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Effects of freezing on the estimated amounts of Tamm–Horsfall glycoprotein in urine, as determined by radioimmunoassay

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Freeze-drying or freezing of salt-free solutions of human Tamm–Horsfall glycoprotein appeared to lead to changes in the structure of the latter, changes that increased its ability to bind with antibody raised, in rabbits, against it. This alteration in avidity of the glycoprotein was observed irrespective of whether antiserum was raised against freeze-dried or non-frozen antigen. The implications of this finding for the radioimmunoassay of the glycoprotein in urine samples were studied. Appropriate treatment for urine samples, before assay, was devised. The amount of Tamm–Horsfall glycoprotein excreted was shown to range from 30 to 138 mg in normal males and 43 to 126 mg in normal females per 24 h.

It is generally accepted that the hyperosmolarity in the medulla of the kidney results from passage of Cl^- ions with their accompanying Na^+ ions across the single cell layer of the lumen of the thick ascending limb of the loop of Henle, a region of the nephron with relatively high impermeability to water (Roche & Kokke, 1973), and it has been hypothesized that Tamm–Horsfall glycoprotein may contribute to the effect (Sikri *et al.*, 1979; Hoyer *et al.*, 1979).

There is tentative evidence that Tamm–Horsfall glycoprotein, a renal product (Cornelius *et al.*, 1965; McKenzie & McQueen, 1969; Schenk *et al.*, 1971; Sikri *et al.*, 1979), may be involved in a number of pathological conditions. These include acute pyelonephritis (Hanson *et al.*, 1976; Larsson *et al.*, 1978) and certain cases of renal tubular acidosis (Tsantoulas *et al.*, 1974). Urinary casts, produced in the nephrotic syndrome, are composed to a large extent of aggregated Tamm–Horsfall glycoprotein (McQueen, 1962; Fletcher *et al.*, 1970). Precipitation of the glycoprotein is suggested to be the first step in urinary stone formation (Hallson & Rose, 1979), and it is possible also that the oligoanuric phase of acute renal failure could result from intratubular precipitation of the substance (Patel *et al.*, 1964). Interstitial renal deposits of the substance were found in a large number of patients with a variety of tubulointerstitial diseases (Zager *et al.*, 1978).

Several immunoassay procedures for the determination of Tamm–Horsfall glycoprotein have been developed (Grant & Neuberger, 1973; Bichler *et al.*, 1973; Mazzuchi *et al.*, 1974; Avis, 1977; Wieslander *et al.*, 1977; Samuell, 1978; Goodall & Marshall, 1978). In these methods the comparative standard for quantification was invariably a preparation of Tamm–Horsfall glycoprotein, which had been freeze-dried. The results reported in the present paper show that freezing salt-free solutions of the glycoprotein, a step in the freeze-drying procedure, leads to changes in the substance, which alter its ability to interact with antibody raised against it. The implications of this finding for the radioimmunoassay of the glycoprotein in urine have been studied.

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Experimental

Isolation of human Tamm–Horsfall glycoprotein

The glycoprotein was isolated from pooled adult urine (Tamm & Horsfall, 1950, 1952; Goodall & Marshall, 1978). Some samples of the final salt-precipitated glycoprotein were dialysed against water at 4°C, shell frozen and freeze-dried, using CO_2/methanol, and these are referred to as FD-Tamm–Horsfall glycoprotein. Other samples of the salt-precipitated glycoprotein were dialysed against water at 4°C and the dialysed solutions were used directly. At no time were they subjected to freezing. These are referred to as NF-Tamm–Horsfall glycoprotein, and preparations made in this way were always used within a maximum of 2 or 3 days.

Preparation of solutions of freeze-dried Tamm–Horsfall glycoprotein

This was carried out by periodic swirling of

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weighed amounts of the glycoprotein in water at 40°C for 2h.

Spectrophotometric determination of Tamm–Horsfall glycoprotein solutions

This was done on appropriately diluted samples of Tamm–Horsfall glycoprotein solutions (after shell-freezing (see below)). Measurements were made in the u.v. region with a Unicam SP.500 spectrophotometer. A value of 10.8 for $A^{1\%}_{1\,cm}$ at a wavelength of 277nm (Finnigan *et al.*, 1971) was used for calculation of the concentrations of the glycoprotein in the solutions before freezing.

