Localization by Immunofluorescence and by Light- and Electron-Microscopic Immunoperoxidase Techniques of Tamm–Horsfall Glycoprotein in Adult Hamster Kidney

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1. Tamm–Horsfall glycoprotein was isolated from hamster urine and antiserum against it was produced in rabbits. Immunoglobulin G was isolated from the antiserum. 2. Indirect methods of immunofluorescence staining were applied to kidney sections previously fixed by both perfusion and immersion methods. Tamm–Horsfall glycoprotein was identified associated with only the cells of the ascending limb of the loop of Henle and the distal convoluted tubule. Maculae densae were free of the glycoprotein. 3. Indirect immunoperoxidase procedures with light microscopy were applied to kidney sections. The results extended those found by immunofluorescence by showing that the glycoprotein is largely associated with the plasma membrane of the cells. Macula densa cells were shown to be free of the glycoprotein, although the luminal surface of the remaining cells in the transverse section of the nephron at that region was shown to contain it. 4. A variety of immunoelectron-microscopic techniques were applied to sections previously fixed in a number of ways. Providing periodate/lysine/paraformaldehyde was used as the fixative, the glycoprotein was often seen to be present not only on the luminal surface of the cells of the thick ascending limb of the loop of Henle and of the distal convoluted tubule, but also on the basal plasma membrane, including the infoldings. 5. 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. We suggest that Tamm–Horsfall glycoprotein operates as a barrier to decrease the passage of water molecules by trapping the latter at the membrane of the cells. Our hypothesis requires the glycoprotein on the basal plasma membrane also.

The Tamm–Horsfall glycoprotein is of considerable interest. It occurs in the urine of a number of species of placental mammals (Tamm & Horsfall, 1950, 1952; Mia & Cornelius, 1966; Wallace & Nairn, 1971; Dunstan *et al.*, 1974; Masuda *et al.*, 1977), and its main, if not its only, organ of origin is the kidney (Cornelius *et al.*, 1965). The human glycoprotein inhibits haemagglutination induced *in vitro* by myxoviruses (Tamm & Horsfall, 1950, 1952), but the hamster and rabbit glycoproteins do not (Bloomfield *et al.*, 1977), and the function of the glucoprotein is unknown.

A number of studies have been made by immunofluorescence methods of the localization of Tamm–Horsfall glycoprotein in kidney slices. The results have shown considerable disagreement, and the glycoprotein was reported to be present in the cells of the proximal convoluted tubule (Keutel, 1965;

Cornelius *et al.*, 1965), in those of the loops of Henle (Friedmann, 1966; Pollak & Arbel, 1969; Wallace & Nairn, 1971), of the thick ascending limbs of the loops of Henle (McKenzie & McQueen, 1969; Schenk *et al.*, 1971; Hoyer *et al.*, 1974) of that part of the renal tubule in the region of the macula densa (McKenzie & McQueen, 1969; Schenk *et al.*, 1971), of the distal convoluted tubule generally (Friedmann, 1966; McKenzie & McQueen, 1969; Hoyer *et al.*, 1974), of the tubules generally (Masuda *et al.*, 1975) and of the collecting ducts (Herman, 1963; Pollak & Arbel, 1969). The brush border and smooth endoplasmic reticulum of the cells of the proximal convoluted tubule were also suggested to contain the glycoprotein from the results of experiments done with ferritin-labelled antiserum (Pape & Maxfield, 1964).

The discrepancies noted made it desirable to reinvestigate the problem with the use of a number of procedures. Hamster kidneys were fixed by a variety of techniques before localizing the glycoprotein in slices by immunofluorescent methods and by immunoperoxidase techniques with the use of both

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light microscopy and electron microscopy. In all the experiments described, the indirect method for the localization of the antigen was used.

Preliminary data have been reported (Foster *et al.*, 1979; Foster & Sikri, 1979).

Experimental

Materials

Horseradish peroxidase (type VI, RZ approx. 3.0) was from Sigma (London) Chemical Co., London S.W.6, U.K., and 3,3'-diaminobenzidine was from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Hamster Tamm-Horsfall glycoprotein

This was isolated from hamster urine by a procedure involving, finally, chromatography on Sepharose 4B (Dunstan *et al.*, 1974). The preparation was freeze-dried. It showed one band on electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate (Fig. 1).

