Diabetologia (1995) 38: 525–535

Diabetologia

Tamm–Horsfall glycoprotein in streptozotocin diabetic rats: a study of kidney in situ hybridization, immunohistochemistry, and urinary excretion

R. Rasch[1], O. Torffvit[2], S. Bachmann[3], P. K. Jensen[4], N. O. Jacobsen[5]

[1] Department of Cell Biology, Institute of Anatomy, University of Aarhus, Denmark
[2] Department of Internal Medicine, University Hospital of Lund, Sweden
[3] Department of Anatomy and Cell Biology, Ruprecht-Karls-Üniversität, Heidelberg, Germany
[4] Department of Ophthalmology, Aarhus University Hospital, Kommunehospitalet Aarhus Denmark
[5] Institute of Pathology, Aarhus University Hospital, Kommunehospitalet Aarhus Denmark

Summary Tamm–Horsfall glycoprotein, present only in the kidney thick ascending limb of Henle's loop, was studied here in streptozotocin diabetic rats. Tamm–Horsfall glycoprotein mRNA in situ hybridization was performed on snap-frozen left kidneys; the right kidneys were perfusion-fixed with 4 % paraformaldehyde and embedded either in paraffin, for Tamm–Horsfall glycoprotein immunohistochemistry, or in Epon for stereologic measurements. The length of the thick ascending limb of Henle's loop and the amount of glycogen were measured and the ultrastructure of the cells was evaluated. Urinary excretion of Tamm–Horsfall glycoprotein, calcium, magnesium and albumin was measured. After 10 and 50 days' duration of diabetes, kidney weight increased 20 and 41 %, respectively and the length of the thick ascending limb of Henle's loop increased 28 and 56 %, respectively, compared with controls. Substantial glycogen accumulations were present in the thick ascending limb of Henle's loop, and electron microscopy revealed a significant decrease in organelles and basolateral membranes. After 10 and 50 days' duration of diabetes, in situ hybridization of Tamm–Horsfall glycoprotein mRNA revealed a fourfold decrease, and the immunostaining for Tamm–Horsfall glycoprotein showed a threefold decrease as measured by densitometry. However, urinary Tamm–Horsfall glycoprotein excretion rate was increased fivefold and urinary concentration about twofold. Urinary calcium excretion increased threefold and magnesium twofold, but urinary albumin excretion was not significantly increased. The increased amount of Tamm–Horsfall glycoprotein, calcium and magnesium in the urine in diabetes occurs here concomitant with severe cellular damage in the thick ascending limb of Henle's loop. [Diabetologia (1995) 38: 525–535]

Key words Diabetes mellitus, kidneys, thick ascending limb of the loop of Henle, Tamm–Horsfall glycoprotein.

Tamm–Horsfall protein (THP) was first discovered in human urine by Tamm and Horsfall in 1950 [1] and was later also found in rats [2] and hamsters [3]. THP is produced in the thick ascending limb of Henle's loop (TAL) [4], where a substantial part of calcium (Ca) and magnesium (Mg) is reabsorbed [5, 6]. The presence of THP can be demonstrated in the basolateral and apical membranes of the same cells, in vesicles and lysosomes [7, 8]. The relative abundance of THP in the urine [9] indicates a possible physiological role of the protein in the TAL or in the urinary system. It has been proposed that THP is active in maintaining the relative water impermeability of the TAL [10], and the association with plasma membranes could indicate a connection with chloride transport [11]. THP inhibits virus haemagglutination [1], and an antibacterial action has been suggested since certain bacterial strains adhere

Received: 27 April 1994 and in revised form: 11 November 1994

Corresponding author: Dr. R. Rasch, Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C, Denmark

Abbreviations: THP, Tamm–Horsfall protein; TAL, Thick ascending limb of Henles loop, AE, Armanni–Ebstein lesion; OSOM, outer stripe of outer medulla; ISOM, inner stripe of outer medulla.

to THP in the urine [12]. Tubular casts contain a core of THP [13, 14]. THP may play an important role in regulating circulating levels of lymphokines, and thus the kidney may be an immuno-regulatory organ, by regulating the level of lymphokines [15, 16]. A significant reduction in THP synthesis and excess Ca excretion is seen in hyperprostaglandin E-syndrome [17]. In spite of this knowledge about THP, its physiological significance is not yet fully established.

In diabetic patients, the presence of glycogen in the nephron was first demonstrated by Armanni and Ebstein in 1877 and designated the Armanni–Ebstein lesion (AE) [18]. In experimental diabetes, AE is present in the TAL [19] with a distribution within the nephron similar to that of the THP in normal kidneys. In cells with the AE lesion, cellular organelles and basolateral cell membrane infoldings decrease substantially [20]. In order to evaluate a possible significance of THP in diabetes and its interrelationship with the glycogen accumulations, the formation, localization, and excretion of THP were determined in rats after 10 and 50 days duration of diabetes. Urinary excretion of Ca and Mg was also determined.

Materials and methods

Animals. Female Wistar rats, body weight 122 ± 3 g (mean ± SD), were separated into three different groups each containing eight animals: i.e. controls; animals that were diabetic for 10 days (D10); and for 50 days (D50).

Diabetes was induced in the rats by i.p. injection of 90 mg streptozotocin (Upjohn Inc., Kalamazoo, Mich., USA) per kg body weight. Diabetes developed on the second day after streptozotocin administration, as verified by glucosuria and blood glucose concentration measurements with Glucometer II (Ames Division, Miles Laboratories, Inc, Elkhart, Ind., USA) and glucostix (Ames). In the D10 animals, diabetes was induced 40 days later than in the D50 animals so that all animals were the same age at the end of the experiment. All diabetic animals were daily treated subcutaneously with small doses of Heat Treated Ultralente bovine insulin, pH 5.5, not for human use (Novo, Copenhagen, DK) [21]. During this 50-day period, body weight and blood glucose concentration were measured once a week, and insulin was administered in doses designed to produce 20 % lower body weight in the diabetic animals.

Kidney tissue preservation. At the end of the experiment, the animals were anaesthetized with pentobarbital, 50 mg/kg body weight, i.p.

Left kidney: after opening the abdomen, the left kidney was exposed, the renal vessels were clamped, and the kidney was removed, wiped, stripped of its capsule and cut perpendicular to the long axis of the kidney. The three middle slices were snap-frozen in isopenthane cooled in liquid nitrogen and were stored in closed vials at –80 °C until mRNA in situ hybridization could be performed.