Raising of antisera

Antisera to the FD and NF forms of Tamm–Horsfall glycoprotein were raised in rabbits by intramuscular injection of emulsions (0.1 ml injected in five dorsal sites) containing equal volumes of the particular glycoprotein (1 mg/ml of water) and complete Freund's adjuvant (Calbiochem, Hereford, U.K.). Two weeks later 0.5 ml of glycoprotein solution (1 mg/ml) was injected intravenously in the ear, and 10 days after this the rabbits were bled. Blood, taken from the ear vein of the immunized rabbits, was added to tubes containing lithium heparin and the tubes were centrifuged for 25 min at 2000 ***g***. The plasma was used directly.

Radioiodination of Tamm–Horsfall glycoprotein

Solutions in water of FD- and NF-Tamm–Horsfall glycoprotein at concentrations of 1 mg/ml were prepared. $Na^{125}I$ solution (1 mCi; bought from The Radiochemical Centre, Amersham, Bucks., U.K.) was made up to 0.2 ml with water. A portion of this solution (0.1 ml) was added to 100 μl of the particular glycoprotein solution. Chloramine-T solution (0.1 ml; 4 mg/ml) in Dulbecco A buffer (Oxoid) was added. The tube was stoppered and the solution was mixed. The reaction was stopped after 30 s by the addition of 0.2 ml of sodium metabisulphite solution (4.8 mg/ml) in the same buffer. KI (0.4 ml; 20 mg/ml in buffer A) was added, followed by 0.1 ml of bovine serum albumin solution (30 mg/ml in buffer). The sample of radiolabelled Tamm–Horsfall glycoprotein was separated on a Sephadex G-200 column (40 cm × 1.5 cm) equilibrated with Dulbecco A buffer. Volumes of 1.5 ml were collected, and the labelled glycoprotein appeared in fractions 8–15. These fractions were pooled and diluted to 50 ml with buffer A. Portions of the solution were diluted to give 10^5 c.p.m./0.3 ml for the assay.

Radioimmunoassay

A solution of either FD- or NF-Tamm–Horsfall glycoprotein at a concentration of 1 mg/ml was serially diluted with buffer A containing 0.0011% sodium dodecyl sulphate to give concentrations of the freeze-dried glycoprotein from 0.006 to 25 μg/ml and those of the non-frozen material from 0.006 up to 200 μg/ml. The solutions were incubated overnight at 37°C.

Diluted rabbit antiserum raised against either FD- or NF-glycoproteins (1:500 dilution in 0.1 M-Tris/HCl buffer, pH 9.4; 0.5 ml), was incubated in round-bottomed (40 mm × 11 mm) polystyrene tubes (3h; 22°C), and the tubes were washed with 2 × 0.6 ml of buffer A.

Aged human serum (diluted 1:10 with buffer A; 0.6 ml) was incubated in the tubes (0.5 h; 22°C) and removed by aspiration. The preincubated Tamm–Horsfall glycoprotein solutions (0.25 ml) were then added, followed by 0.3 ml of a solution of radiolabelled NF- or FD-glycoprotein (10^5 c.p.m.). The stoppered tubes were kept at 37°C for 16 h and the solutions were aspirated to remove unbound antigen. After washing the tubes (2 × 0.7 ml of water) they were assessed for radioactivity (Packard Auto-gamma spectrometer model 3002).

Zero-inhibitor values were obtained from those control experiments in which buffer A (0.25 ml) was substituted for non-radiolabelled glycoprotein. A low control value was obtained from experiments in which the polystyrene tube was not coated with antibody.

