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LABORATORY INVESTIGATION

Analysis of Na^+-D-glucose cotransporter and other renal brush border proteins in human urine

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Analysis of Na^+-D-glucose cotransporter and other renal brush border proteins in human urine. A sensitive quantitative radioimmunoassay is described by which different antigens in the urine can be assayed simultaneously. Urinary excretion of three proteins from proximal tubules was compared: 1) the Na^+-D-glucose cotransporter from brush border membranes and subapical vesicles; 2) a kidney-specific hydrophobic M_r 400,000 polypeptide from intermicrovillar invaginations and subapical vesicles; and 3) villin from microvilli cores. In the normal urine about 50% of the excreted Na^+-D-glucose cotransporter and villin, and about 25% of the M_r 400,000 polypeptide was associated with brush border membrane vesicles, whereas the remaining fractions of the three proteins formed small sedimentable aggregates which contained some cholesterol and fatty acids but no phospholipids. The normal urinary excretion of the Na^+-D-glucose cotransporter was correlated with that of villin and the M_r 400,000 polypeptide. The data show that membrane proteins from the proximal tubule are excreted by the shedding of different brush border membrane areas. They suggest that some microvilli are released in total, and that a large fraction of the brush border membrane proteins is excreted without being associated with a phospholipid bilayer. In an attempt to define protein excretion patterns during kidney malfunctions, the excretion of brush border membrane proteins was analyzed after one intravenous injection of the X-ray contrast medium, iopamidol. No change in villin excretion was observed, but a reversible increase in the excretion of brush border membrane proteins was found in patients without diabetes. With diabetes a more pronounced iopamidol effect on the excretion of brush border membrane proteins and a significant increase in the excretion of villin was observed.

The quantitative analysis of kidney derived antigens in the urine is considered to have the potential to become the most important procedure for an early differential diagnosis of kidney malfunction [1–3]. To achieve this aim: (i) a set of specific, preferably monoclonal antibodies must be available that are directed against well characterized proteins which are localized in defined segments of the nephron; and (ii) sensitive, quantitative and easily performable immunoassays must be established by which the respective antigenic proteins can be detected in the urine.

The antigenic proteins to be analyzed should not occur in the blood, should be present in high concentrations in the kidney and should be kidney-specific. The localization of the antigenic proteins has to be defined in relation to their distribution in the nephron and to their ultrastructural localization. In addition, their presence in the urine of healthy persons should be characterized. The analysis of proteins associated with the luminal membranes of the tubular cells is most interesting since they are in direct contact with the urine. To understand whether or not the appearance of such proteins indicates destruction of luminal membranes, it has to be determined whether they are integral membrane proteins or loosely associated with the membrane. Even more specific information can be obtained if the physiological functions of the analyzed proteins and/or their interaction with nephrotoxic drugs is understood.

In the past, several monoclonal antibodies have been described which react with specific parts of the human nephron [4–12] and some of these have been used to measure antigen excretion in the urine [2, 13–17]. Only a few of the employed antibodies have been sufficiently characterized using sandwich-radioimmunoassays which were established for the individual antibodies [13, 14, 18]. Firstly, in this paper a sensitive quantitative solid-phase radioimmunoassay is described by which different antigens in the urine can be analyzed simultaneously. Secondly, some monoclonal antibodies are introduced for urinary diagnosis which react specifically with integral membrane proteins from the brush border membrane of renal proximal tubules, and thirdly, information on the appearance of these membrane proteins in the urine of healthy individuals is presented. For urinary diagnosis we propose the use of recently prepared monoclonal antibodies against the Na^+-D-glucose cotransporter [19–23] and a monoclonal kidney-specific antibody which is directed specifically against intermicrovillar invaginations of proximal tubules [24]. Our data suggest that the excretion of both types of antigens can be used as a marker for the excretion of the luminal membrane of renal proximal tubules.

Methods

Preparation of brush border membranes

Pig kidneys were obtained from a local slaughterhouse and human kidney material was removed from nephrectomized hypernephroma kidneys. Brush border membrane vesicles

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were isolated from porcine and human kidney cortex as described before with the exception of Ca^{++} was replaced by Mg^{++} [25]. The vesicles were suspended in 10 mM triethanolamine HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA disodium salt, and 10% glycerol and frozen in liquid nitrogen.

Preparation of urine samples

Urine was obtained from healthy volunteers (N = 26, 13 female, 13 male, aged 18 to 39 years) without history of renal disease or exposure to renal toxins, and with normal laboratory values of creatinine clearance, urinary protein, urinary glucose and composition of urinary sediments. The urine was taken as a first-morning sample or collected over a 24 hour period. A total of 0.1% (wt/vol) of NaN_3 was added as a preservative and particulate components were removed by a 10 minute centrifugation at 2000 × g (room temperature).

The 2000 × g supernatants from ten of the first-morning urines (5 female, 5 male) were pooled, concentrated over PM 10-filters (Amicon) with a cut off value of 10 kDa and then centrifuged for one hour at 200,000 × g. The pellet from this centrifugation was removed and the supernatant was centrifuged for another 16 hours (8°C) at 350,000 × g. Protein-free ultrafiltrates of the urine were prepared by filtering the urine first through PM 10-filters and then through YM 5-filters (Amicon) with a cut off value of 5 kDa.

Measurement of antibody binding after protein separation

Polypeptides were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and transferred to nitrocellulose by Western blotting as described before [26]. Polyacrylamide gels were stained with silver [27] and Western blots with Amido black [26]. The immune reaction on Western blots was measured similarly to an earlier described procedure [26]. Thus, the nitrocellulose membrane with the blotted proteins was cut into strips and incubated for two hours (37°C) with phosphate buffered saline (PBS) containing 10% (vol/vol) normal calf serum and 0.5% (wt/vol) Tween 20 (PST buffer). Then, the nitrocellulose strips were incubated for 16 hours (4°C) with culture supernatants which had been diluted 1:2 with PBS containing 10% (vol/vol) newborn calf serum plus 1% (wt/vol) Tween 20, 2 M D-glucose, and 20% (vol/vol) glycerol and contained 10 μg of antibody per ml. The nitrocellulose strips were washed three times (5 min at 37°C) with PST buffer. Detection of bound monoclonal antibodies was performed by subsequent incubations (1 hr at 37°C each) with a) anti-mouse IgM antiserum from rabbit (diluted 1:5000 with PST buffer), b) anti-rabbit immunoglobulin antiserum from goat (diluted 1:1000 with PST buffer), and c) anti-goat IgG from rabbit which was labeled with ^{125}iodine [28] and contained 0.5 × 10^6 cpm per ml. After each antibody incubation the strips were washed three times with PST buffer (5 min at 37°C). Finally the nitrocellulose strips were dried, glued onto paper, and exposed for four hours at −70°C to a Kodak X-Omat S film (Eastman Kodak, Rochester, New York, USA).

Quantitative radioimmunoassay of urinary proteins

To attach proteins and membranes to nitrocellulose 50 μl of urine were mixed with 150 μl of PBS containing 0.0133% SDS and added to wells of a 96-well microfiltration apparatus (BioDot of BioRad, Richmond, California, USA). In control experiments 100 μl of PBS containing different amounts of human brush border membrane vesicles and detergent concentrations, or 25 μl of urinary filtrate with different pH and osmolarity plus 75 μl of PBS and different amounts of brush border membrane vesicles, were added to the wells. After the samples were added and after the fluid had disappeared by gravity flow (about 30 min), a vacuum was applied and each well was washed with 500 μl of PBS. Thereafter, the nitrocellulose sheet was removed from the filtration apparatus and incubated for two hours (37°C) in PST buffer. The reaction of the adsorbed proteins with primary monoclonal antibodies from mouse, with secondary anti-mouse-IgM or anti-mouse-IgG antiserum from rabbit, with tertiary anti-rabbit gammaglobulin antiserum from goat and with quaternary iodine-labeled anti-goat gammaglobulin antiserum from rabbit, was performed as described for the immune reaction of Western blots. After the immune reaction, the nitrocellulose was dried and the single spots were dissected and counted in a gamma counter. Nonspecific binding, which was determined for each experiment by omitting the respective primary antibody, was subtracted. The measurements were performed in triplicate; mean values and standard deviations are presented.

Measurement of protein binding to nitrocellulose

To determine the concentration range in which the amount of brush border membrane proteins adsorbed to the nitrocellulose is proportional to the amount of applied brush border membrane proteins under different experimental conditions, increasing amounts of ^{125}I-labeled human brush border membrane proteins (5000 cpm/μg of membrane protein) were applied to the nitrocellulose and subsequently treated as described for the radioimmunoassay. The ^{125}I-labeled brush border membrane proteins which were applied to the filtration apparatus were suspended in 100 μl of PBS with and without SDS or Tween 20 or Triton X-100, in 100 μl of urinary filtrate at various pH's and osmolarities, or in 100 μl of urinary filtrate containing 10 μg of nonlabeled urinary proteins. To obtain different concentrations of ^{125}I-labeled proteins, 100 μg of human brush border membrane proteins were labeled with ^{125}I [28] and diluted with increasing concentrations of nonlabeled brush border membrane proteins.

Determinations of protein concentration, antibody concentration and N-acetyl-β-D-glucosaminidase activity

Protein was determined after precipitation with 10% (vol/vol) trichloroacetic acid and dissolution in 1 N NaOH and 1% (wt/vol) sodium dodecyl sulfate (SDS) according to Lowry and coworkers [29]. Bovine serum albumin was used as standard protein. Antibody concentrations were determined by radioimmunoassay [19] and N-acetyl-β-D-glucosaminidase activity by an colorimetric assay supplied by Boehringer (Mannheim, Germany).