Right kidney: perfusion fixation of the right kidney was performed retrograde through the aorta with 4 % paraformaldehyde for 4 min at a pressure of 140 mm Hg [22]. Only kidneys that blanched immediately, indicating optimal perfusion, were included in the study. After fixation of the tissue, the left kidney was removed, weighed, and cut in 2-mm slices perpendicular to the long axis of the kidney [23]. Every second 2-mm slice was embedded in paraffin and processed for immunohistochemical staining for THP's protein. From the remaining kidney slices, five 2 × 2-mm randomly selected blocks were cut from the cortex, the outer stripe of the outer medulla (OSOM), and from the inner stripe of the outer medulla (ISOM), stained with osmium, and embedded in Epon (TAAB, Reading, UK).

Measurements of TAL volume fraction in kidney. On paraffin-embedded kidney slices with THP immunohistochemical staining from the control kidneys the volume fraction of TAL in the individual kidney zones was measured with a point count technique.

Quantitation of TAL glycogen accumulations, the Armanni-Ebstein lesion (AE), and the distal tubule (DT) length. Five Epon-embedded blocks were cut from cortex, OSOM and ISOM in each animal on an Ultratome III (LKB, Stockholm, Sweden). Sections 3 μm thick were stained with toluidine blue and periodic acid Schiff for light microscopy. On these sections, the volume fraction of TAL containing glycogen was measured applying a point count technique [24] at a magnification of 400 ×, and the length of the distal tubule was measured by applying a stereologic technique whereby the number of distal tubule profiles is counted within a frame [25] at a magnification of 200 ×.

Two 3-μm-thick Epon sections from each kidney zone per animal were re-embedded in Epon, and thin sections cut for electron microscopy [26]. The sections were stained with lead citrate and uranyl acetate and photographed in a Jeol 100 B electron microscope (Akishima, Tokyo, Japan), at a magnification of 13,000 ×. From these sections, the ultrastructure of the glycogen-containing cells is described.

THP staining procedure. The perfusion-fixed, paraffin-embedded tissue was sectioned in 4-μm-thick slices and immunostained with anti-Tamm–Horsfall glycoprotein antiserum (Behringwerke, Marburg, Germany) raised in rabbits against uromucoid [27]. The antiserum was diluted 1:200. The site of the antigen-antibody reaction was visualized by the peroxidase-antiperoxidase method described by Sternberger [28] and conventional staining controls were employed.

In situ hybridization. Cryostat sections 7 μm thick were obtained with a cryostat microtome and mounted on glass slides coated with poly-L-lysine (Sigma, St Louis, Mo., USA). The sections were then fixed in 4 % paraformaldehyde and stored in 70 % ethanol at 4°C until further use. In situ hybridization of the kidney tissue sections was performed according to Bachmann et al. [4].

Video-based densitometry. The mRNA autoradiography and immunoperoxidase-stained renal sections from five animals in the three groups were studied in a light microscope equipped with a × 10 objective, as described by Flyvbjerg et al. [29]. An image was produced directly onto the face plate of a video camera (MTI-CCD 72; Dage, Michigan City, Ind., USA). The threshold slice level of 90 for creation of binary images (black and white only) was chosen as the level clearly discriminating between pigment located in the TAL and pigments used as counterstain. The same level was used for discriminating between the autoradiographic grains and background. The microscopist was unaware of the treatment groups from which each sample was obtained and all preparations were digitized

during one session. Three non-overlapping images were obtained from each region: cortex; OSOM; ISOM; and inner medulla. In the inner medulla, however, it was possible to obtain only one field without overlap. For each image a histogram distribution of grey values was obtained. The three histograms obtained from each animal for each region were averaged and used in a two-way analysis of variance components between groups and regions.

Urine collection. The animals were placed in metabolic cages for 4 h (08.00–12.00 hours) during the last 3 days of the experiment. The collected urine was stored without preservatives at –20 °C in 2 ml polypropylene plastic tubes. Water intake was not measured.

Preparation of rat Tamm–Horsfall protein. Tamm-Horsfall protein was prepared by precipitation with 0.58 mol/l NaCl as described previously [30]. However, the final preparation from rat urine contained a large amount of salt crystals if the following procedure was not used. The rat urine was dialysed before preparation and the final solution was dialysed four times instead of three times as for human urine. NaCl was added to the pool of urine mixed with an equal volume of distilled water to reach a concentration of 0.58 mol/l, pH 5–6. The precipitate formed after 24 h was collected by centrifugation (7000 × *g* for 30 min). The pellet was dissolved in distilled water and, after centrifugation (7000 × *g* for 30 min), the precipitation process was repeated with the solute. The final solution was dialysed four times against distilled water and freeze dried.

Preparation of human THP. The preparation of human THP followed the steps above but without need for dialysis before precipitation with NaCl, and the final solution needed to be dialysed only three times.

Electrophoresis. SDS-PAGE was performed as described by Laemmli [31] in 3–22 % linear gradient gels. These were stained with silver according to the method of Morrisey [32]. Both human and rat THP showed a similar single band corresponding to a mol. wt. of 94,000 dalton, corresponding to the monomeric molecules.

Preparation of antibodies against rat Tamm–Horsfall protein. Polyclonal antibodies were produced in the rabbits. Rat THP (120 μg) with Freund's complete adjuvant (Sigma) was injected subcutaneously in the neck at three places (100 μl) in two rabbits. Six and 12 weeks later, 120 μg THP in Freund's incomplete adjuvant was injected. To make the antibodies react with both human and rat THP, a dose of the human THP (100 μg) was given five months later in incomplete Freund's adjuvant.

Enzyme-linked immunoassay. The assay for THP followed the same steps as previously described [30]. The plates were coated with human THP. Before analyses, urine and standard were diluted in distilled water in order to de-aggregate THP. Urine was diluted ten times, and a standard serial dilution of THP, 64–4000 μg/l, was analysed on each plate. The assay showed no difference in the standard curve whether the plates were coated with human or rat THP. Consequently, as human THP is much easier to collect and prepare, we used this antigen for the rat assay. Before analysis, urine and standard were diluted in distilled water. Urine dilution of 1:10 was used. The polyclonal antibody was diluted 1:1000 (v/v) in ELISA buffer and 100 μl was added to the 100 μl of test/standard sample. Thereafter, 50 μl of swine-anti-rabbit alkaline phosphatase conjugate (Dako-patts, Copenhagen, Denmark), diluted 1:250 (v/v) was added. The mixture was incubated at room temperature for 3 h. After washing, enzyme activity was determined as previously described [30]. Samples were analysed in duplicate and the coefficient of variation was calculated from 24 single measurements of the same urine on each plate. The intra-assay variation was calculated on 3 days on 3 × 24 = 72 cumulative measurements.