Estimation of Tamm–Horsfall glycoprotein concentration in urines

Collections of normal urine over 24 h periods were made over NaN_3 from AAG (female; 25 years; height 1.62 m; weight 58.9 kg), MRD (male; 32 years; height 1.80 m, weight 72.1 kg) and GHT (male; 42 years; height 1.77 m; weight 73.0 kg). The urine volumes were 2300, 1960 and 2060 ml respectively. Portions of the urines were dialysed against three changes of water for 24 h at 4°C. The dialysed urines were each divided into three parts. The first batch (2 ml) was freeze-dried after the addition of 0.1 ml of [^{14}C]alanine solution (781 c.p.m.). The dried residue was taken up into 2 ml of water. The ^{14}C counts were found to be 800, 760 and 820 c.p.m. respectively for the three samples, indicating little, if any, loss on freeze-drying. The second samples were shell frozen: 1 ml was frozen in a tube of about 2.5 cm diameter for 5 min in solid CO_2/methanol. Each of the last three fractions was kept at 4°C until assayed.

Radioimmunoassay was carried out on all fractions, after dilution, with appropriate preparations of FD- or NF-glycoproteins, radiolabelled samples of these, and antiserum raised against one or other of them.

Results

Standard curves obtained with FD- and with NF-glycoproteins

An examination of standard curves obtained in the radioimmunoassay with the use of either NF- or FD-Tamm–Horsfall glycoproteins reveals a number of interesting features. In the first place the curves obtained are not superimposable (Fig. 1), larger amounts of the NF-glycoprotein than of the FD-glycoprotein being required to inhibit binding of a given quantity of radiolabelled Tamm–Horsfall glycoprotein: in the centre portions of the curves, almost ten times as much NF-glycoprotein can be seen to be required to decrease the amount of binding of the radiolabelled material by 50% as FD-glycoprotein. The results shown in Fig. 1 were obtained with antiserum that had been raised against FD-glycoprotein, as was also the sample of radioiodinated glycoprotein used. The concentration of FD-glycoprotein required to decrease the amount of radiolabelled glycoprotein bound by 50% of that found in the zero-inhibitor control was 1200ng/ml in the experiment shown (Fig. 1), and other values obtained in similar experiments, in which the radiolabelled glycoprotein, the unlabelled glycoprotein and that used for raising antiserum were all freeze-dried preparations, were of the same order of magnitude (Table 1). The relatively large standard deviation emphasizes the need to construct a standard curve for each particular assay; it is probably due to different degrees of iodination that the radiolabelled glycoprotein had undergone during the labelling process. The corresponding value in which NF-glycoprotein was used to derive the results shown (Fig. 1) was 16600ng/ml, and values obtained in other experiments were similar (Table 1).

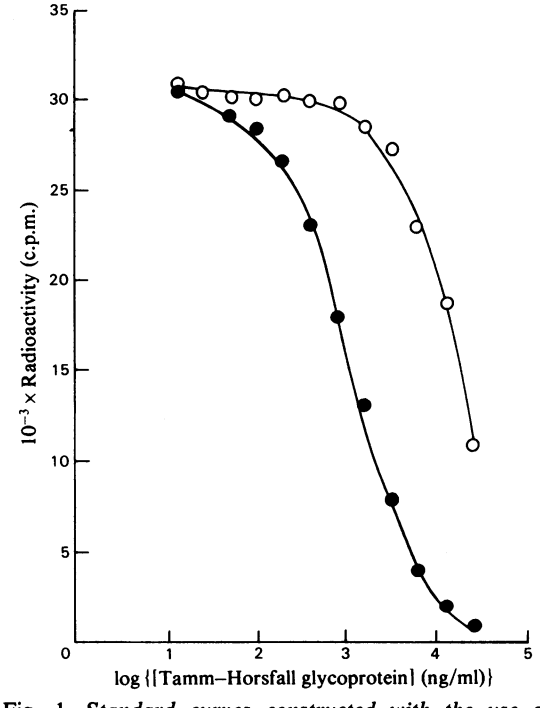

Fig. 1. *Standard curves constructed with the use of antiserum (at a dilution of 1:500) raised in rabbits against FD-Tamm–Horsfall glycoprotein*
Non-radiolabelled glycoprotein was either FD-glycoprotein (●) or NF-glycoprotein (○). The sample of radioiodinated glycoprotein was FD-material.