Lipid analysis of brush border membranes and urine fractions

Lipid analysis was performed by high-performance thin layer chromatography (HPTLC) according to a procedure recently described for alkyllysolipids [30]. Briefly, total lipids were extracted from brush border membranes or from urine pellets which were suspended in H_2O [31]. The organic phases of the

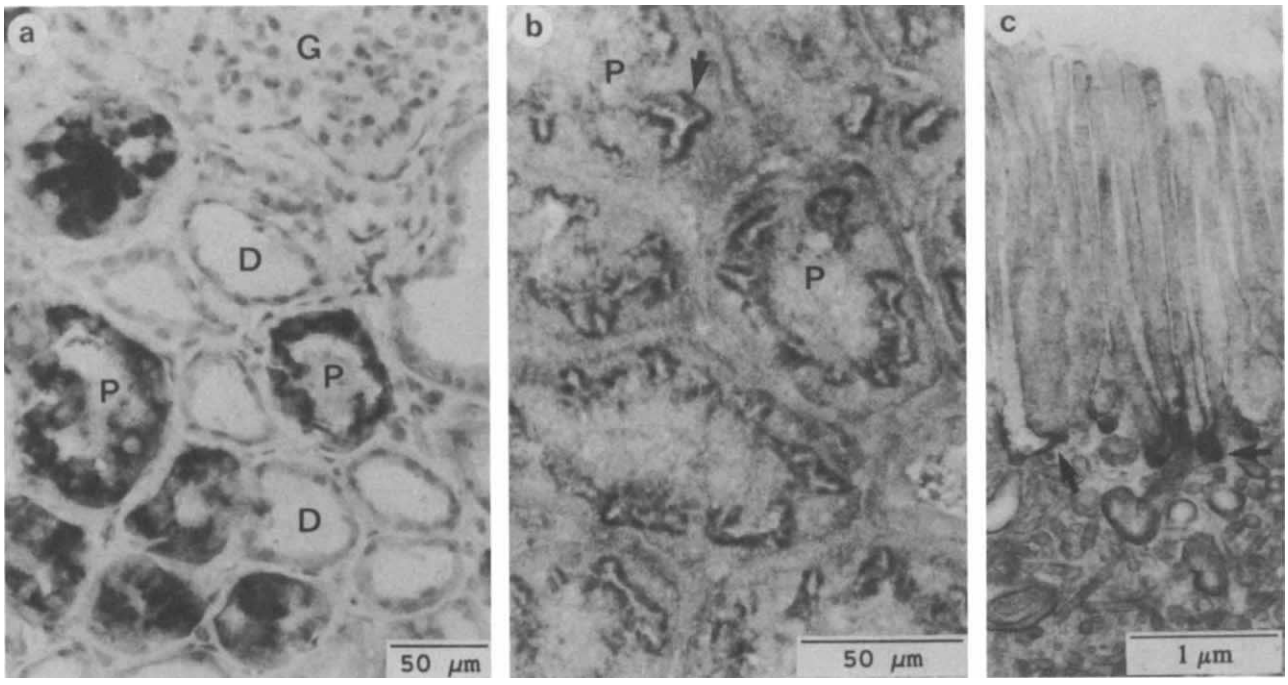

Fig. 1. *Immunostaining of proximal tubules from human* (**a**) *and porcine* (**b,c**) *renal cortex with monoclonal antibody N4A4.* The reaction with N4A4 was performed on cryosections (a,b) or with fixed tissue slices (c), and the immune reaction was visualized by secondary antibodies which were coupled to alkaline phosphatase (a) or to peroxidase (b,c). The light microscopic sections (a,b) demonstrate the specific reaction of N4A4 with proximal tubules (P) and no reaction with distal tubules (D) and glomeruli (G). The section in a shows some nonspecific tissue staining since it was counterstained (**Methods**). The electron micrograph in c obtained after pre-embedding immunostaining, demonstrates that the immune reaction is confined to the basal parts of the microvillar membranes and to endocytotic vesicles with connections to the tubular lumen (arrows in c). In controls where the monoclonal antibody was omitted or replaced by an nonspecific IgG_1-antibody the above-described immune reactions were not observed (not shown).

extracts were dried under nitrogen and the samples were resolved in 200 μl of $CHCl_3$:CH_3OH:H_2O (30:60:8, vol/vol) and streaked automatically to the HPTLC plates with a Linomat IV (CAMAG, Berlin, Germany). For chromatography of triglycerides, cholesterols and free fatty acids a mobile phase of hexane: diethylether (1:1, vol/vol) was used and the chromatography of phospholipids was performed with $CHCl_3$:CH_3OH:triethylamine:H_2O (30:35:34:8, vol/vol). To quantify the different major lipid species, different concentrations of pure synthetic dipalmitoyl phosphatic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidyl-L-serine and sphingomyelin were spotted on the HPTLC plates. After charring, the lipids were quantified using a CD 60 TLC scanner (Desaga, Heidelberg, Germany) as described recently [30].

Immunohistochemistry

To prepare sections for light microscopic immunoreactions, small pieces of renal cortex from a human (Fig. 1a) or pig (Fig. 1b) were rapidly frozen in liquid propane and 6 μm thick cryosections were cut. The sections were thaw-mounted onto glass slides, dried, fixed with acetone and blocked with bovine serum albumine as described recently [32]. Then, the sections were incubated one hour (22°C) with tenfold diluted hybridoma supernatant of the monoclonal antibody N4A4. Antibody binding was either visualized with alkaline phosphatase-coupled (Fig. 1a) or with peroxidase-coupled (Fig. 1b) antibodies.

Labeling with alkaline phosphatase and counterstaining of the sections was performed as described by Dako Diagnostik (Hamburg, Germany). Briefly, the sections were incubated with anti-mouse IgG antiserum from rabbit and with a complex of alkaline phosphatase plus mouse antibodies against alkaline phosphatase (APAAP-complex). The alkaline phosphatase activity was analyzed with naphthol as biphosphate and newafuchsin, and the sections were counterstained with hemotoxylin.

For peroxidase labeling the sections were incubated (30 min, 22°C) with peroxidase-labeled anti-mouse IgG antiserum from sheep which was diluted (1:30) with PBS containing 1% (wt/vol) BSA. After a further wash with PBS (15 min), peroxidase activity was demonstrated as described by Graham and Karnovsky [33].

For electronmicroscopic immunostaining (Fig. 1c) small pieces of pig kidney cortex were fixed by incubating for two hours (22°C) with 20 mM NaH_2PO_4, 80 mM Na_2HPO_4 plus 140 mM NaCl, pH 7.2 containing 4% (vol/vol) paraformaldehyde plus 0.1% (vol/vol) glutaraldehyde. The fixed tissue was cut into 100 μm thick slices and incubated overnight in PBS containing 0.05 M NH_4Cl plus 1% (wt/vol) bovine serum albumin (BSA). The slices were incubated for three hours (22°C) with hybridoma supernatant of the monoclonal antibody N4A4. The slices were washed, reacted with peroxidase-labeled anti-mouse IgG serum from sheep, and peroxidase activity was revealed as described above. The slices were then fixed in cacodylate buffer containing 1.5% (vol/vol) glutaraldehyde, postfixed with 1%

Table 1. Properties of monoclonal antibodies against the Na^+-D-glucose cotransporter

Antibody	M_r of antigenic polypeptides		Immunohistochemical localization of antigenic sites in rat kidney	Effects on the renal Na^+-D-glucose cotransporter
	Rat kidney	Pig kidney		
R1C4	75000 47000	75000 60000 47000	Proximal tubules: entire brush border membrane, apical vesicles, lysosomes Descending limbs of Henle's loops: apical membrane Collecting tubules: apical membrane	Phlorizin binding: inhibition D-glucose transport: no effect
R4A6	75000	75000	Proximal tubules: entire brush border membrane, apical vesicles, lysosomes Collecting ducts: apical membrane	Phlorizin binding: stimulation D-glucose transport: no effect
V6D1	75000 47000	75000 60000 47000	Not determined	Phlorizin binding: no effect D-glucose transport: stimulation

The antibodies R1C4 and R4A6 have been described earlier [19] and the monoclonal antibody V6D1 was recently obtained after immunization of mice with the M_r 75,000 polypeptide component of the Na^+-D-glucose cotransporter (unpublished data). The molecular weights of the antigenic polypeptides were determined in Western blots and the antibody effects on Na^+-D-glucose cotransport and on phlorizin binding were measured as described before [19]. The localization of the antigenic sites of antibody R1C4 and R4A6 has been described recently [20].

(wt/vol) OsO_4, contrasted with uranyl acetate and embedded in Spurr's resin [34]. Ultrathin sections were cut, placed on Formvar-coated nickel grids and analyzed by electron microscopy.

Electron microscopy of sediments

Sediments from the human urine and isolated brush border membrane vesicles were fixed for three hours (22°C) with 0.1 M cacodylate buffer containing 2.5% (vol/vol) glutaraldehyde, enclosed in agar-agar and fixed further with 1% (wt/vol) OsO_4. Then, the samples were dehydrated with increasing concentrations of ethanol, passed through propyleneoxide and embedded in Spurr's resin. Ultrathin sections were prepared and analyzed in a Philips EM 300 electron microscope.