Standard curve for THP. The standard curves of serial dilutions (64–4000 μg/l) for human and rat antigen were parallel and inhibited the antibody to similar degree. The human THP was easier to prepare and consequently this was used for the standard. The dilution curve of rat urine was similar to the THP standard curve, thus confirming that the antigen measured was actually THP.

Sensitivity of the assay. The detection limit was 64 ng/ml or 6.4 ng. The intraassay coefficient of variation was 17 % and the interassay variation was 27 % at urine concentration of 3.8 mg/l (diluted 1:10), which fell at the 50 % intercept of the standard curve. The intraindividual coefficient of variation was 31.8 % in diabetic rats and 44.6 % in control rats.

Human and rat THP was added in amounts of 1, 2, 5, 7 and 9 μg to urine samples with concentrations of 4.1 and 3.1 mg/ml. The mean recovery of the amount added was 117 % (range 93–147 %) and 100 % (75–121 %).

Rat albumin assay. The assay was performed as previously described for human albumin [33]. Rat albumin (Sigma) and clonal rabbit anti-rat (Nordic Immunolog Laboratories, Tillberg, The Netherlands) were used. The latter was diluted 1:2,000 (v/v). The detection limit was 16 μg/l or 1.6 ng. The intraassay and the interassay coefficients of variation were 11.8 and 12.8 %. The dilution curve of urine was parallel to the standard curve. The mean recovery of added albumin (48, 91, 166, 286 and 444 μg) to urine (31 mg/l) was 102 % (range 93–114 %).

Ca and Mg measurements. Urine Ca and Mg were analysed by a colorimetric technique (EKTA chem-analyzer 700 XR-C; instrument KODAK, Rochester, NY, USA).

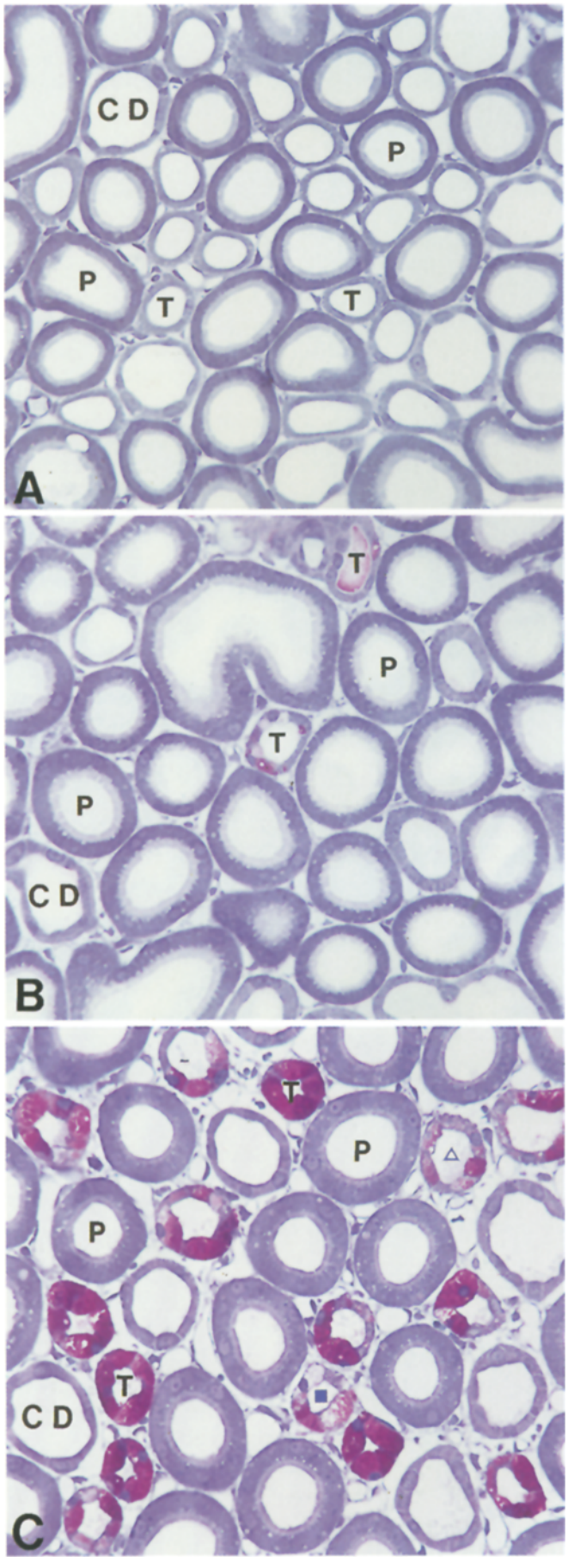
Statistical analysis

The values in the different groups of rats were first compared with the Kruskall Wallis' test and, if a significant difference was found, the values were compared with the Mann–Whitney's test. When comparing data on different kidney zones in the rats we used Friedman's two-way analysis of variance and, if significant differences were found, Wilcoxons' signed rank test. The frequency distribution of urinary Tamm–Horsfall protein concentration and excretion rate and the albumin excretion rate is known to become Gaussian on $\log^{10}$ transformation [30] and the values are therefore given as geometric mean x/ tolerance factor. All tests were two-tailed and the level of significance $p < 0.05$.

Results

Animals. Control animals and D50 animals weighed 122 ± 3 g (mean ± SD) at the start of the experiment. When diabetes was induced in the D10 animals, 40 days later, these animals weighed 204 ± 12 g.

At the end of the experiment the body weight in the controls was 241 ± 12 g. The body weight of the D10 was 203 ± 9 g and in the D50 animals 193 ± 7 g. The blood glucose concentration during the experi-



ment was 5.8 ± .6 mmol/l in the control animals and at the termination of the experiment it was 17 ± 1.7 mmol/l in D10 and 19 ± 1.4 mmol/l in D50 animals. Blood glucose concentration after 4 h fasting in the metabolic cages was significantly lowered, from 19 ± 1.7 to 15 ± .5 mmol/l, but the animals were still glycosuric.

Kidney weight. Kidney weight increased significantly from 1004 ± 67 mg in the controls to 1207 ± 110 mg in D10, $p < 0.0004$ and 1416 ± 102 mg in the D50 animals, $p < 0.0004$.

TAL volume fraction in kidney zones. In normal animals the volume fraction of TAL was 4 ± 0.2 % in the cortex, 12 ± 0.6 % in the OSOM, and 24 ± 2.3 % in the ISOM.