In another series of experiments antiserum raised in rabbits against NF-glycoprotein was used, and some results are described in Fig. 2. The radioiodinated material was prepared from a freeze-dried sample of the glycoprotein. In these experiments also, larger amounts of the NF-glycoprotein than of the FD-glycoprotein were found to be needed to inhibit binding by similar amounts of radiolabelled FD-Tamm–Horsfall glycoprotein. Concentrations of FD-glycoprotein of 1220ng/ml (Table 1) were needed to decrease binding of the labelled glycoprotein by 50%, but a concentration of 19400ng/ml (Table 1) of the NF-glycoprotein was required.

Radioimmunoassay of Tamm–Horsfall glycoprotein in normal urine

The implication of the data reported was tested by the determination of the amounts of Tamm–Hors-

Table 1. *Concentrations (ng/ml) of FD- and NF-Tamm–Horsfall glycoprotein solutions required to decrease by 50% the amount of radiolabelled glycoproteins bound in the control experiments*

Antisera were raised in rabbits against either FD- or NF-glycoprotein. The radiolabelled glycoprotein was in all cases prepared from an FD-sample. Numbers of samples refer to different preparations of glycoproteins.

Nature of glycoprotein used in assay	Antibody raised against: FD-glycoprotein	NF-glycoprotein
FD-glycoprotein	1455 ± 600 (S.D.; $n = 13$)	1580, 960, 1120 (mean 1220)
NF-glycoprotein	16600, 14800, 14750, 13800 (mean 15000)	19500, 18100, 20500 (mean 19400)

fall glycoprotein in urine. All samples were dialysed against water exhaustively, and they were subsequently divided into groups of three. One was freeze-dried and the material dissolved in water, a second in each case was shell-frozen and the remaining one was kept at 4°C. Assays were then made on each of the three solutions, with appropriate samples of FD- or NF-glycoproteins for deriving the standard curves, for use in raising antibody and for preparation of the radioiodinated samples. The results show (Table 2) that closely similar results were obtained for each of the three treated samples of any given urine.

Radioimmunoassay of random normal 24h urines from a number of individuals was carried out after shell-freezing samples, with FD-glycoprotein as standard, for preparing the radiolabelled material and for raising antibody. The values are given in Table 3.

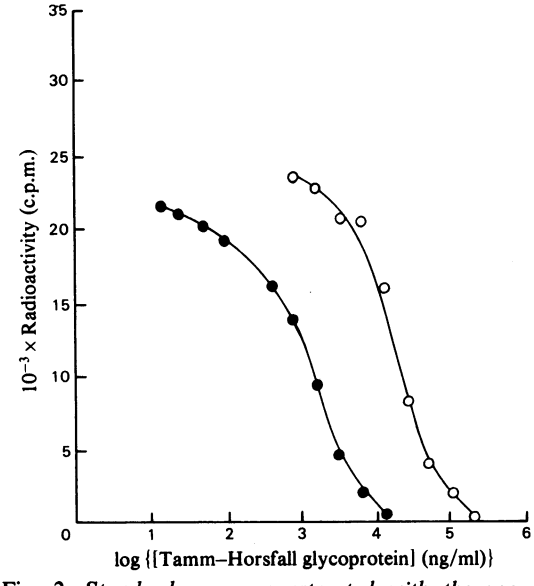

Fig. 2. *Standard curves constructed with the use of antiserum (at a dilution of 1:500) raised in rabbits against NF-Tamm–Horsfall glycoprotein*
Non-radiolabelled glycoprotein was either FD-glycoprotein (●) or NF-glycoprotein (○). The sample of radioiodinated glycoprotein was FD-material.

Discussion

There is a change in the structure or degree of aggregation of the Tamm–Horsfall glycoprotein as a result of shell-freezing at low temperatures, and this results in the FD-glycoprotein becoming a better competitor, relative to radiolabelled glycoprotein, for antibody raised against the Tamm–Horsfall glycoprotein. Thus in the standard curves derived from results in which the degree of inhibition of binding of the labelled glycoprotein is plotted against concentration of Tamm–Horsfall glycoprotein in the assays, that line for the FD-material is shifted to the left. Ten times as much NF-glycoprotein as FD-substance is needed to inhibit binding of a given amount of radioactive tracer glycoprotein. The results found were similar regardless of whether the antiserum had been raised against FD-glycoprotein or against NF-glycoprotein (Figs. 1 and 2). The curves in each case were parallel, and this suggests that the antigenic sites of the FD- and NF-glycoproteins used in establishing the standard curves