Monoclonal antibodies

In the present study we employed six monoclonal antibodies which are directed against proteins from the brush border of renal proximal tubules (R1C4, R4A6, V6D1, 11A3D6, N4A4 and ID2C3) and one monoclonal antibody which binds to Tamm-Horsfall protein (CL1032) which is synthesized by cells of the thick ascending limb of Henle's loop [35–37].

The antibodies R1C4, R4A6, V6D1 and 11A3D6 are directed against the Na^+-D-glucose cotransporter, an integral membrane protein which is mainly localized in the brush border membrane of proximal tubules. The IgG_{2b} antibody 11A3D6 was prepared and supplied by Dr. Lever [21–23]. This antibody was shown to stimulate Na^+-dependent phlorizin binding in pig kidney brush border membranes [21] and to increase FITC labeling of a renal polypeptide with a molecular weight around 75,000 [22], which has been identified as a component of the Na^+-D-glucose cotransporter [22, 38]. The properties of the IgM-antibodies R1C4, R4A6 and V6D1, which were prepared and characterized by the authors, are summarized in Table 1. Thus, it has been shown that R1C4, R4A6 and V6D1 react with M_r 75,000, M_r 60,000 and/or M_r 47,000 polypeptides which have been identified as components of the Na^+-D-glucose cotransporter [19, 20, 32, 39]. Furthermore, in kidney and intestine V6D1 stimulates Na^+-D-glucose cotransport [19, 32, unpublished data] and R1C4 and R4A6 alter specific high-affinity phlorizin binding to the Na^+-D-glucose cotransporter [19, 32]. The antibodies which were raised against the renal porcine transporter cross react with the renal and intestinal Na^+-D-glucose cotransporter from rat [20, 32]. Immunohistochemical investigations showed that the antigenic sites of R1C4, R4A6 [20] in kidney are mainly localized in brush border membranes and in subapical vesicles of all three segments of the proximal tubules. In the thin ascending limbs of Henle's short and long loops, in the distal tubules and in the interstitial renal tissue no antigenic sites of these antibodies were detected. However, in addition to the proximal tubules, some antigenic sites were also found in the luminal membranes of short and long loops of descending limbs of Henle, and in luminal membranes of intercalated and principal cells of collecting ducts [20].

The monoclonal IgG_1 antibody N4A4 which has been raised after immunization with porcine renal brush border membranes is directed against a M_r 400,000 glycoprotein and binds exclusively to renal proximal tubules [24]. The antibody cross reacts with rat, pig and man and shows an absolute specificity for renal tissue (unpublished data). Light microscopic and electron microscopic immunohistochemistry shows that N4A4 binds to the brush border membranes but not to the basolateral membranes of renal proximal tubules (Fig. 1). The antibody does not react with glomerula (Fig. 1a), Henle's loop, distal tubules (Fig. 1a) and collecting ducts. In the proximal tubule N4A4 reacts specifically with the intervillous region of the brush border membrane and with subapical vesicles (Fig. 1b,c).

A monoclonal IgG_1 antibody against villin from chicken (ID2C3) [11] which recognizes porcine, chicken and human villin was obtained from Immunotech S.A. (Marseille, France). The antibody binds specifically to the central core of microvilli in renal proximal tubules. In Western blots of human brush border membrane proteins the antibody reacts specifically with a M_r 95,000 polypeptide (unpublished data).

A monoclonal IgG antibody against human Tamm-Horsfall

protein (CL1032) was obtained from Cedarlane Laboratories Limited (Hornby, Canada).

Materials

Nitrocellulose paper (0.45 μm pore size) was obtained from Bio Rad Laboratories (Munich, Germany), ^{125}iodine (16 Ci/mg) from Amersham Buchler (Braunschweig, Germany), newborn calf serum from Gibco (Eggenstein, Germany), anti-rabbit-immunoglobulin antiserum from goat by Behring Werke (Marburg, Germany) and anti-mouse-IgM antiserum from rabbit from ICN Biomedicals (Eschwege, Germany). Anti-mouse-IgG antiserum from rabbit, anti-goat-immunoglobulin antiserum from rabbit, alkaline phosphatase complexed with mouse antibody against alkaline phosphatase (APAAP-complex), the reagents for detection of alkaline phosphatase and for counterstaining of the tissue with hematoxilin were delivered by Dako Diagnostik (Hamburg, Germany). Mouse myeloma IgM, peroxidase-labeled anti-mouse IgG antiserum from sheep, the molecular mass markers β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), trypsinogen (24 kDa) and synthesized phospholipids were purchased from Sigma (Munich, Germany). All other chemicals were obtained as described before [26].

Results

Monoclonal antibodies against the Na^+-D-glucose cotransporter in pig and rat cross react with humans

This study tested whether the monoclonal antibodies R1C4, R4A6, V6D1 and 11A3D6, which interact with the Na^+-D-glucose cotransporter of pig and rat, cross react with the human Na^+-D-glucose cotransporter. The antibodies bind to porcine renal brush border membrane polypeptides with apparent molecular weights of 75,000, 60,000 and/or 47,000 [19, 22]. The polypeptides have been identified as components of the porcine renal Na^+-D-glucose cotransporter [22, 38, 39]. In rat, antibodies R1C4, R4A6 and V6D1 cross react with M_r 75,000 and M_r 47,000 polypeptides, and bind specifically to brush border membranes of renal proximal tubular cells and of enterocytes from small intestine [20, 32]. Furthermore, V6D1 alters Na^+-D-glucose cotransport into brush border membrane vesicles from porcine kidney (unpublished data) and from rat jejunum [32], whereas R1C4, R4A6 and 11A3D6 alter high-affinity phlorizin binding to the porcine renal Na^+-D-glucose cotransporter [19, 21, 32]. In lanes a and b of Figures 2 and 3 the protein patterns of porcine and human brush border membranes are compared after silver-staining of SDS polyacrylamide gels and after staining of Western blots with Amido black. Both experiments show that the protein patterns of brush border membranes from human and porcine kidney are similar. Comparison of lanes a and b in Figure 4 reveals that the antibodies R1C4, V6D1, R4A6 and 11A3D6 show a similar reaction with Western blots of brush border membranes from pig and human. Thus, in pig and human R1C4 and V6D1 react specifically with polypeptides with apparent molecular weights of 75,000, 60,000 and 47,000, whereas R4A6 and 11A3D6 bind mainly to a M_r 75,000 polypeptide. These data strongly suggest that the antibodies R1C4, V6D1, R4A6 and 11A3D6, which interact with the Na^+-D-glucose cotransporter from rat and pig (Table 1), cross react with the human Na^+-D-glucose cotransporter.

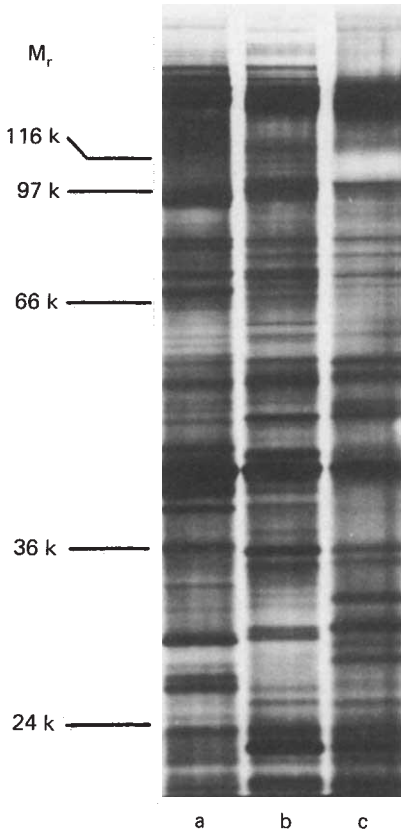

Fig. 2. *Comparison of proteins in porcine and human renal brush-border membranes and in human urine.* Renal brush border membranes from pig (**a**), man (**b**), and an urinary sediment from man obtained after 1 hour centrifugation at 200,000 × g (**c**) were separated by SDS gel electrophoresis and stained with silver as described in **Methods**. The positions of the molecular mass marker proteins at M_r 116,000 (β-galactosidase), M_r 97,000 (phosphorylase b), M_r 66,000 kDa (bovine serum albumin), M_r 36,000 kDa (glyceraldehyde-3-phosphate-dehydrogenase) and M_r 24,000 kDa (trypsinogen) are indicated.