Antiserum

Antiserum to hamster Tamm-Horsfall glycoprotein was raised in New Zealand White rabbits. Injections were made intramuscularly of 1 ml of an emulsion containing equal volumes of glycoprotein

(1 mg/ml in water) and complete Freund's adjuvant (Calbiochem, Hereford, U.K.). A total of at least three injections was given at fortnightly intervals. Blood was taken from the ear vein of the rabbits between 7 and 10 days after the last injection and the serum was collected.

Isolation of immunoglobulin antibody

Rabbit anti-(hamster Tamm-Horsfall glycoprotein) immunoglobulin G. This was isolated from the samples of antiserum by the procedure of Sober & Peterson (1958). It was dialysed against water and the solution was freeze-dried. The product was dissolved in the same volume of water as the serum from which it was derived and the solution was stored in small portions at -20°C . For use in the experiments to be described, a 10-fold dilution of the aforementioned solution was made with phosphate-buffered saline (Dulbecco A buffer; Dulbecco & Vogt, 1954).

Fluorescein-labelled sheep anti-(rabbit immunoglobulin) serum. This was bought from Wellcome Laboratory, Pirbright, Surrey, U.K. The freeze-dried material (7.6 mg of protein on the basis of the biuret method; 2.95 mg of antibody protein) was dissolved in 1 ml of Dulbecco A buffer; it was diluted eight times before use.

Sheep anti-(rabbit globulin) immunoglobulin G. This was isolated (Sober & Peterson, 1958) from antiserum purchased from Gibco-Bio-Cult, Paisley PA3 4EP, Scotland, U.K. For some experiments the combined fractions from the DEAE-cellulose column containing immunoglobulin G were dialysed against either Dulbecco A buffer or Tris/saline buffer, pH 7.6 (0.046 M-Tris/0.139 M-NaCl), and diluted 2-fold in the respective buffers. For other experiments the immunoglobulin G obtained from the column was dialysed against water and the solution was freeze-dried.

Preparation of conjugates of horseradish peroxidase with sheep anti-(rabbit globulin) immunoglobulin G. Method 1. Peroxidase (10 mg) was coupled to 5 mg of immunoglobulin G by the procedure of Avrameas & Ternynck (1971) in which glutaraldehyde was used for linking the enzyme to the antibody.

Method 2. Peroxidase (5 mg) was converted into poly-(*p*-N-dinitrophenyl)peroxidase before linking of the latter to immunoglobulin G (5 mg) by the method of Nakane & Kawaoi (1974).

Soluble complex of horseradish peroxidase and rabbit anti-(horseradish peroxidase) immunoglobulin (Sternberger *et al.*, 1970) was. This kindly provided by Dr. L. A. Sternberger, Chemical Systems Laboratory, U.S. Army Armament Research and Development Command, Aberdeen Proving Ground, MD, U.S.A.

The solution as supplied contained the equivalent of 0.93 mg of horseradish peroxidase and 2.91 mg of antiperoxidase per ml. For use in the experiments it

Fig. 1. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Marshall & Zamecnik, 1969) of the Tamm-Horsfall glycoprotein

was diluted 40-fold with Tris/HCl buffer, pH 7.6, containing NaCl (0.046 M-Tris/0.139 M-NaCl), which also contained 1% normal sheep serum. This solution is referred to as the PAP reagent below.

Fixation of kidneys

Immersion fixation. After intramuscular injection of hamsters with Nembutal (0.2 ml), the kidneys were removed and cut into small pieces, which were immersed for 16 h at 22°C in aqueous formaldehyde solution containing Ca^{2+} , described below as formol/ Ca^{2+} [1 vol. of commercial formaldehyde solution (40%, w/v) neutralized with phosphate buffer and 1 vol. of 10% CaCl_2 + 8 vol. of water].

The kidneys were washed in running water for 2–4 h and dehydrated [1 h successive immersion in aq. 50%, 70% and 90% (v/v) ethanol followed by absolute ethanol (several changes)]. They were cleared by immersion in chloroform overnight and finally embedded in paraffin wax.