Table 2. *Radioimmunoassay of Tamm–Horsfall glycoprotein (T–H) of urines that had been dialysed against water*
Samples from 24h collections were divided into three, one of which was freeze-dried and the residue dissolved in water (FD), the second of which was shell-frozen (SF) and the remaining one was kept at 4°C (NF). Determinations were then carried out on the various solutions, with appropriate antisera and preparations of Tamm–Horsfall glycoprotein. Details are given in the text. Results are means ± S.D. ($n = 6$).

	mg/24h		
Urine ...	FD	SF	NF
Standard T–H ...	FD	FD	NF
^{125}I-labelled T–H ...	FD	FD	NF
Antibody raised against ...	FD	FD	NF
Subject			
A. A. G.	89 ± 7	75 ± 11	87
	95 ± 7*	80 ± 12*	93*
M. R. D.	88 ± 12	87 ± 13	91
	80 ± 11*	79 ± 12*	83*
G. H. T.	90 ± 9	93 ± 8	107
	83 ± 8*	86 ± 7*	98*

* Values corrected to a body surface area of 1.73 m^2.

Table 3. *Daily Tamm–Horsfall glycoprotein urinary excretion (mg/24 h) by a number of normal men and women*
The assays were carried out on shell-frozen samples of urine, and FD-glycoprotein was used as the comparative standard, for preparing the radiolabelled material and for raising antibodies. The urine for four individuals was collected on more than one day. Values are means ± S.D. ($n = 6$)

Subject	Age (years)	Height (m)	Weight (kg)	Amount of Tamm–Horsfall glycoprotein (as determined)	Amount of Tamm–Horsfall glycoprotein (corrected to 1.73 m^2 body area)
Males					
P. B.	26	1.77	71.2	37 ± 11	34 ± 10
				30 ± 10	28 ± 9
B. A.	24	1.75	67.1	32 ± 4	31 ± 4
D. J.	24	1.85	74.0	60 ± 9	52 ± 8
A. R.	40	1.72	66.7	72 ± 7	70 ± 7
R. C.	23	1.75	57.0	79 ± 7	81 ± 7
G. H. T.	42	1.77	73.0	79 ± 5	73 ± 5
				93 ± 8	86 ± 7
R. G.	40	1.83	67.0	114 ± 10	105 ± 9
M. R. D.	32	1.80	72.1	117 ± 14	107 ± 13
				119 ± 14	108 ± 13
				88 ± 12	80 ± 11
M. R.	35	1.83	73.1	126 ± 7	112 ± 6
C. T.	25	1.83	73.0	138 ± 6	122 ± 5
Females					
J. M.	20	1.59	54.0	59 ± 7	55 ± 8
L. T.	19	1.60	58.9	57 ± 8	62 ± 9
A. A. G.	25	1.62	58.9	89 ± 7	95 ± 7
				107 ± 4	114 ± 4
				43 ± 4	46 ± 4
A. G.	25	1.77	65.3	101 ± 10	97 ± 10
F. S.	39	1.60	59.3	126 ± 12	136 ± 13

were the same, but were more exposed in the former substance (Ekins, 1974).

The implications of these data for the radioimmunoassay of Tamm–Horsfall glycoprotein in dialysed samples of urine are obvious. As might have been predicted, if samples of dialysed urine that had not been freeze-dried were assayed for Tamm–Horsfall glycoprotein, with the use of FD-glycoprotein as the comparative standard, the values found were artefactually low (about 10% of the results described in Table 2, Goodall & Marshall, 1978). However, if determinations of the Tamm–Horsfall glycoprotein concentration in dialysed urine, and in dialysed freeze-dried urine, were done with standards of the glycoprotein that had been treated in the same manner in each case, the results found were the same (Table 2).

If measurements of the glycoprotein are to be made on large numbers of samples of urine, it is desirable for practical reasons to avoid the need to freeze-dry the urines. For this reason, some samples of urine were assayed for the glycoprotein after dialysis against water followed by freezing under the same conditions as preceded freeze-drying of standard Tamm–Horsfall glycoprotein. The results obtained on samples of urine treated in this way were the same as those found on freeze-dried samples of urine, when FD-glycoprotein was used as the comparative standard.