Glycogen accumulations. Controls contained no glycogen in TAL, Figure 1A. In the D10 animals, the TAL cells contained focal accumulations of glycogen, but the basic structure of the cells was preserved. In D10, the fraction of glycogen in the TAL profiles was 10.7 ± 0.3 % in the cortex and 3.5 ± 0 % in the OSOM, Fig. 1B. In the D50 rats (Fig. 1C), the 73 ± 4.2 % glycogen dominated the cells of the cortical TAL to such an extent that very few organelles were seen compared with controls (Fig. 2). The few mitochondria were located along the plasma membrane and the cell had lost its basolateral interdigitations. Glycogen accumulations were also present in the OSOM, 7.8 ± 0.2 %. No glycogen was present in the ISOM.

Tubule length. The total length of the distal tubule per kidney increased from 188 ± 17 meters (m) in the controls to 240 ± 29 m and 294 ± 37 m in the D10 and D50, $p < 0.0009$ in D10 and D50 compared with controls.

Fig. 1. **A** Light micrograph of cortex from a normal animal. Proximal tubules (P), thick ascending limbs of Henle's loop (T) and collecting ducts (CD) are seen. No glycogen accumulations are present. Staining PAS and toluidine blue. Magnification × 400. **B** Light micrograph of cortex from a 10-day diabetic animal. Proximal tubules (P), thick ascending limbs of Henle's loop (T) and collecting ducts (CD) are seen. In the T, glycogen accumulations appear as red. Magnification × 400. **C** Light micrograph of cortex from a 50-day diabetic animal. Proximal tubules (P), thick ascending limbs of Henle's loop (T) and collecting ducts (CD) are seen. In the T, glycogen accumulations appear as red occupying the whole TAL profile. TAL cells marked with △ have a varying amount of glycogen, and in TAL cells marked with ■ the glycogen has been washed out during preparation and the cells appear empty. Glycogen cannot be seen at the light microscope level in normal animals with the method applied. Glycogen accumulations, however, appear red and they are very pronounced in the cortical region after 50 days duration of diabetes. Magnification × 400

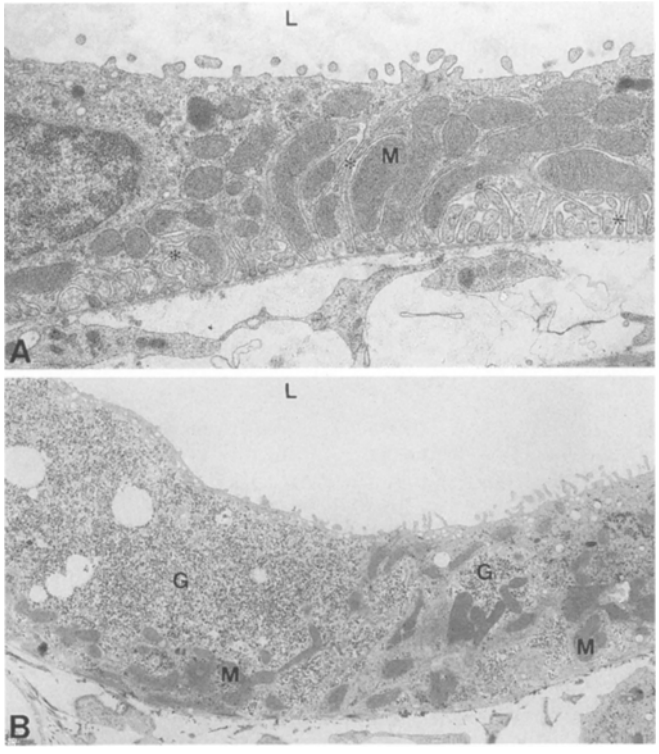


Fig. 2. **A** Electron micrograph from a normal TAL. Glycogen is seen as individual particles indistinguishable from ribosomes, which also are seen as individual particles or as attached to the rough endoplasmatic reticulum. L, lumen; M, mitochondria; * basolateral membrane, magnification × 13,000. **B** Electron micrograph of TAL from an animal with 50 days duration of diabetes. Two cells are shown. In the cell to the left, glycogen particles are seen in large amounts. The mitochondria are located in the periphery of the cell and no basolateral infoldings of the plasma membrane can be seen. In the cell to the right, glycogen accumulations, G, can be seen between mitochondria. L, lumen; M, mitochondria; nearly all the particles seen on the micrograph are glycogen. Magnification × 13,000

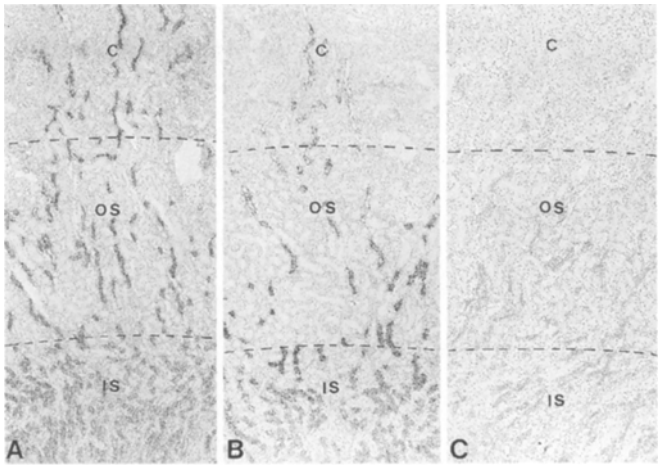


Fig. 3 A–C. In situ hybridization of THP mRNA at low magnification in cortex (C), outer stripe of outer medulla (OS) and inner stripe of outer medulla (IS) shows the autoradiographic grains over the cells of the thick ascending limb of Henle's loop (TAL) in the medullary rays in the cortex. More profiles with grains are seen in the OS and most in the IS, in accordance with the fractions of TAL in the kidney zones in normal animals. (**A**) In the 10-day diabetic animal (D10), (**B**) and the 50-day diabetic animals (D50), (**C**) a decrease in autoradiographic grains is obvious over the TAL cells in the C, OSOM and ISOM. Magnification × 50

Densitometry of THP mRNA and THP tissue staining. No change is seen in the ISOM. In Fig. 3 the distribution of the mRNA at low magnification can be seen. At this magnification very few silver grains are seen after 50 days' duration of diabetes. Figures 4 shows a larger magnification of the OSOM, here, too, the decrease in number of autoradiographic grains is very obvious. Figure 5 shows the decrease in the densitometric recordings in the cortex, OSOM, and ISOM. After only 10 days, the decrement is already significant in the cortex and OSOM.

As shown in Figs. 6A and 7A, THP immunostaining is an excellent marker of the entire outline of the TAL. A significant fall in staining intensity in the cortex after both 10 and 50 days of diabetes in all zones can be seen in Figs. 6B, C and 7B, C. The results of the measurements are shown in Fig. 8.