Perfusion fixation. This was carried out with apparatus modified from that of Rossi (1975). Animals were anaesthetized by intramuscular injection of 0.2 ml of Nembutal, and fixed by vascular perfusion for 10 min at 140 mmHg pressure through the abdominal aorta (Maunsbach, 1966). A variety of fixatives was used as follows: (a) solutions of paraformaldehyde (1% or 2%, w/v) containing glutaraldehyde (0.05–0.5%, v/v) in 0.2 M-sodium cacodylate buffer, pH 7.4; (b) the sodium periodate/lysine/paraformaldehyde fixative of McLean & Nakane (1974), in which the paraformaldehyde concentration varied from 1 to 2% (v/v); and (c) solutions of formol/ Ca^{2+} (see above).

Immediately after perfusion, kidneys were removed from the animals and small specimens, which were excised from different levels of cortex and medulla, were placed in the respective fixative for 2–4 h. Samples from kidneys fixed as in (a) were washed with 0.2 M-sodium cacodylate buffer, and those fixed as in (b) with 0.05 M-sodium phosphate buffer, pH 7.2, containing 15% (w/v) sucrose. They were either dehydrated at this stage and embedded in paraffin wax for light microscopy or were used for immunoelectron microscopy. Samples from kidneys fixed as in (c) were dehydrated for light microscopy.

Light-microscopy technique by immunofluorescence

Sections (5 μm thick) were cut from the paraffin-embedded material, and the wax was dissolved with xylene, followed by washing with ethanol, aqueous ethanol solutions and Dulbecco A buffer. The sections were immersed in small volumes of the 10-fold diluted rabbit anti-(hamster Tamm-Horsfall glycoprotein) immunoglobulin G (see above) for 10 min at 22°C, and then washed (6 $\times$ 2 min each) with Dulbecco A buffer. The slices were then immersed in fluorescein-labelled sheep anti-(rabbit immunoglo-

bulin) antibodies (8-fold dilution; see above) for 10 min at the same temperature and exhaustively washed (6 $\times$ 2 min) with Dulbecco A buffer. The slices were mounted in the same buffer under sealed cover slips.

The preparations were examined with a Leitz microscope fitted with a Leitz dark-ground condenser and a quartz/ I_2 light source provided with a Turner filter (Gillet and Siebert FITC 3). A barrier filter (Wratten B15) was incorporated into the microscope. Alternatively, a Gillet and Siebert conference microscope fitted with a Zeiss IV FL epifluorescence condenser with an HBO 50 W Hg lamp, and a Zeiss-recommended FITC specific filter set with blue excitation at 450–490 nm was used. Illumination, exposure time and photographic processing were standardized throughout. Photographs were taken on Ilford HP4 and HP6 (ASA 400) films.

Light-microscopic technique with the use of peroxidase

Pieces of dehydrated kidney were embedded in paraffin wax and 5 μm sections were cut, mounted and treated as above to remove the wax and finally washed with Dulbecco A buffer. The sections were treated (20 min, 4°C) with 1% (v/v) H_2O_2 in methanol to inactivate endogenous peroxidase (Burns, 1975). The methanol was removed by washing (3 $\times$ 5 min at 22°C) with Dulbecco A buffer.

The sections were then treated with undiluted normal sheep serum (20 min, 22°C) to minimize non-specific staining. They were washed after this and subsequent steps with Dulbecco A buffer, unless otherwise stated, and the 10-fold diluted rabbit anti-(hamster Tamm-Horsfall glycoprotein) immunoglobulin G solution was applied (30 min, 22°C) before washing again. Sheep anti-(rabbit immunoglobulin) immunoglobulin G (the 2-fold dilution in Dulbecco A buffer) was next applied followed by washing, before application of the 40-fold diluted PAP reagent.

The sections were thoroughly washed, before staining histochemically for peroxidase activity with 3,3'-diaminobenzidine and H_2O_2 (20 min) by the procedure of Graham & Karnovsky (1966). They were washed with water thoroughly and taken through graded water/ethanol mixtures to absolute ethanol, cleared in xylene, and mounted in XAM resin.

Control sections were prepared in which the anti-(Tamm-Horsfall glycoprotein) immunoglobulin G was either omitted altogether or replaced with normal rabbit serum.

Observations were made and photographs were taken with a Leitz microscope.

Immunoelectron microscopy

Small pieces of fixed kidney were placed for 1 h in a mixture consisting of 1 vol. of dimethyl sulphoxide

(Kuhlmann & Miller, 1971) and 9 vol. of either 0.2M-sodium cacodylate buffer, pH 7.4, or 0.05M-sodium phosphate buffer, pH 7.2, depending on the buffer used in fixation (see above). They were then rapidly frozen in liquid N₂ and sections were cut at -20°C at a thickness of 10–30 µm (Avrameas & Bouteille, 1968; Kuhlmann & Miller, 1971). They were washed in Dulbecco A buffer for about 30 min, and then one of the following procedures (I, II or III) was followed.