Detection of glucose transporter components in the urine

It was tested whether the urine of healthy volunteers contains protein components which react with our monoclonal antibodies directed against components of the human Na^+-D-glucose cotransporter. After centrifugation of cell-free urine at 200,000 × g (**Methods**) a sediment was obtained which contained 3.6 ± 1.1% of the total protein in the urine. A comparison of lanes b and c in Figure 2 shows that the polypeptide composition of the 200,000 × g sediment is similar to the composition of human brush border membrane vesicles. Since this sediment contained mainly vesicles (Figs. 5a,b) and has a similar lipid composition as isolated human brush border membrane vesicles it is supposed to represent mainly membrane material from shedded microvilli of proximal tubules. Comparison of Figures 3 and 4 shows that nonspecific IgM antibodies did not react with Western blots of brush border membranes and of the urine sediment, whereas our monoclonal antibodies bound to specific

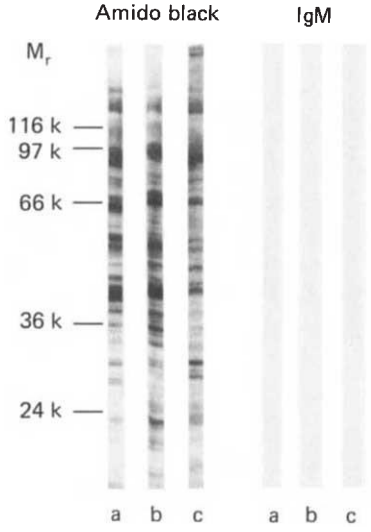

Fig. 3. *Demonstration that brush border membrane proteins and urinary proteins in Western blots did not react nonspecifically with IgM.* Brush border membranes from pig (**a**), brush border membranes from human (**b**), and an urinary sediment from human obtained after one hour of centrifugation at 200,000 × g (**c**) were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. The Western blots were stained with Amido black (indicated by "Amido black") or reacted with 50 μg/ml of IgM as primary antibody followed by secondary and tertiary antibodies and by iodine-labeled quaternary antibodies (indicated by "IgM").

polypeptides. The monoclonal antibodies against the Na^+-D-glucose cotransporter bound to polypeptides with apparent molecular weights of either 60,000 plus 47,000 (R1C4) or 47,000 (V6D1) or 75,000 (R4A6, 11A3D6). Since in purified brush border membranes and in the urine sediment all the monoclonal antibodies against the Na^+-D-glucose cotransporter exhibit the strongest reaction with polypeptides of identical molecular weights, the data suggest that the urine contains brush border membranes with intact Na^+-D-glucose cotransporters.

Quantification of the brush-border membrane proteins in the urine

To investigate the urinary excretion of the Na^+-D-glucose cotransporter and other brush border membrane proteins in more detail, a quantitative radioimmunoassay was established in which several proteins could be analyzed simultaneously. In the assay urinary proteins were adsorbed to a defined area of nitrocellulose, and the antigenic brush border membrane proteins were analyzed by the addition of: a) specific monoclonal murine antibodies; b) anti-mouse IgM or anti-mouse IgG antiserum from rabbit; c) anti-rabbit immunoglobulin antiserum from goat; and d) iodine-labeled anti-goat IgG antiserum from rabbit. To determine the range and experimental conditions in which brush border membrane proteins could be analyzed quantitatively, the adsorption of brush border membrane proteins to nitrocellulose and the antigen antibody reaction on the adsorbed protein was investigated.

Figure 6 shows that under the experimental conditions described in **Methods**, the amount of brush border membrane proteins adsorbed to the nitrocellulose increased linearly up to 5 μg of applied protein. Since the addition of detergent to urinary samples prevents protein aggregation in the urine we tried to find a detergent which may be added without altering the adsorption of brush border membranes to the nitrocellulose. Figure 6a shows that protein adsorption was not altered by 0.01% (wt/vol) SDS but was significantly reduced by 0.01% (wt/vol) Triton X-100 or 0.01% (wt/vol) Tween 20. To determine the interaction of urinary proteins with the adsorption of brush border membrane proteins, the adsorption of brush border membrane proteins was measured in urinary filtrate (pH 6.5, 0.5 Osm) with and without additional urinary proteins. The adsorption of brush border membrane proteins in the presence of 0.01% (wt/vol) SDS was not altered by 0.1 mg/ml of urinary proteins (Fig. 6b). Next it was tested whether the osmolarity or pH of the urine effects the adsorption of brush border membrane proteins to the nitrocellulose. Figure 6b shows identical adsorption of brush border membrane proteins from PBS and urinary filtrate (pH 6.5, 0.5 Osm) and Table 2 indicates that in our assay the adsorption is independent from the osmolarity (0.16 to 1.04 Osm) and the pH (pH 5.5 and 7.5) of the urine.

After it had been established that brush border membrane proteins could be adsorbed to nitrocellulose in the presence of 0.01% (wt/vol) SDS we tested whether the SDS present during adsorption alters the antibody reactions which are performed later in the absence of SDS (**Methods**). It was found that treatment of brush border membranes with small concentrations of SDS increased the antigen-antibody reactions studied in this paper. Figure 7 shows that the antibody binding to the Na^+-D-glucose cotransporter (for example, R4A6 and 11A3D6) and to villin (ID2C3) was maximally increased with 0.01% (wt/vol) SDS, whereas the binding of N4A4 was maximally increased with 0.005% (wt/vol) SDS. Since 0.01% (wt/vol) SDS did not reduce the antigen-antibody reaction of the antibodies used in this study, 0.01% (wt/vol) SDS was added routinely in the experiments.

Next we investigated whether our analysis of protein excretion is disturbed by proteolysis of the brush border membrane proteins which may be dependent on the pH of the urine. We incubated human brush border membranes for 24 hours at 37°C in urinary filtrates with different pH and analyzed the protein composition of the membranes and the integrity of the M_r 75,000 polypeptide component of the Na^+-D-glucose cotransporter. Figure 8 shows that after 24 hours of incubation at 37°C the polypeptide composition of brush border membranes was not significantly altered and the M_r 75,000 polypeptide was not degraded. Thus, proteolysis may not significantly disturb the analysis of the excretion of brush border membrane proteins.

The range in which antibody binding increased linearly with the amount of brush border membrane protein added to the test was determined for the antibodies used in this study. It was found that antibody binding increased linearly up to an amount of brush border membrane protein of 1 μg. In Figure 9 this is shown for monoclonal antibodies against the Na^+-D-glucose cotransporter. Note that the absolute amount of antibody binding per brush border membrane protein varied with different antibodies. Thus, for quantitative determinations in the urine the samples had to be diluted until the antibody binding was lower than the binding of the respective antibody to 1 μg of brush border membrane protein. So as not to exceed the binding capacity of the nitrocellulose a maximum of 10 μg of

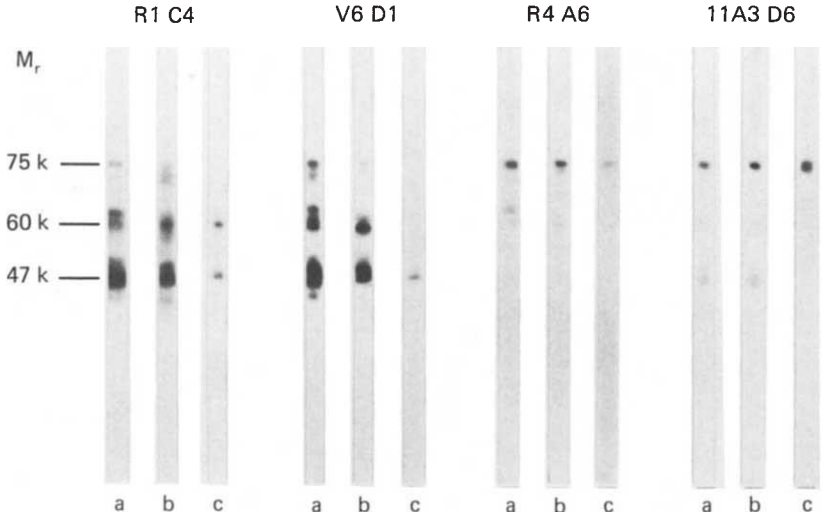

Fig. 4. *Demonstration that antibodies directed against the porcine Na^+-D-glucose cotransporter bind to polypeptides with the same molecular weights in porcine and human brush border membranes and in the human urine.* Brush border membranes from pig (**a**), brush border membranes from human (**b**), and a urinary sediment from human obtained after centrifugation at 200,000 × g (**c**) were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose as in Fig. 3. The Western blots were reacted with the hybridoma supernatants of R1C4, V6D1, R4A6 and 11A3D6 as indicated. The apparent molecular weights of three components of the Na^+-D-glucose cotransporter are indicated.

total urinary protein was applied in quantitative measurements. The data validate our test system as appropriate to analyze brush border membrane proteins in the urine quantitatively.

Analysis of the regular excretion of brush border proteins

To promote an understanding of how brush border membrane proteins are excreted in healthy individuals the sedimentation of total protein and lipids and of individual membrane proteins was studied. In addition the daily excretion of the Na^+-D-glucose cotransporter components was compared with the excretion of an M_r 400,000 polypeptide from the brush border membrane, of villin and of Tamm-Horsfall protein. For the sedimentation experiment the morning urine of ten healthy volunteers was collected, large particles were removed by low-spin centrifugation and the urine was concentrated 14-fold over PM-10 filters. Since 73% of the polypeptides in the urine passed the filters the predominant fraction of urinary proteins have a molecular mass smaller than 10 kDa. In the filtrate no binding activity of the antibodies R4A6, V6D1, N4A4 and ID2C3 was detected. The concentrated urine was centrifuged for one hour at 200,000 × g, the obtained pellet was removed and the supernatant was centrifuged for another 16 hours at 350,000 × g. In the pellets and final supernatants the amounts of total protein and of different urinary proteins were determined by the above-described radioimmunoassay. The total protein was measured according to Lowry and coworkers [29], the Na^+-D-glucose cotransporter was analyzed with the monoclonal antibodies R4A6 and V6D1, and a M_r 400,000 membrane protein from the intermicrovillar invaginations of proximal tubules was analyzed with the monoclonal antibody N4A4. Villin was determined with the monoclonal antibody ID2C3 [11]. Lipids were extracted from the pellets, separated by thin layer chromatography and analyzed quantitatively as described in **Methods**.