In normal kidneys, the densitometric measurements showed that the autoradiography for mRNA and immunostaining for THP was least pronounced in the cortex, as a consequence of the small percentage of TAL present here (4%). THP is most pronounced in the OSOM and less pronounced in the ISOM. The reason for lower intensity in the ISOM, where most TAL tissue is actually present (24%) is that the intensity of the staining decreases from OSOM to ISOM. However, when calculated as total amount of TAL tissue, the distribution between cortex-OSOM and ISOM is 1: 1.2: 1.8, since the volume fraction of cortex in the kidney is 50%, of OSOM 30%, and of ISOM only 15% [20], indicating that the overall decrease in cortical THP is considerable.

Urine excretion. The urine volume at the termination of the experiment was 13 ± 6.6 ml/24 h in the controls. It was 20.8 ± 13.1 in the D10 animals and 34.2 ± 34 in the D50 animals (Table 1).

Urinary THP excretion rate and concentration. The THP excretion rate was increased threefold after 10 days and fivefold after 50 days duration of diabetes (Table 1). The THP concentration was also increased in the diabetic rats after 10 days duration of diabetes but not after 50 days duration of diabetes (Table 1). The THP excretion rate ($r = 0.58$; $p < 0.001$), but not the THP concentration, was correlated to diuresis.

Urinary albumin excretion. In control rats, the albumin excretion rate was 167 μg/day (61–648) (median, range). The excretion rate was not significantly increased after 10 days of diabetes, 107 μg/day (39–145) or 50 days of diabetes, 213 μg/day (23–2690).

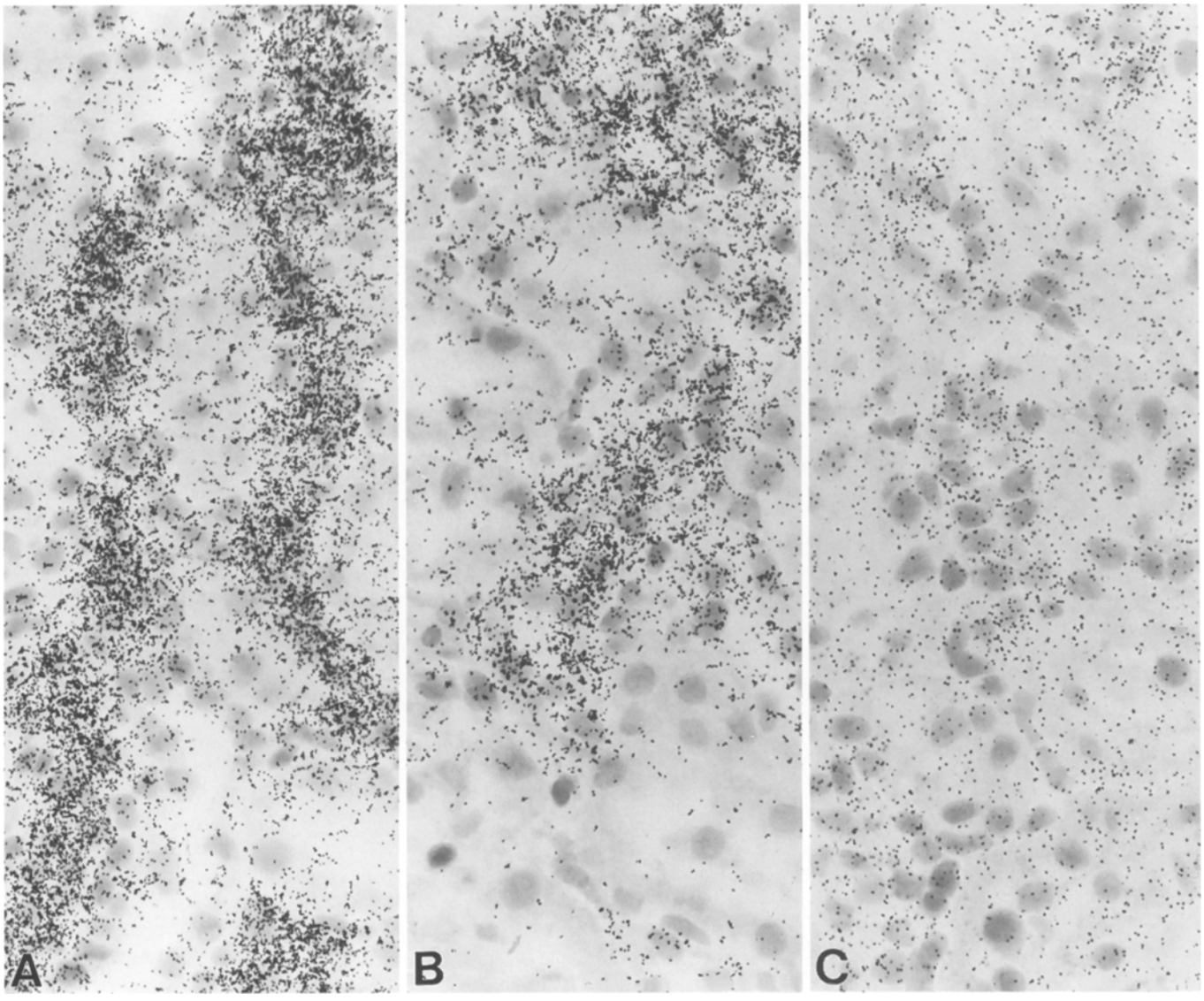


Fig. 4 A–C. In situ hybridization of THP mRNA at higher magnification from the outer stripe of outer medulla. The amount of autoradiographic grains over the cells of the thick ascending limb of Henle's loop (TAL) is pronounced in the control, (**A**). In the 10-day diabetic animals (**B**) fewer grains are seen, and in the 50-day diabetic animals (**C**), a pronounced reduction of the amount of grains in the TAL is seen. Magnification × 600

Urinary Ca and Mg excretion. The Ca excretion increased significantly after both 10 and 50 days duration of diabetes (Table 1). The Mg excretion showed the same pattern (Table 1). The THP excretion correlated to the Ca excretion ($r = 0.71$, $p < 0.001$) and to the Mg excretion ($r = 0.71$, $p < 0.001$).

Discussion

A few months of experimental diabetes led to glycogen accumulations in TAL, decrease in tissue THP mRNA and tissue THP immunostaining; at the same time there was an increase in THP, Ca, and Mg excretion in the urine. Although classic symptoms of nephropathy with increased urinary albumin excretion rate were not yet present at this stage, the kidney structure was abnormal.