Procedure I. This was based on that of Avrameas & Ternynck (1971). The slices were immersed for periods of 6–18 h at 4°C in rabbit anti-(Tamm-Horsfall glycoprotein) immunoglobulin G and then washed for 2 h in three changes of Dulbecco A buffer with constant stirring. They were immersed for 6–18 h at 4°C in the Dulbecco A solution of the conjugate composed of sheep anti-(rabbit globulin) immunoglobulin G with horseradish peroxidase, and then washed as before with Dulbecco A buffer before being histochemically stained for peroxidase with 3,3'-diaminobenzidine and H₂O₂ (Graham & Karnovsky, 1966). They were washed with water.

Procedure II. This was based on that of Nakane & Kawaoi (1974). The slices were treated as before with rabbit anti-(Tamm-Horsfall glycoprotein) immunoglobulin G and washed with Dulbecco A buffer before immersion in the Dulbecco A-diluted solution of sheep anti-(rabbit globulin) immunoglobulin G-horseradish peroxidase conjugate formed with the use of NaIO₄ and KBH₄. The slices were then treated as in procedure I.

Procedure III. This was based on that of Sternberger *et al.* (1970). The kidney sections were immersed for 1 h at 4°C in a 1 in 20 (v/v) dilution of normal sheep serum in Tris/HCl buffer, pH 7.6 (0.046M in Tris) containing 0.139M-NaCl, and then without washing in rabbit anti-(Tamm-Horsfall glycoprotein) immunoglobulin G solution for 18 h at 4°C. They were washed in several changes of the

Tris/NaCl buffer and then immersed for 18 h at 4°C in a solution of Tris/NaCl buffer containing 1% normal sheep serum of sheep anti-(rabbit globulin) immunoglobulin G. After washing with several changes of the Tris/NaCl buffer, the slices were immersed for 18 h at 4°C in the 40-fold diluted PAP reagent, followed by further washing with the Tris/NaCl buffer. Histochemical staining for peroxidase was carried out as before.

The various slices histochemically stained for peroxidase were washed with water and post-fixed in OsO₄ solution (Palade, 1952) for 1 h before washing again with water. They were dehydrated as above and embedded in Taab's resin (Taab Laboratories, Emmer Green, Reading, Berks., U.K.). Ultra-thin sections (0.05 µm) were cut on a Huxley Ultramicrotome (Cambridge Instrument Co.), mounted on uncoated 200-mesh copper grids, and viewed without further heavy-metal staining with a Miles MR 60C electron microscope at 60 KV.

Results

Studies by immunofluorescence

Immunofluorescent staining of precut sections showed that the immunogen was clearly localized in the ascending limb of the loop of Henle (Plates 1a–1d and 2b) and in the distal convoluted tubule (Plates 1c, 1d, 2a and 2c), with the notable exception of the macula densa (Plate 2a). The glomeruli, proximal convoluted tubules, thin descending limb of Henle's loop and collecting ducts did not fluoresce. Nuclei were not stained.

Several fixation techniques were used and the results in all cases were similar in exhibiting staining of the cells of the ascending limb of the loop of Henle and the distal convoluted tubule. But the most intensely and evenly stained reacting tubules were

EXPLANATION OF PLATE 1

Fluorescein staining for Tamm-Horsfall glycoprotein

(a) and (b) show thick ascending limbs of Henle's loops in the medulla after formol/Ca²⁺ immersion fixation (a) or formol-Ca²⁺ perfusion fixation (b). Magnification 240× in both (a) and (b). (c) and (d) show fixed section of kidney cortex with fluorescent distal convoluted tubules and ascending limbs of Henle's loops after formol/Ca²⁺ immersion fixation (c; magnification 320×) or fixation by perfusion with formol/Ca²⁺ (d; magnification 240×).