After centrifugation of the concentrated urine for one hour at 200,000 × g, 4% of the total urinary protein was found in the sediment (Table 3). This sediment contained mainly membrane vesicles with diameters between 0.05 and 0.5 μm (Figs. 5 a and b). Table 4 shows that the sediment contains cholesterols, fatty acids, shingomyelins, phosphatidylcholines, phosphatidylserines and phosphatidylethanolamines and that the lipid composition is similar to the lipid composition of brush border membrane vesicles which were isolated from human kidneys. The comparison of the ultrastructure (Fig. 5), the protein composition (Fig. 2) and the lipid composition (Table 4) of the 200,000 × g sediment and isolated human brush border membranes indicate that the 200,000 × g sediment mainly contains brush border membranes from renal proximal tubules.[1] Thus, part of the Na^+-D-glucose cotransporter appears to be excreted by shedding of microvilli of the proximal tubule into the urine.

Since the Na^+-D-glucose cotransporter is a membrane-spanning protein which should not be soluble in water and since only about 50% of the total Na^+-D-glucose cotransporter in the urine was sedimented at 200,000 × g (Table 3, R4A6, V6D1), we questioned whether part of the transporter in the urine may form small aggregates which do not sediment at 200,000 × g, but might be sedimented at prolonged centrifugation with higher force. In fact, when the 200,000 × g supernatant was centrifuged 16 hours at 350,000 × g a pellet was obtained which consisted mainly of amorphous material (Fig. 5c). This pellet contained about 40% of the Na^+-D-glucose cotransporter in the urine (Table 3, R4A6, V6D1). It was mainly composed of protein but contained also some cholesterols and fatty acids (Table 4). Since no phospholipids were detected the components of the Na^+-D-glucose cotransporter in this sediment appear to be excreted independently from the shedding of microvilli.

A detailed inspection of Table 3 shows that after centrifugation of the urine the Na^+-D-glucose cotransporter distributed

[1] The differences between the lipid composition of the 200,000 × g sediment and the human brush border membranes may reflect: i) lipid exchange during the preparation of brush border membrane vesicles and/or of the vesicles in the urine; ii) different origin of the vesicles from the brush border membrane, such as, from the tips of the microvilli (200,000 × g sediment) and from the total microvilli (purified brush border membrane vesicles); or iii) contamination of the vesicles with other membranes, such as, contamination of purified brush border membrane vesicles with subapical vesicles and of the 200,000 × g sediment with luminal membranes from other parts of the nephron.

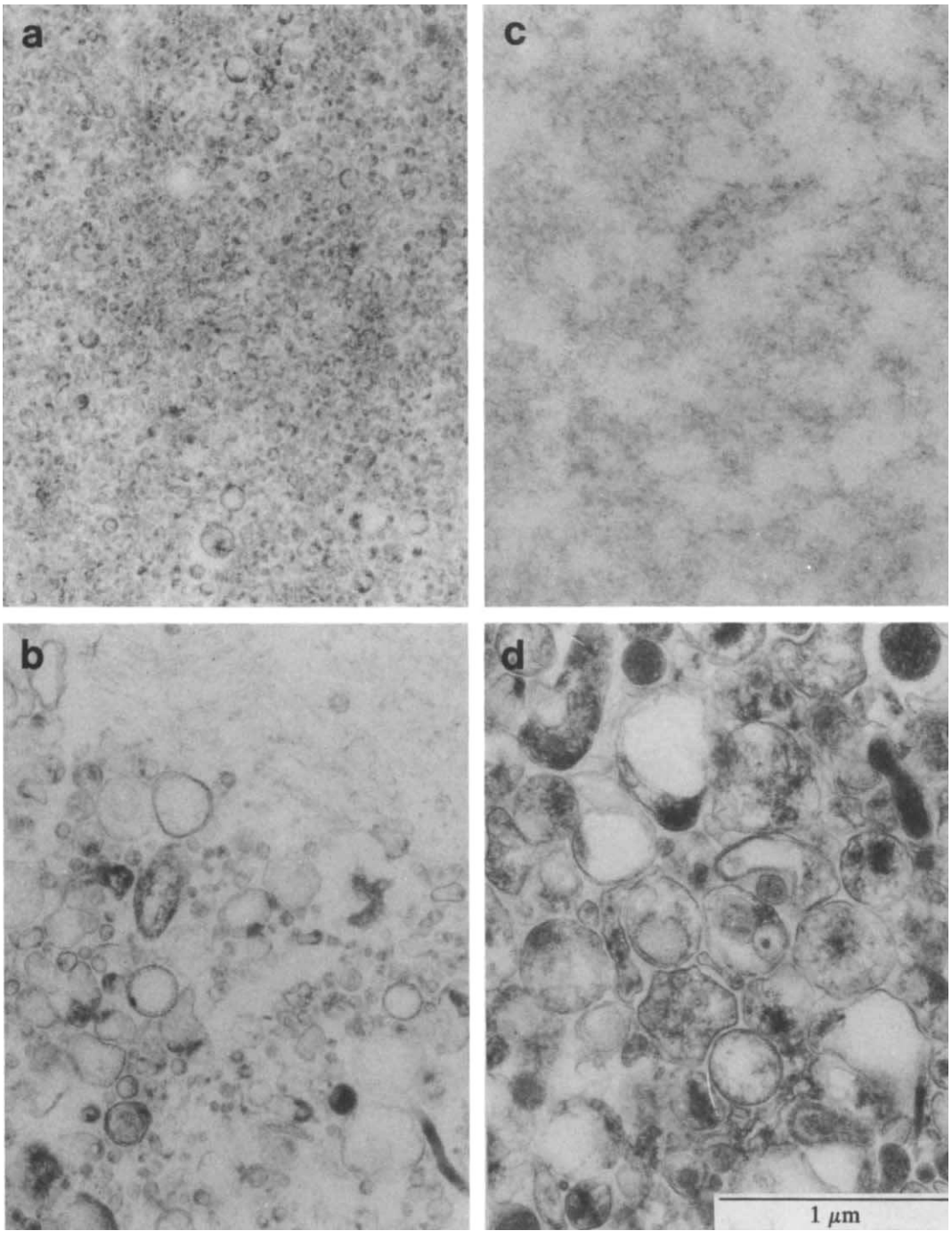

Fig. 5. *Demonstration that the human urine contains vesicles* (**a,b**) *with a similar appearance as purified human renal brush border membrane vesicles* (**d**) *and amorphous material which sediments after prolonged high-spin centrifugation* (**c**). Urine of healthy volunteers from which particulate material had been removed was centrifuged for one hour at 200,000 × g and the obtained supernatant was centrifuged for 16 hours at 350,000 × g. The sediments and brush border membrane vesicles, which had been purified from human renal cortex after precipitation with Mg^{++} [25], were fixed, embedded and prepared for electron microscopy as described in **Methods**. Sections through the upper and lower part of the 200,000 × g sediment are shown in a and b respectively; c) shows a section through the homogeneous 350,000 × g sediment and d) a section through a pellet of human brush border membrane vesicles.

differently than the M_r 400,000 polypeptide or villin. After a one hour centrifugation at 200,000 × g, about 47% of the Na^+-D-glucose cotransporter (R4A6, V6D1), 55% of villin (ID2C3) but only 25% of the M_r 400,000 polypeptide (N4A4) were sedimented. After 16 hours of centrifugation of the 200,000 × g supernatant at 350,000 × g, about 40% of the Na^+-D-glucose cotransporter or villin, and 61% of the M_r 400,000 polypeptide were sedimented. Thus, 12 to 14% of the Na^+-D-glucose

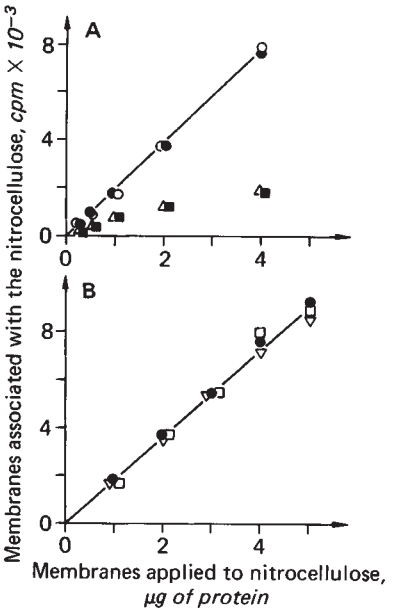

Fig. 6. *The quantitative radioimmunoassay: Effects of detergents (A) and urinary proteins (B) on the binding of brush border membranes to nitrocellulose.* Different amounts of ^{125}I-labeled porcine renal brush border membrane proteins (abscissa) were added to nitrocellulose in the presence of different detergents (A) or in the presence of filtered urine with and without additional urinary proteins (B), and the binding of radioactivity to the nitrocellulose (ordinate) was measured. The brush border membranes were suspended in 100 μl of PBS without detergent (○), of PBS plus 0.01% (wt/vol) SDS (●), of PBS plus 0.01% (wt/vol) Triton X-100 (△), of PBS plus 0.01% (wt/vol) Tween 20 (■) and in 100 μl of filtered urine (pH 6.5, 0.5 Osm) plus 0.01% (wt/vol) SDS (□) or of filtered urine (pH 6.5, 0.5 Osm) plus 0.01% (wt/vol) SDS and plus 10 μg of urinary proteins (▽). The nitrocellulose was incubated as described for the quantitative radioimmunoassay with the exception that the antibodies were omitted, and the radioactivity associated with the nitrocellulose was measured. Mean values of triple measurements and calculated regression lines ($r^2 > 0.99$) are presented.