The structural abnormalities in experimental diabetes start with kidney growth within days [34–36] and the growth has been studied in detail after a few months of diabetes [37]. It is systematic, i.e. the volume fraction in the individual tissue components in the diabetic kidneys is about the same compared with control animals, with the exception that glomeruli are relatively smaller [37]. In diabetic animals, the distal tubule becomes longer [20], its diameter is increased, and the cells are higher. The growth of the cells in the diabetic distal tubule is characterized by a 16% increase in cell size and a 36% increase in cell number [35], this in fact also occurs in the proximal tubule. If diabetes is treated meticulously from the onset of the disease, it is possible to prevent renal structural deterioration [38, 39]. After months' dura-

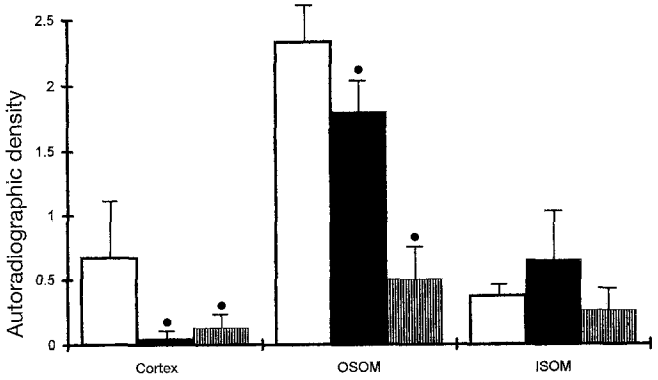


Fig. 5 Total mRNA, as assessed by densitometric measurements of the autoradiographic grains over the tissue, in cortex, outer stripe of outer medulla (OSOM) and inner stripe of outer medulla (ISOM). The x-axis shows the kidney zones in the three groups, and the y-axis the autoradiographic density as grey level. A decrease in the amount of autoradiographic grains can be seen in the cortex and the OSOM in diabetic animals. Control animals □, in 10-day diabetic animals ■ and in 50-day diabetic animals ▨. • $p < 0.04$ compared with controls

tion of diabetes and hyperglycaemia it is possible to nearly normalize kidney weight in diabetic animals by normalization of blood glucose concentration, and the AE shows total regression after only 15 days intensive insulin treatment [37, 40].

In *normal* rats, TAL is present in the ISOM, OSOM, and cortex. Ultrastructurally, the normal TAL shows an axial heterogeneity [41–43]. The TAL cells in the OSOM and ISOM are higher, have more basal cellular invaginations, more mitochondria, and NA^+,K^+-ATPase staining of the membranes compared with the cells in the cortex, and throughout the normal TAL glycogen is not seen as accumulations. The TAL ends abruptly, either at the macula densa or shortly after [43]. In the TAL, the active transport of Na^+ out of the cells without concomitant water transport is the foundation of the countercurrent multiplier system which forms the concentration gradient from cortex to medulla in the kidney.

In normal rats, THP is formed continuously in the TAL [13, 44]. Of all the cells in the body, only the TAL cells in the kidney form THP. Turnover studies indicate a half life of 9 h in rabbits [45] and 3–168 h [9] in humans. It is produced in the rough endoplasmic reticulum, passes through the Golgi apparatus and vacuoles, and is then inserted into the cell membranes, into both the apical and the basolateral membranes [8, 10]. THP is attached to the outside of the cell membrane, where it binds covalently to glycosylphosphatidylinositol [46–48]. The lipid-linked THP is released into the urine after loss of its hydropic anchor, probably by action of a phospholipase or a protease [48]. THP is then released into the urine where it constitutes about half of the total amount of proteins present in normal urine. In the TAL, THP probably acts as a water barrier that allows ion movement across the TAL [49]. However, neither the reason for the formation, its presence in and on the TAL cells, nor the function of the THP in the urine is fully understood.

The production of the THP is independent of urinary volume and transport rate of solutes in the TAL [2] in normal rats but the excretion of urinary THP increases after increase in protein intake [2].

In the TAL, 25 % of the kidney Ca reabsorption takes place. Ca is reabsorbed by a passive, paracellular, voltage, lumen positive, driven mode and by a transcellular pathway in the cortex [5]. The major part of Mg reabsorption takes place in the TAL [6], and although the reabsorption mode is different for the two ions, they have in common that the reabsorption in the TAL is in part transcellular and influenced by hormones.

In the *diabetic* kidney, only the TAL cells exhibit massive glycogen accumulations. No other cells in the organism accumulate that much glycogen, except liver cells; but the latter are not in a degenerative state and they accumulate α particles, whereas the glycogen in the TAL accumulates in β particles [50]. Normal kidneys, like the liver, participate in blood glucose concentration regulation, albeit much less, and this function is probably located in the collecting duct [51].

In D10, small accumulations of glycogen are present in cortex and OSOM TAL (Fig. 6). In D50, many severely affected cells are loaded with glycogen in cortex, fewer in OSOM, and they are not present in ISOM (Fig. 7).

Table 1. Tamm–Horsfall protein (THP) concentration and excretion rate. Excretion rate of calcium and magnesium and diuresis in control rats (C) and in rats with 10 (D10) and 50 (D50) days duration of diabetes

	Controls	D10	D50
n	12	12	12
THP concentration (μg/ml)	1.5 ×/1.5	2.9 ×/1.6[a]	2.6 ×/2.9
THP excretion rate (μg/day)	15.7 ×/1.7	45.4 ×/1.6[b]	78.7 ×/2.9[b]
Calcium (μmol/day)	85.4 ± 34	207.7 ± 101[b]	296.5 ± 107[c]
Magnesium (μmol/day)	199.8 ± 65	266.6 ± 95	341.6 ± 128[a]
Diuresis (ml/day)	13.0 ± 6.6	20.8 ± 13.1	34.2 ± 13.4[c]

[a] $2p < 0.01$, [b] $2p < 0.001$ compared with control rats. [c] $2p < 0.05$ compared with rats with 10 days duration of diabetes. Values are given as geometric mean ×/ tolerance or as mean ± SD