EXPLANATION OF PLATE 2

Fluorescein staining for Tamm-Horsfall glycoprotein

(a) shows staining in the cells of the distal convoluted tubule (DCT), but its absence in the macula-densa (MD) cells. Other abbreviations: G, glomerulus. The formol/Ca²⁺ immersion fixation method was used. Magnification 360×. (b) shows the thick ascending limbs of Henle's loops in the medulla. Fixation was by perfusion with paraformaldehyde (2%) containing glutaraldehyde (0.5%). Magnification 240×. (c) shows the distal convoluted tubules in the cortex. Fixation was as in (b). Magnification 200×. (d) shows a paraffin section (5 µm thick) of renal cortex treated by the light-microscopic immunoperoxidase technique. Fixation was by perfusion with formol/Ca²⁺. Cells of the distal convoluted tubules (DCT) are stained, with the exception of the macula-densa (MD) region. Other abbreviations: G, glomerulus. Magnification 240×.

obtained with materials fixed by perfusion with formol/ Ca^{2+} (Plates 1b and 1d). Immersion fixation with this fixative, although also yielding intensely and evenly stained reactive tubules, was a less valuable procedure because the quality of fixation of the kidney was, in general, not so good. Frequently the tubules collapsed, resulting in a closed lumen (Plates 1a and 1c).

The results obtained after fixation with paraformaldehyde/glutaraldehyde showed greater staining of the luminal border in the medullary ascending limb of Henle's loop than of the remainder of the cell body (Plate 2b). The overall fluorescence was somewhat patchy. On the other hand, staining of sections from the cortex suggested a decreased degree of fluorescence on the luminal border compared with elsewhere in the cell (Plate 2c). In some of the fluorescent tubules from the cortex, small streaks of fluorescent material could be seen to be arising from the basement-membrane side of the cells and passing towards the luminal side (Plate 2c), suggesting staining of the basal plasma membrane with its infoldings.

The macula-densa region of the renal tubule adjacent to the glomerulus was readily identified, because of its slightly taller, but narrower, cells, and consequently with more closely packed nuclei (Plate 2a). Confirmation was obtained by switching to phase-contrast optics. These macula-densa cells, wherever observed, were always negative for the specific staining for Tamm-Horsfall glycoprotein.

Studies with immunoperoxidase at the light-microscopic level.

The examination of 5 μm -thick paraffin sections of material fixed in formol/ Ca^{2+} or periodate/lysine/paraformaldehyde and stained with the PAP reagent

showed a strong apparently cytoplasmic reaction of Tamm-Horsfall glycoprotein in cells of both the ascending limb of the loop of Henle and of the distal convoluted tubule, again with the notable exception of its macula densa (Plate 2d).

Further results were obtained when sections of kidney that had been fixed and treated in the following way were examined. Kidney fixed by perfusion with paraformaldehyde/glutaraldehyde was treated in 30–40 μm -thick cryostat sections by procedure II. Tamm-Horsfall glycoprotein was present on the luminal surface of cells of the thick ascending limb of Henle's loop and of the distal convoluted tubule. (Plate 3). Once again, the macula densa is free of the glycoprotein.

Another feature, not so clearly evident after the use of immunofluorescence reagents, was the lessened intensity of periluminal Tamm-Horsfall glycoprotein staining in the distal convoluted tubule near the macula densa compared with the other portions.

Studies by immunoelectron microscopy

All the results showed that although Tamm-Horsfall glycoprotein was invariably associated with the cells of the ascending loop of Henle and the distal convoluted tubule, it was always absent in proximal convoluted tubules (Plate 4a), the thin loops of Henle (Plate 4b), the collecting ducts (Plate 5a) and likewise in glomeruli and maculae densae (Plate 5b).

Examination of sections that had been fixed by perfusion with paraformaldehyde/glutaraldehyde and treated by any one of the three procedures used for electron microscopy (see the Experimental section) gave results showing the presence of Tamm-Horsfall glycoprotein on the luminal border of the cells of the ascending limb of the loop of Henle (Plate 4b) and of the distal convoluted tubule (Plates 4a and 5a).

EXPLANATION OF PLATE 3

Araldite section 1 μm thick cut from a thicker section of renal cortex that had been treated with immunoperoxidase reagent

The thin section after counterstaining with Azure II was examined by light microscopy. Tamm-Horsfall glycoprotein may be seen intensely on the luminal border of distal convoluted tubule cells (DCT) far removed from the macula densa. The glycoprotein is absent from the luminal surface of the macula-densa cells (MD), but can be seen to be present on the luminal surface of the cells opposite to the macula-densa region. Other abbreviations: PCT, proximal convoluted tubule; G, glomerulus. Fixation was by perfusion with paraformaldehyde/glutaraldehyde. Magnification 550 $\times$.

