specific areas of the kidney by histological methods (Hoyer *et al.*, 1979; Sikri *et al.*, 1979). The affinity-purified antibodies used in the present study were shown by immunofluorescence (McGiven *et al.*, 1978) and immunoperoxidase techniques to react specifically with these areas, i.e. tubular casts and distal tubular cells of normal human kidney. They also bind specifically to purified THP, as demonstrated by Western blotting. Furthermore, they show an identical specificity to a number of monoclonal antibodies against THP.

It has been reported that THP (Avis, 1977; Dawnay *et al.*, 1980) or material with which antibodies to THP cross-react (Lynn & Marshall, 1981) is present in normal human serum. The results from Western-blotting experiments with our affinity-purified anti-THP antibodies suggest that they react specifically with this glycoprotein or one with very similar biochemical properties and an identical histological distribution. Furthermore, we have recently shown that the affinity-purified antibodies used in this radioimmunoassay do not cross-react with a wide range of normal biological fluids, including human serum or plasma, and show a high degree of specificity for primate urine (Taylor & Hunt, 1983). It seems possible that some of the reactivity towards material reported to be present as THP in normal serum could be removed by prior affinity purification of the antibodies used for these immunoassays.

Radiolabelling of THP with ^{125}I results in an unstable product (Grant & Neuberger, 1973; Dawnay *et al.*, 1980, 1982). The use of solid-phase immunoassays with radiolabelled antibody avoids this problem. Under our assay conditions, provided the urine is not frozen after dialysis, reasonably consistent results can be obtained. Freezing of dialysed urine before analysis appears to alter the protein in such a way as to make its apparent concentration more variable. Aggregation 'artefacts' may be minimized by the high dilution (1:200) of urine being measured and the use of phosphate-buffered saline containing Tween-20, a non-ionic detergent shown to minimize non-specific reactivity and a common additive in many solid-phase immunoassay systems (Engvall & Perlmann, 1972).

Our solid-phase radioimmunoassay is rapid and simple to perform, with the standard curve between 30 and 600ng/ml (Fig. 4) showing good reproducibility from plate to plate and day to day. The affinity-purified antibodies used to coat the PVC plates retain their reactivity for at least 6 weeks provided that the plates are stored dry at 4°C. ^{125}I-labelled antibodies are stable for up to 4 weeks if stored as portions and frozen at −20°C after the addition of 1% human serum albumin. Urine samples may be collected and frozen until required for assay. There does not appear to be a great difference between THP concentrations in fresh or frozen urine samples assayed after dialysis (Table 1).

Other immunoassays for the determination of 24h THP excretion in normal urine have given values of from 30.3mg/24h (Wieslander *et al.*, 1977) to 84.7mg/24h (calculated from Goodall & Marshall, 1980). Of these, the method of Goodall & Marshall (1980), applied to a smaller sample than in the present study, appears to have the greatest sensitivity, showing a standard curve with linearity down to 160ng/ml. The present method has a similar curve, with the linear section extended to 30ng/ml, thus increasing the sensitivity approx. 5-fold. The striking similarity between the mean 24h excretion rates measured by these two methods is also noteworthy.

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