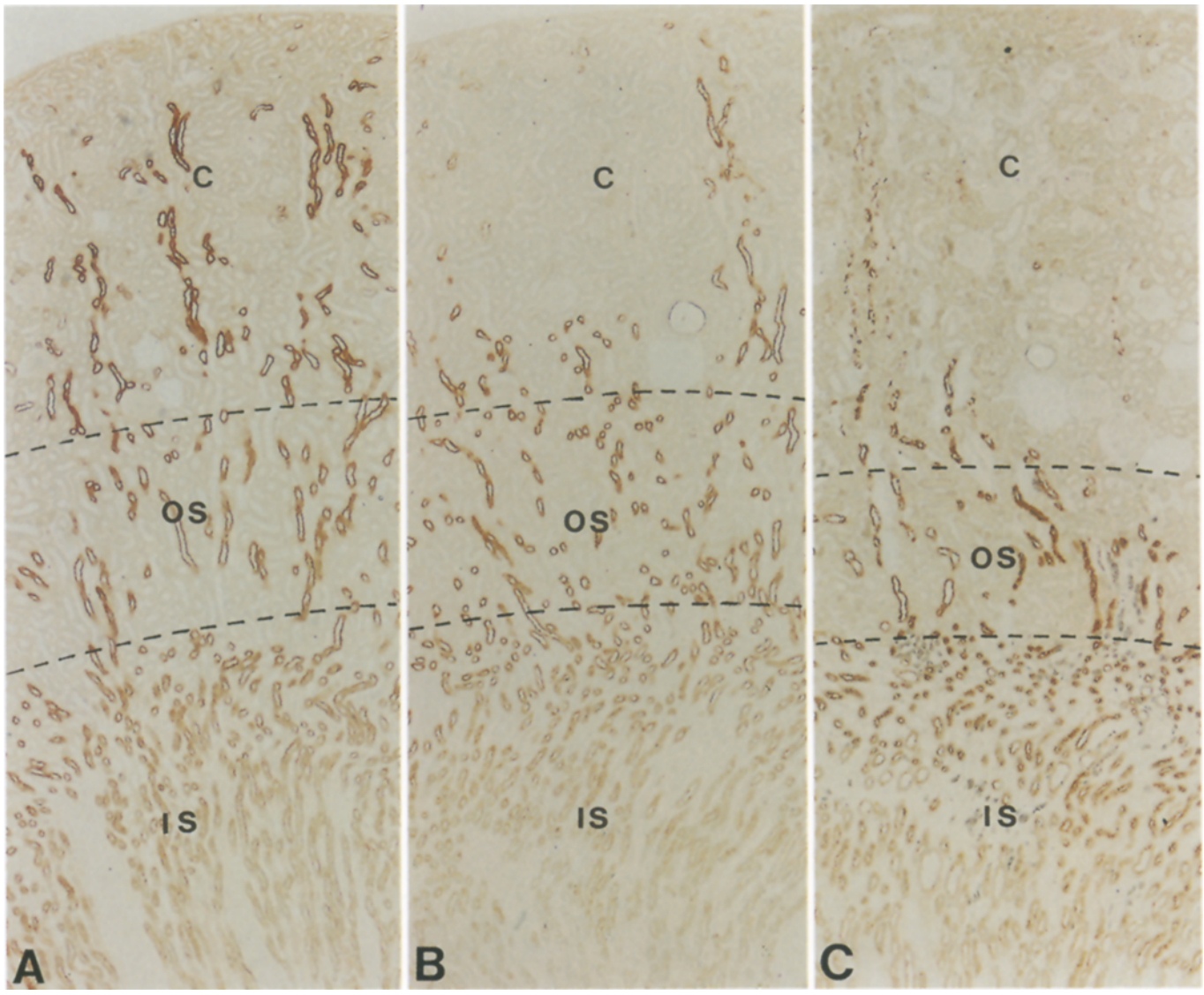


Fig. 6A–C. Immunohistochemical peroxidase staining of THP in cortex (C), outer stripe of outer medulla (OS) and inner stripe of outer medulla (IS) without counterstaining. The brown reaction product from the immunohistochemical procedure reveals the localization of THP in kidney tissue to be in the thick ascending limb of Henle's loop (TAL). In cortex, the THP is seen only in the medullary rays. In OS, more profiles are seen but most TAL profiles are present in the IS, as also documented in the measurements of the TAL fraction in the various kidney zones. In the 10-day diabetic animals (D10) the staining in the cortex, OS and IS is decreased, and in the 50-day diabetic animals (D50) a pronounced decrease in staining is seen. Magnification × 25

In normal rats, both the mRNA and the THP immunohistochemical staining are most pronounced where the glycogen accumulations appear in diabetes, and the decrease in THP mRNA and immunohistochemical staining occurs in the same cells in diabetes, and thus the heterogeneity of the normal TAL is reflected in the distribution of the abnormalities in the diabetic TAL.

Kidney weight and THP urinary excretion rate increased, and part of the increment in THP excretion can possibly be explained by the 40 % increase in kidney weight in diabetes. The decrease in densitometric measurements of mRNA and immunohistochemical staining is not due merely to a smaller volume fraction of TAL cells in diabetes, since volume fraction of distal tubules in diabetes is the same as in controls [37], and the explanation must thus be sought in the abnormalities within the cells of the TAL.

In diabetic animals, the food intake is increased about 40 % compared with controls [52]. The protein content in the fodder is 18 %, the increase in protein intake is much smaller than the increment in protein intake that has been shown to provoke an increase in THP urinary excretion [53].

The rats in the present study had normal excretion rate of albumin and would therefore not fulfill the criteria of incipient diabetic nephropathy with microalbuminuria, and thus an increase in THP excretion