EXPLANATION OF PLATE 4

Identification of Tamm-Horsfall glycoprotein by immunoelectron microscopic methods

(a) shows the presence of the glycoprotein on the luminal surface of cells of the distal convoluted tubule (DCT), and its absence from the surface of those of the proximal convoluted tubule (PCT). Fixation was by perfusion with paraformaldehyde/glutaraldehyde and staining was done by the method of Nakane & Kawaoi (1974). Magnification 12000 $\times$. (b) shows the presence of the glycoprotein on the luminal surface of the thick ascending limb of the loop of Henle (ALH) and its absence on the thin descending limb of Henle's loop (TDLH). Fixation was carried out as in (a). Magnification 10000 $\times$.

Tamm-Horsfall glycoprotein was found to occur as far along the distal convoluted tubule as its junction with the collecting duct (Plate 5a). This junction can be recognized by the change in the ultrastructure of the cells, those of the distal convoluted tubule having their mitochondria arranged roughly perpendicular to the basement membrane. On the other hand, the cells of the collecting ducts have their mitochondria randomly distributed and are also extremely vacuolated.

Material that had been fixed by perfusion with the periodate/lysine/paraformaldehyde reagent gave information additional to that already described, namely that there is Tamm-Horsfall glycoprotein not only on the luminal side of the cells of the distal convoluted tubule and of the ascending limb of the loop of Henle, but also on the basal plasma membrane, including its invaginations (Plates 6a and 6b).

Discussion

The results of the present studies performed by the sandwich technique with immunofluorescence show that the Tamm-Horsfall glycoprotein is present in hamster kidney slices only in the ascending limb of the loop of Henle and the distal convoluted tubule. There was generalized fluorescence that cannot be fully interpreted, in part because relatively thick sections were used, and the light microscope has a relatively low resolving power. The glycoprotein was absent in the proximal convoluted tubule, a finding that confirms the results of most other workers. It is possible that some of the previous data suggesting that the glycoprotein occurs within cells of the proximal convoluted tubule of human kidney were

due to autofluorescence, which in our experience is quite marked in the hamster, especially with unfixed cryostat sections.

Other workers have also reported the presence of Tamm-Horsfall glycoprotein in the cells of the ascending limb of Henle's loop and the distal convoluted tubule, but have particularly described staining of the macula-densa region of the nephron (McKenzie & McQueen, 1969; Schenk *et al.*, 1971; Wallace & Nairn, 1971; Hoyer *et al.*, 1974). Our results show that in those sections where macula-densa cells were clearly identified, they did not fluoresce and therefore lack Tamm-Horsfall glycoprotein. A previous report concerning these particular cells in human kidney, in which a claim to the contrary was made by Wallace & Nairn (1971), may require reconsideration, for an examination of the photograph shown in support of their claim does not reveal the morphological characteristics of a macula densa.

Collecting ducts were found to be negative by immunofluorescence, although this part of the nephron has been reported to contain material cross-reacting with uromucoid (Pollak & Arbel, 1969; Masuda *et al.*, 1977). The absence of the glycoprotein in the cells of the collecting duct was confirmed by the results obtained by immunoperoxidase techniques at both the light- and electron-microscopic levels. Indeed, by the last mentioned method, the junction of the distal convoluted tubule with the collecting duct acted as a dividing line between cells producing the glycoprotein and those not doing so (Plate 5a).

The observations made by immunofluorescence were extended by the data obtained by immunoperoxidase methods with the light microscope. In particular, the macula densa of the tubule was shown

EXPLANATION OF PLATE 5

A section of renal cortex showing the junction of the collecting duct and the distal convoluted tubule (a) and a section showing the absence of the Tamm-Horsfall glycoprotein on the luminal surface of the macula-densa (MD) cells and its presence on the other cells of the distal convoluted tubule (DCT) at that point (b)

In (a) Tamm-Horsfall glycoprotein is seen to be present on the luminal surface of the cells of the distal convoluted tubule (DCT) and absent on those of the collecting duct (CD). Other abbreviation: PCT, proximal convoluted tubule. Fixation and staining procedures were carried out as in Plate 4(a). Magnification 6000 $\times$. In (b) fixation was by perfusion with paraformaldehyde/glutaraldehyde and the immunoperoxidase technique of Nakane & Kawaoi (1974) was used for staining. Abbreviation: G, glomerulus. Magnification 8000 $\times$.