Table 2. Effect of osmolarity and pH on the adsorption of human brush-border membrane vesicles to nitrocellulose

Properties of urinary filtrates		Adsorption of brush-border membrane protein to nitrocellulose
pH	Osmolarity	*cpm* × 10^{-3}/μg *of protein*
5.5	1.04	1.91 ± 0.11
5.5	0.56	1.86 ± 0.12
5.5	0.16	1.81 ± 0.08
7.5	1.04	1.86 ± 0.05
7.5	0.56	1.91 ± 0.12
7.5	0.16	1.82 ± 0.11

Fifty microliters of urinary filtrates of varying pH and osmolarity containing different amounts of ^{125}I-labeled human brush border membrane vesicles (1 to 5 μg of protein) were diluted with 150 μl of PBS containing 0.0133% SDS and added to nitrocellulose as described in **Methods**. The nitrocellulose was incubated, the associated radioactivity was determined and the correlation between the amount of protein applied to the nitrocellulose and the amount of analyzed radioactivity was calculated by linear regression analysis as in Fig. 6. Under all conditions correlation coefficients larger than 0.98 were obtained. The calculated amounts of radioactivity bound on the nitrocellulose per μg of applied brush border membrane protein are presented together with the respective standard deviations.

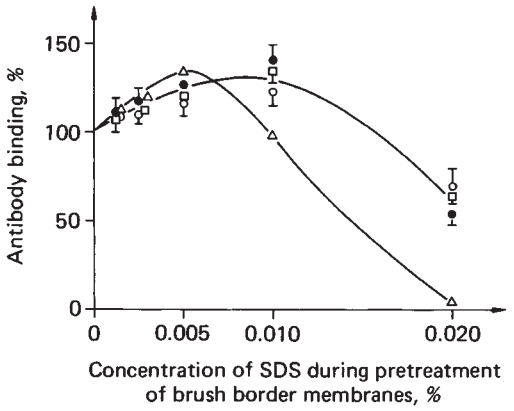

Fig. 7. *The quantitative radioimmunoassay: Effects of SDS present during the adsorption of brush-border membranes to nitrocellulose on antigen-antibody reactions measured in the absence of SDS.* Fifty microliters of filtrated urine containing 1 μg of human brush border membrane proteins was mixed with 150 μl of PBS containing different concentrations of SDS. The samples with the SDS concentrations indicated on the abscissa were applied to nitrocellulose. The reaction of the adsorbed proteins with antibodies R4A6 (●), 11A3D6 (○), ID2C3 (□) and N4A4 (△) was measured as described in **Methods**.

cotransporter or the M_r 400,000 polypeptide and 6% of villin remained in the supernatant after 16 hours centrifugation at 350,000 × g. The data show that the Na^+-D-glucose cotransporter, the antigen of N4A4 and villin are not soluble in the urine. About half of the Na^+-D-glucose cotransporter and a quarter of the M_r 400,000 polypeptide are associated with shedded microvilli. Most of the remaining Na^+-D-glucose cotransporter and the M_r 400,000 polypeptide in the urine form sedimentable aggregates which are associated with some cholesterol and fatty acids.

To determine how the daily excretion of Na^+-D-glucose cotransporter components varies in comparison with the excretion of other proteins from the proximal tubule, antigenic proteins of R4A6, V6D1, 11A3D6 and N4A4, and villin were measured in the urines of 26 healthy volunteers (50% female). In addition the excretion of Tamm-Horsfall protein from the thick ascending limb of Henle's loop [35–37] was determined immunochemically and the excretion of enzymatic activity of the lysosomal enzyme N-acetyl-β-D-glucosaminidase [40–42] was measured. The respective mean values and standard deviations of (a) the urine volumes collected over 24 hours, (b) the creatinine clearance related to a body surface of 1.73 m^2, and (c) the total excreted protein were 1696 ± 654 ml, 108 ± 19 ml × min^{-1} and 52 ± 14 mg. The standard deviations in Table 5 indicate that the daily excretion of the components of Na^+-D-glucose cotransporter, of antigenic protein of N4A4 and of villin varied considerably between different individuals. When we corrected for different affinities of the monoclonal antibodies by normalizing the respective antibody binding in the urine against the binding to 1 mg of renal brush border membrane protein, a similar amount of excreted Na^+-D-glucose cotransporter was determined with antibodies R4A6, V6D1 and 11A3D6 (Table 5). The amount of excreted antigen of N4A4 was about 50% and that of excreted villin about 20% of the excreted Na^+-D-glucose cotransporter.

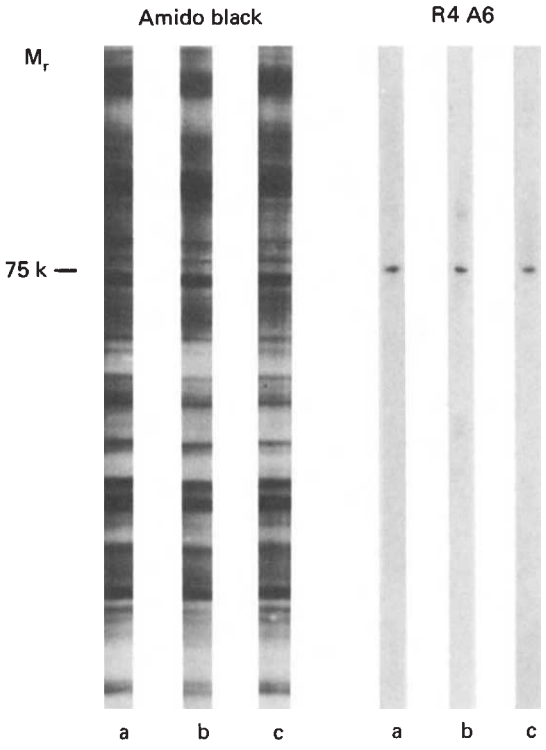

Fig. 8. *The quantitative radioimmunoassay: Attempt to evaluate proteolysis of brush border membrane proteins in the urine.* Human brush border membranes were separated by SDS polyacrylamide gel electrophoresis, blotted to nitrocellulose and either stained with Amido black (indicated by "Amido black") or reacted with hybridoma supernatant of R4A6 (indicated by "R4A6"). The brush border membranes were either directly applied to electrophoresis (**a**), or previously incubated 24 hours (37°C) in filtered urine with a pH of 5 (**b**) or with a pH of 7.4 (**c**).

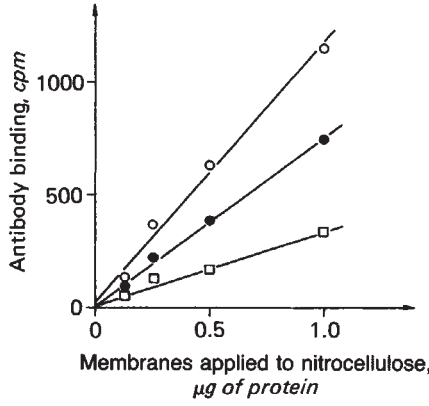

Fig. 9. *The quantitative radioimmunoassay: Correlation between the amount of brush border membranes added to the test and antibody binding.* One hundred microliters of PBS buffer containing 0.01% (wt/vol) SDS and the protein amounts of porcine renal brush border membranes, which are indicated on the abscissa, were added to nitrocellulose and reacted with the monoclonal antibodies R4A6 (○), R1C4 (□) and V6D1 (●) as described in **Methods**. Mean values of triple measurements and calculated regression lines ($r^2 > 0.98$) are presented.

To determine whether the physiological release of different proteins from the brush border occurs on the same or on different routes, we tested whether the varying amounts of

Table 3. Sedimentation of components of the Na^+-D-glucose cotransporter and of other proteins in the urine

Sample	Fraction of antigenic sites of				Fraction of protein
	R4A6	V6D1	N4A4	ID2C3	
	% of total amount in non-concentrated urine				
Sediment after 200,000 × g	49	45	25	55	4
Sediment after 350,000 × g	39	42	61	39	13
Supernatant after 350,000 × g	12	13	14	6	10

The morning urine of healthy volunteers was concentrated after particulate material had been removed. Then the concentrated urine was centrifuged for 1 h at 200,000 × g and the obtained supernatant for another 16 h at 350,000 × g. In the non-concentrated and concentrated urine, the 200,000 × g-sediment and the 350,000 × g-sediment, the amounts of antigenic sites of R4A6, V6D1, N4A4, ID2C3 and the total protein were determined as described in **Methods**. The antibodies were directed against the Na^+-D-glucose cotransporter (R4A6, V6D1), against a M_r 400,000 polypeptide from the intervillar region and from subapical vesicles of proximal tubules (N4A4) or against villin (ID2C3).