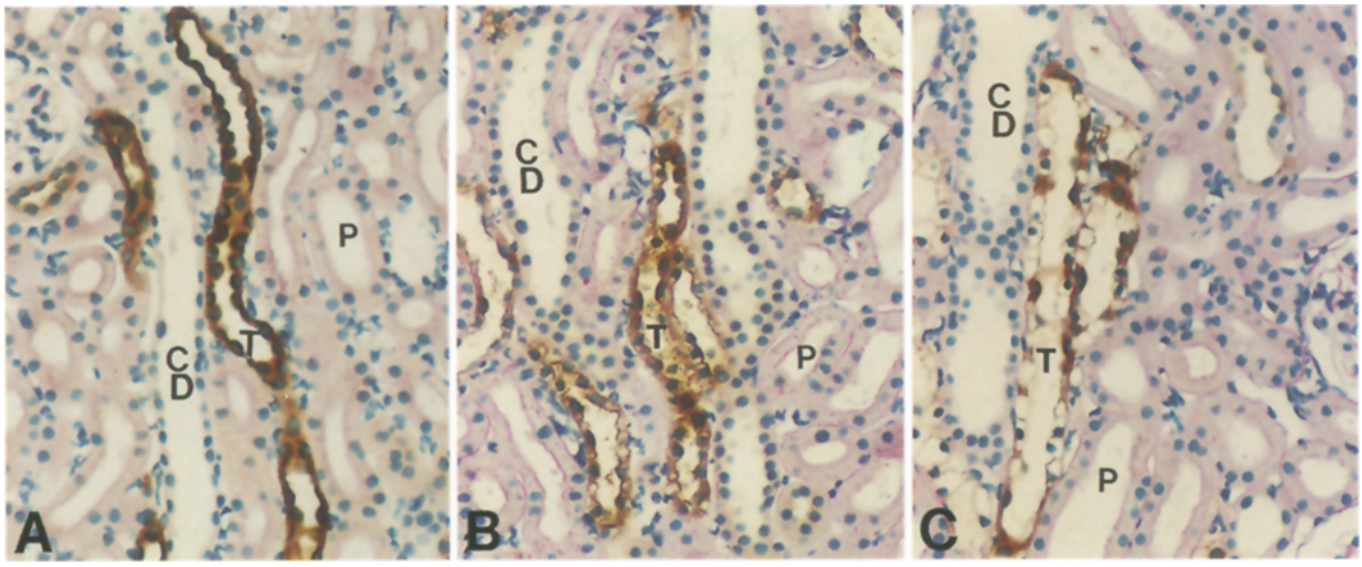


Fig. 7A–C. Immunohistochemical peroxidase staining of THP in cortex tissue sections which are also stained with PAS and haematoxylin. The brown reaction product from the immunohistochemical procedure shows THP in kidney tissue to be located in the thick ascending limb of Henle's loop (TAL). All the TAL cells are darkly stained throughout in the controls (**A**). In the 10-day diabetic animals, (**B**) the staining in the TAL cells (T) is less pronounced, and in the 50-day diabetic animals (**C**), many cells in the TAL (T) appear totally devoid of THP immunohistochemical staining whereas others contain THP staining. The empty-appearing TAL cells are the glycogen containing cells, but the glycogen washes out during the tissue embedding procedure for paraffin. P, proximal tubules; T, TAL; CD, collecting ducts. Magnification × 300

rate is an earlier indicator of structural kidney abnormalities than urinary albumin excretion rate.

The paradox that tissue THP and mRNA are decreased and yet an increase in THP urinary excretion is seen could have different explanations. Retention of THP on the TAL cells could be decreased. The THP molecule in diabetes, as it appears in the urine, has a different composition, reduced surface charge density [54], and increased glucose content [55] which could possibly contribute to a decreased binding capacity to glycosyl-phosphatidylinositol on the cell membranes [46]. Some of the increased amount of THP, however, may come from within the ISOM. No glycogen was found in the ISOM, and considering that nearly half of the total amount of TAL is located here, without accumulations of glycogen and with only a small decrease in THP mRNA and tissue immunostaining, it is possible that this region to some extent may compensate for a decreased formation of THP in the cortex and OSOM.

Many abnormalities are present in the diabetic kidney and, taking the serious damage of the TAL into consideration, this may influence the rest of the kidney.

The AE lesion causes a decrease in mitochondria and basolateral cell membranes, and diabetes may hereby affect the activity of the NA^+,K^+-ATPase which is localized in the membranes. The decrease in THP in diabetes may affect the water impermeability in this segment of the nephron [10, 49]. The severe cellular damage of the TAL cells may influence the reabsorption of substances in the TAL. The increased Ca and Mg excretion in the urine point to such a decreased ability to reabsorb these ions. Since the cellular damage is localized in the TAL which is just proximal to the macula densa and the juxtaglomerular apparatus and hereby the autoregulation of the kidney via the tubuloglomerular feedback it is possible that the abnormalities in the glomerular filtration rate [56–58] known to be present in the diabetic kidney originate from the TAL. The abnormal THP, with a possible lack of ability to stay attached to the cell membrane, may provoke an immunologic response. THP functions as a glycoprotein that specifically binds to and regulates cytokines, including interleukin-1 and tumour necrosis factor [15, 59] and it has been proposed that the THP could act as a specific ligand regulating circulating levels of interleukin (IL-1α, IL-1β) and tumour necrosis factor thus playing an important role for the circulating lymphokines and for the naturally occurring antibodies making the kidney a major immunoregulatory organ [60]. In diabetes there are accumulations of immunoglobulins in the mesangium [61], and the abnormal TAL in diabetes may possibly be the structure that initiates the immunological reactions in the glomeruli that may be of significance for the development of diabetic nephropathy.

The present study indicates that the earliest sign of a possibly reversible diabetic nephropathy may be an increased THP, Ca and Mg excretion rate which according to the present investigations also indicates the presence of enlarged kidneys with severe abnormalities in the TAL.

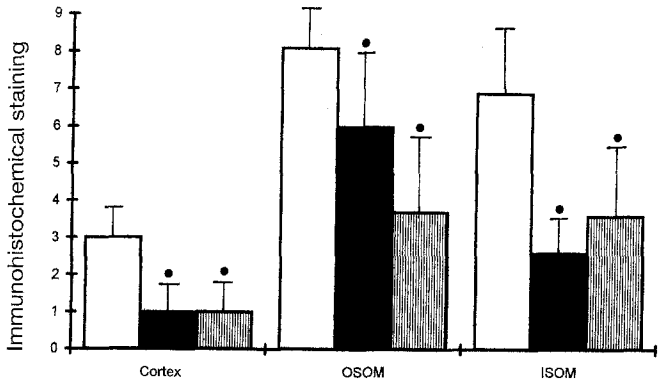


Fig. 8 Total tissue THP peroxidase immunostaining as measured by densitometry of the brown staining in cortex, outer stripe of outer medulla (OSOM) and inner stripe of outer medulla (ISOM) in control animals □, in 10-day diabetic animals ■ and in 50-day diabetic animals ▥. The x-axis shows the kidney zones in the three groups, and the y-axis the immunostaining as grey level. It can be seen that the total dye in controls is most pronounced in the OSOM, less is seen in the ISOM, least in the cortex where the fraction of TAL only is 3.5 %. In the diabetic animals, a significant fall is seen in the cortex, OSOM and ISOM, • $p < 0.04$ compared with controls. The values given are the mean values from all animals measured, the tolerance factor (X/) can be seen in the text

Acknowledgements. We thank Ms. H. Weiling and Ms. B. Grann for technical assistance. The study has been supported by the Novo Foundation, the Danish Diabetes Association, the Danish Medical Research Council and Fonden til Lægevidenskabens Fremme, the Swedish Trygg-Hansa Research Foundation, the Swedish Society of Medicine, the Royal Physiographic Society, Lund, the Medical Faculty, University of Lund, the Tore Nilsson Foundation, Sweden. Deutsche Forschungsgemeinschaft Ba 700/10/1.

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