EXPLANATION OF PLATE 6

The presence of Tamm-Horsfall glycoprotein on both the luminal surface and basal plasma membrane, including its invaginations (BI), of cells of the distal convoluted tubule

Basal lamella (BL) is negative. Fixation was by perfusion with periodate/lysine/paraformaldehyde reagent. In (a) staining was carried out by the method of Nakane & Kawaoi (1974). Magnification 17000 $\times$. In (b) staining was carried out by the method of Sternberger *et al.* (1970). Magnification 12000 $\times$.

to be free of the glycoprotein (Plates 2*d* and 3). The photograph showing overall cytoplasmic staining of the cells of the distal convoluted tubule (Plate 2*d*) is of a 5 μm -thick paraffin section that was subsequently treated with the reagents. It should be compared with the result depicted in Plate 3, which represents a 1 μm -thick section cut from a 30–40 μm -thick cryostat section that had been treated by the immunoperoxidase procedure before embedding in Araldite. In the latter type of experiment staining of the Tamm–Horsfall glycoprotein was restricted to the luminal surface of the cells, a result that is related to the method of fixation, or of application of the labelled antibody. It is also noteworthy that the luminal surface of the remaining cells in the transverse section of the nephron cut at the level of its macula densa, like all other cells of the distal convoluted tubule, shows the presence of the glycoprotein (Plate 3).

Further important information was obtained by the studies made with immunoelectron-microscopic methods. Providing periodate/lysine/paraformaldehyde was used as the fixative, the glycoprotein was often seen to be present not only on the luminal surface of the cells of the thick ascending limb of the loop of Henle and of the distal convoluted tubule, but also on the basal plasma membrane, including the infoldings. Staining of this part of the cell was not seen when other fixatives were used. It seems likely from these results that the plasma membrane of the cells in question is generally associated with the Tamm–Horsfall glycoprotein, rather than just the cell surface exposed to the tubular urine.

Three important features emerge from these studies. Tamm–Horsfall glycoprotein was localized only in the cells of the thick ascending limb of the loop of Henle and of the distal convoluted tubule as far as its junction with the collecting duct. The glycoprotein is associated with the plasma membrane of the cells in question, both on the luminal surface and on the basal side including its infoldings. The glycoprotein is absent in cells of the maculae densae.

From the present observations and those of others concerning the high viscosity of Tamm–Horsfall glycoprotein solutions (Curtain, 1953; Stevenson, 1968; Stevenson *et al.*, 1971), it is tempting to postulate the following role for Tamm–Horsfall glycoprotein in the normal mammalian kidney. The ability of the substance to produce highly viscous solutions suggests that the aggregated molecules of the Tamm–Horsfall glycoprotein can entrap water molecules in a relatively fixed structure. If it is assumed that a similar entrapment of water molecules may also occur in the glycoprotein whilst the latter is still associated with the cell surface, one can envisage a situation in which a barrier of relatively stationary water molecules, occurring within an ordered structure, is present on the surface of the

cells of the thick ascending limb of the loop of Henle and of the distal convoluted tubule. This might be expected to lead to a relatively impermeable barrier to the water molecules, but not to the dissolved small solute molecules within the urine in the tubules. A selective passage of molecules across the basal plasma membrane might also be expected to result from the presence of the substance associated with that surface and so prevent loss of water from the particular tubular cells. 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 we suggest that Tamm–Horsfall glycoprotein may produce the effect. The presence of the glycoprotein associated with the surface of the cells of the distal convoluted tubule might be expected to decrease the permeability to water of this region, although in the rat at least this is believed to be regulated by vasopressin (Pitts, 1974).

The absence of the glycoprotein on macula-densa cells adds weight to the hypothesis because if these cells function as a sensor for the Cl^- or Na^+ concentration of the urine at that part of the nephron, as is generally believed (Latta, 1973), it is reasonable to expect their surface to be exposed directly to the urine in the tubules.

The possibility that the Tamm–Horsfall glycoprotein is in some way involved in electrolyte and water transport in the kidney has been suggested before (Lewis *et al.*, 1972; Schwartz *et al.*, 1973), although no specific hypothesis has been put forward.

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