Table 4. Comparison of the lipid composition of urinary sediments and isolated brush border membranes

Lipid fraction	Brush-border membrane vesicles	200,000 × g sediment	350,000 × g sediment
Triglycerides	28 ± 3	ND	ND
Cholesterols	133 ± 13	185 ± 19	8 ± 1
Fatty acids	46 ± 5	44 ± 4	15 ± 12
Lysophosphatidylcholines	ND	ND	ND
Sphingomyelins	187 ± 20	250 ± 25	ND
Phosphatidylcholines	140 ± 12	51 ± 1	ND
Phosphatidic acids	19 ± 2	ND	ND
Phosphatidylserins	84 ± 9	250 ± 25	ND
Phosphatidylinositols	47 ± 5	ND	ND
Phosphatidylethanolamines	140 ± 15	130 ± 14	ND

From purified brush border membranes of human kidneys and from urine sediments which were obtained after one hour of centrifugation at 200,000 × g and after 16 hours of centrifugation of the 200,000 × g supernatant at 350,000 × g, lipids were extracted and analyzed for different lipid species (**Methods**). Mean values and standard deviations of lipid concentrations were calculated from 3 estimations and are presented as nmol of lipid per mg of protein. Nondetectable lipid species are indicated by ND.

brush border membrane proteins and of villin excreted by different individuals are correlated to each other, and whether they are correlated to the excretion of Tamm-Horsfall protein from the distal tubule (antibody CL1032) or to the excretion of the lysosomal enzyme N-acetyl-β-D-glucosaminidase. There was no significant correlation between the excretion of Tamm-Horsfall protein from the distal tubule (CL 1032) and the excretion of any of the above-mentioned proteins from the proximal tubule (Table 6, Fig. 10d). No significant correlation was obtained between the excretion of enzymatic activity of N-acetyl-β-D-glucosaminidase (NAG) and the excretion of Na^+-D-glucose cotransporter components ($r_{NAG/R4A6} = 0.06$, $r_{NAG/11A3D6} = 0.08$) or the excretion of villin ($r_{NAG/ID2C3} = 0.17$). A high correlation ($r = 0.74$) was obtained between the excretion of the antigenic sites of antibody R4A6 and 11A3D6 (Table 6, Fig. 10a). Also the correlation between the excretion

Table 5. Comparison of the daily excretion of Na^+-D-glucose cotransporter components with the excretion of other proteins from the microvilli of renal proximal tubules

Antibodies	Daily excretion of antigenic sites	
	Sites in the urine $cpm \times 10^{-6}$	Sites in the urine per sites in 1 mg of brush border membrane protein
R4A6	38.1 ± 24.1	10.4 ± 6.6
V6D1	121.8 ± 63.6	13.1 ± 6.9
11A3D6	40.6 ± 19.2	15.2 ± 7.2
N4A4	16.5 ± 12.6	5.7 ± 4.4
ID2C3	24.1 ± 9.0	2.1 ± 0.8

The urine of 26 healthy volunteers was collected over 24 hours and the total amounts of antigenic sites of several antibodies were determined as described in **Methods**. The antibodies were directed against the Na^+-D-glucose cotransporter (R4A6, V6D1, 11A3D6), against a M_r 400,000 polypeptide from the intervillar region and from subapical vesicles of proximal tubules (N4A4) or against villin (ID2C3). Either the total amount of cpm is presented, which was measured for antibody binding in the different urines, or the total amount of cpm normalized by the amount of cpm which was determined for antibody binding to purified brush border membrane vesicles containing 1 mg of protein. Mean values and standard deviations of the data obtained from the 26 urines are presented.

Table 6. Correlation between the daily excretion of components of the Na^+-D-glucose cotransporter and of other excreted renal proteins

Antigenic sites of	Antigenic sites of			
	11A3D6	N4A4	ID2C3	L1032
R4A6	0.74	0.55	0.49	0.07
11A3D4	–	0.37	0.61	0.03
ID2C3	0.61	0.32	–	0.13
CL1032	0.03	0.26	0.13	–

In the collected 24 h urines of 26 healthy volunteers (age 28 ± 7) the total amounts of D-glucose cotransporter (antigen of R4A6 or 11A3D6), of a M_r 400,000 polypeptide (antigen of N4A4), of villin (antigen of ID2C3) and of Tamm-Horsfall protein (antigen of CL1032) were determined. The correlations between the excretion of the different proteins which varied considerably in the different urines was calculated. The obtained correlation coefficients are presented.

of the glucose transporter components and villin ($r_{R4A6/ID2C3}$ = 0.49, $r_{11A3D6/ID2C3}$ = 0.61) was significant. This suggests that either a large fraction of the Na^+-D-glucose cotransporter is excreted by shedding of total microvilli (see ID2C3 in Table 5) and/or that shedding of microvilli membranes is followed by the release of the microvilli core. Some correlation was also found between the excretion of the M_r 400,000 protein from the intervillar region and either villin ($r_{N4A4/ID2C3}$ = 0.32) or components of the Na^+-D-glucose cotransporter ($r_{N4A4/R4A6}$ = 0.55, $r_{N4A4/11A3D4}$ = 0.37). This suggests that shedding of luminal membrane from the proximal tubule includes the intervillar membrane region and/or leads to the excretion of subapical vesicles from tubular cells. Such an excretion of subapical vesicles is also supported by the finding that the excretion of NAG, which is found in lysosomes and subapical vesicles, was correlated with the excretion of the M_r 400,000 protein ($r_{NAG/N4A4}$ = 0.58) which has been localized to the intervillar membrane and to membranes of subapical vesicles (Fig. 1).

Excretion of brush border proteins during medical treatment

After having characterized the normal excretion of different brush border membrane proteins we subsequently investigated

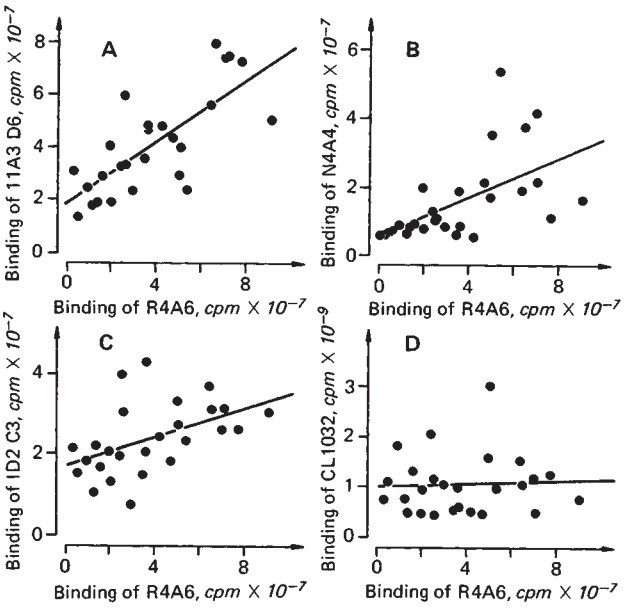

Fig. 10. *Correlation between the urinary excretion of different renal proteins.* In 26 healthy volunteers the daily excretion of antigenic proteins of several antibodies (against the Na^+-D-glucose cotransporter: R4A6 and 11A3D6, against the M_r 400,000 polypeptide from the intermicrovillar region: N4A4, against villin: ID2C3, and against Tamm-Horsfall protein: CL1032) was analyzed as described in **Methods**. The daily excretion of the different antigens is indicated by the binding of the different antibodies which is given in cpm. The graphs show the correlations between the antigen excretion measured with R4A6 on one hand and with 11A3D6 (**A**), N4A4 (**B**), ID2C3 (**C**) and CL1032 (**D**) on the other. Mean values of triple measurements and calculated regression lines with the correlation coefficients of 0.74 (A), 0.55 (B), 0.49 (C) and −0.07 (D) are presented.

whether or not the above-described antigens could be used as markers of kidney malfunction. Therefore, we studied whether or not kidney diseases and/or medical treatments correlated with specific excretion patterns of the above-described brush border proteins. Preliminary experiments on patients after renal transplantation and/or cyclosporine treatment (data not shown) and on patients after angiography with iopamidol (Fig. 11) suggest that this was the case. Figure 11 shows the excretion of villin (antigen of ID2C3), of the Na^+-D-glucose cotransporter (antigen of R4A6) and of the antigen of N4A4 in four patients who received a single injection of 135 ml of iopamidol for coronary angiography. Two patients (a,b) had no accompanying disease, and two (c,d) suffered from diabetes mellitus type II and were being treated with oral antidiabetica. Although none of the patients developed a significant decrease of creatinine clearance and/or increase of urinary protein excretion after iopamidol injection, a significant increase of brush border membrane proteins was observed between one and two days after the iopamidol injection. In patients without diabetes the urinary excretion of the Na^+-D-glucose cotransporter and of the antigen of N4A4 was increased about threefold over the basal level after iopamidol injection, whereas the excretion of villin was not altered significantly. In patients with diabetes the basal level of the excretion of villin, the Na^+-D-glucose cotransporter, and the antigen of N4A4 was significantly higher than in patients without diabetes. In patients with diabetes the

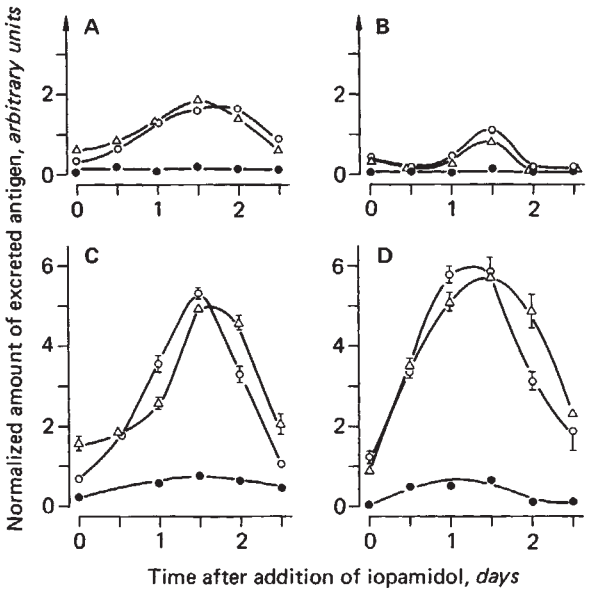

Fig. 11. *Effects of a single injection of 135 ml iopamidol on the excretion of brush border proteins.* Four patients with angina pectoris (**A**: 35 years old, female, body weight of 60 kg, no additional disease; **B**: 53 years old, male, body weight of 80 kg, no additional disease; **C**: 64 years old, female, body weight of 65 kg , diabetes mellitus type II; **D**: 45 years old, female, body weight of 74 kg, diabetes mellitus type II) obtained a single intravenous injection of 135 ml iopamidol. Before the injection (time 0) and in 12 hour intervals after the injection urine was collected and particulate components were removed by 10 min centrifugation at 2000 × g. Then the protein and creatinine concentration was determined and the antigenic sites of ID2C3 (●), N4A4 (○) and R4A6 (△) were measured by the quantitative radioimmunoassay described in **Methods**. The normalized amounts of excreted antigens on the ordinate indicate the amounts of antibody binding to 50 μl of urine which are divided (i) by the binding of the respective antibody to 1 μg of human brush border membranes and (ii) by the creatinine concentration in the respective urine.