We have shown (Goodall & Marshall, 1978) that if urine itself is frozen, or if it is dialysed against Dulbecco A or Tris/maleate buffer (0.05 M in Tris, pH 7.2) before freezing, then the apparent amount of Tamm–Horsfall glycoprotein present remains unaltered. It seems essential that dialysis is effected against water, before freezing. Moreover the method of freezing is important. Slow freezing (25 ml at −20°C for 10–30 min) of solutions leads to the glycoprotein being in a state that assays at 20–40% of the true amounts present when FD-glycoprotein is used as the comparative standard. A system of quick freezing was also used earlier: this was shell-freezing of 1 ml of a solution in a 1 cm wide tube for 30–60 s in solid CO_2/methanol (Goodall & Marshall, 1978). The resultant product assayed for amounts of about 60–65% of the true amount of glycoprotein present in the solution. The present conditions of shell-freezing are more effective, so that if a 1 ml volume of the urine is shell-frozen in solid CO_2/methanol for 5 min in a 2.5 cm wide tube, the product assays virtually quantitatively. The greater surface area and/or longer time of freezing now used seems necessary to convert the glycoprotein into its most active form.

Our results show that the best procedure to adopt for radioimmunoassay of Tamm–Horsfall glycoprotein in urine is the following. A measured volume of urine, preferably from a 24h sample, should be thoroughly dialysed against water and 1ml volumes should be shell-frozen in a wide tube for 5min in a solid CO_2/methanol mixture. Dilutions of the thawed sample of urine of 10-, 20-, 40- and 80-fold should be assayed with the use of FD-glycoprotein as the comparative standard. These dilutions of urine are in a large number of cases likely to give Tamm–Horsfall concentrations in the diluted samples of between 500 and 6000ng/ml. The values selected, from those obtained, as being valid should be those derived from the relatively straight centre portion of the standard curve, and this gives a range of FD-glycoprotein concentration for the results shown in Fig. 1 from about 160 to about 4000ng/ml, although there is some shift in the curve from one experiment to another (Table 1). It is essential therefore to derive a new standard curve for each series of estimations.

It may be noted that storage of samples of urine may be safely done in the deep freeze, without prior dialysis. These at some later time should be thawed in cold water, and the urines then treated in the manner described above (Goodall & Marshall, 1978).

It was reported previously (Grant & Neuberger, 1973) that freezing of urines led to artefactually high results for the concentrations of Tamm–Horsfall glycoprotein, when the latter was assessed by radioimmunoassay (with FD-glycoprotein as standard). In our experience the values obtained after freezing are more likely to approximate to the actual amounts of glycoprotein present, depending on the method of freezing of the urine. Radioimmunoassay of sera for variants of pregnancy-specific β_1-glycoprotein is reported to give artificially low values (Shultz-Larsen *et al.*, 1979), and it may be that the problem of freezing should be considered.

The amounts of Tamm–Horsfall glycoprotein excreted daily by a group of individuals (Table 3) show that there is a considerable range of values, from 30 to 138mg in males and from 43 to 126mg in females. One individual, a female (A. A. G.), was found to excrete amounts that differed over a 2.5-fold range from one day to another. Before satisfactory conclusions can be drawn, both the amount excreted daily under a variety of physiological conditions and any circadian rhythm of excretion need examination.

The maximum present values are about twice those found previously by radioimmunoassay (Grant & Neuberger, 1973), although one might have expected to have observed greater differences. Our ranges of values are close to these reported by Mazzuchi *et al.* (1974), who used the method of radial immunodiffusion. Previous methods of quantification of the glycoprotein, in which precipitation from urine was the first step (Anderson & MacLagen, 1955; Boyce *et al.*, 1961; McKenzie *et al.*, 1964) may lead to less reliable results, in part because aggregated and non-aggregated forms of the glycoprotein have different solubilities in salt solutions (Bichler *et al.*, 1973), and the proportions in urine are unknown.

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