application of iopamidol led to a much more drastic increase of the excretion of the Na^+-D-glucose cotransporter and of the antigen of N4A4, and also led to a significant increase of the excretion of villin (Figs. 11c,d). The data suggest that analysis with the antigen of N4A4, of the Na^+-D-glucose cotransporter and of villin can be used to determine the individual nephrotoxic effect of iopamidol.

Discussion

In the present study a quantitative radioimmunoassay is described by which the urinary excretion of proteins from brush border membranes of renal proximal tubules can be analyzed. This assay is very sensitive and allows the simultaneous analysis of different antigens. Previously, for the analysis of urinary antigens either precipitation assays [43, 44] or sandwich assays [13, 15, 18, 45, 46] were used. The precipitation assays require a large amount of primary antibody and for the sandwich assays two different antibodies are needed, one for the fixation of the antigen and the other for the detection. At variance to the assay described in this paper, the precipitation and sandwich assays have to be established separately for every antigen-antibody reaction and certain types of antigen are not detected. Thus, the precipitation assays do not detect soluble polypeptides which do not contain multiple epitopes of the primary antibody, and the sandwich assays do not detect antigens which are not immobilized by the first antibody. This may happen if antigens are localized on membrane vesicles. In the radioimmunoassay described in this paper, urine is added to a defined area of nitrocellulose and an amount of antigen is immobilized that is proportional to the urinary antigen concentration. Then the fixed antigen is reacted with the specific monoclonal antibodies which are detected by a cascade of secondary antibodies. The specific monoclonal antibodies can be added as hybridoma supernatant and the test is sensitive enough to detect proteins from the renal proximal tubules in non-concentrated urine of healthy individuals. The test is easily performed, detects soluble proteins as well as those which are associated with membrane vesicles, and more than 10 different antibodies can be tested simultaneously. Thus, if a set of monoclonal antibodies has been selected, which are relevant for the differential diagnoses of kidney malfunction, the described assay may be used to establish a diagnostic kit that can be employed for routine nephrologic investigations.

For many years it has been recognized that the quantification of renal antigens in the urine may be of high diagnostic value in kidney malfunctions [1–3], and many attempts have been made to correlate kidney dysfunctions and urinary antigen excretion [2, 13–16]. In most studies tubular and glomerular leasions could be distinguished, however, no differentiation between specific types of kidney disorder was obtained [2, 13–16]. This failure is not surprising since an analysis of several well-characterized antigens from different parts of the renal nephron, which may be required for a differential diagnosis, had not been performed. Thus, previous investigators have measured either the excretion of deaminase binding protein which is a glycoprotein with a molecular mass of 120 kD [2, 13, 15], or the excretion of several poorly-characterized antigens from different parts of the nephron [14, 16]. In case of deaminase binding protein the problems exist that high concentrations of this protein are not only found in kidney but also in other tissues, and that deaminase binding protein is not only associated with cell membranes but is also present in the cytoplasm [7, 47, 48]. Thus, although deaminase binding protein is confined to the proximal tubule and to the initial portion of the loop of Henle in kidney [9, 10], its urinary excretion may be increased by different pathogenetic mechanisms. From the analysis of proteins from different nephron parts only limited information can be obtained if the proteins are not characterized on a ultrastructural and molecular level, since the excretion of different types of proteins from different subcellular origin may have different diagnostic meanings. Therefore, it has to be known whether hydrophilic or hydrophobic proteins are excreted and whether the proteins are derived from the cytosol, from elements of the cytoskeleton or from cell membranes.

In the present paper the urinary excretion of three proteins from the brush border of renal proximal tubules is investigated and compared with the excretion of Tamm-Horsfall protein which is secreted in the distal tubule. One protein is the Na^+-D-glucose cotransporter, which is an integral membrane protein and spans the brush border membrane [49, 50]. In pig and humans three polypeptide components of the renal Na^+-D-glucose cotransporter with apparent molecular weights of 75,000, 60,000 and 47,000 have been identified [19,

39, this paper] and the total molecular weight of the transporter is about 300,000 [51–53]. The M_r 75,000 polypeptide has been generally accepted as a subunit of the Na^+-D-glucose cotransporter, whereas it is under discussion whether the M_r 60,000 and M_r 47,000 polypeptides are additional subunits of the transporter or proteolytic splitting products of the M_r 75,000 polypeptide [19]. High concentrations of the Na^+-D-glucose cotransporter are found in small intestine and in kidney where the transporter is localized in the brush border membrane of proximal tubules and in subapical vesicles below this membrane. Some transporter molecules were also found in the luminal membrane of descending limbs of loops of Henle and of collecting ducts [20]. However, since the total luminal membrane area of the proximal tubules is more than 30 times larger than the total luminal membrane area of the descending limbs of Henle's loops and of the collecting ducts [54][2], and the transporter concentration in the proximal tubule is higher than in the other nephron segments [20], Na^+-D-glucose cotransporter components in the urine should be mainly derived from proximal tubules. The second protein investigated in this study is a M_r 400,000 polypeptide which has been found in pig, human and rat and is specific for kidney where it is exclusively localized at intervillar invaginations and subapical vesicles of proximal tubules [24, unpublished data]. This protein is probably identical to a M_r 400,000 protein which has been recently identified in the human kidney [55]. The M_r 400,000 polypeptide is supposed to be an integral membrane protein since it is only released from brush border membranes by Triton X-100 when the membranes are completely solubilized (unpublished data). The localization of this polypeptide in the proximal tubule appears to be identical to the localization of the M_r 330,000 antigen of rat Heyman nephritis [55, 56, unpublished data]. However, the Heyman nephritis M_r 330,000 antigen is not kidney specific and is also found in glomeruli of rat [56]. The third investigated urinary protein was villin which occurs in the microvilli core of enterocytes and of epithelial cells from renal proximal tubules. Similar to the two other proteins, villin is found in proximal but not in distal renal tubules [57, 58]. It has a molecular weight of 95,000 and can be only released where the integrity of the brush border membrane is destroyed.

Our data show that the urine of healthy individuals contains membrane proteins from brush border membranes of renal proximal tubules. About half of the observed membrane proteins are associated with membrane vesicles and about 40% of the membrane proteins form aggregates together with other proteins, cholesterol and fatty acids. The components of the Na^+-D-glucose cotransporter associated with the excreted membrane vesicles have about the same molecular weights as the respective polypeptides in the intact brush border membrane. Neither was a substantial degradation detected in the Na^+-D-glucose cotransporter components associated with the excreted protein aggregates (data not shown). The data indicate that shedding of brush border membranes contributes significantly to the excretion of membrane proteins from the microvilli of proximal tubules. The membrane segments released into the urine differ in size and most of them form vesicles (Figs. 5a and b). Since the vesicles contain components of the Na^+-D-glucose cotransporter, of villin and of the M_r 400,000 polypeptide, they are derived from intermicrovillar membrane regions as well as from the whole microvilli. The presence of villin from the cores of the microvilli in the fraction of sedimentable urinary vesicles and the correlation between the excretion of proteins from the microvilli membrane and of villin suggests that total microvilli are also released into the urine. It is probable but not proven that some of the small vesicles in the urine (Fig. 5a) are derived from subapical vesicles which are released when the integrity of the luminal membrane is disrupted.

The origin of the aggregated membrane proteins in the urine which are sedimented after 16 hours of centrifugation at 350,000 $\times$ g is not well understood. These proteins probably do not originate from shedded brush border membranes since they are not associated with phospholipids or lysophospholipids (Table 4). They may be excreted independently from phospholipids by some unknown mechanism. After their excretion these membrane proteins may associate with other urinary proteins as, for example, villin (Table 3).

The excretion of proteins from the brush border of renal proximal tubules varies considerably in healthy persons. After the physiological range of protein excretion was determined we started to investigate the excretion during medical treatments and kidney dysfunctions. The results obtained so far indicate that Na^+-D-glucose cotransporter components and the M_r 400,000 brush border membrane polypeptide described above are diagnostic markers which are more sensitive indicators for the destruction of the integrity of brush border membranes of renal proximal tubules than villin. Thus, in patients with angina pectoris a single dose of iopamidol did not increase the excretion of villin, but the excretion of the Na^+-D-glucose cotransporter and of the M_r 400,000 membrane polypeptide was increased (Fig. 11 a,b). When patients with angina pectoris in addition suffered from diabetes type II their basal urinary excretion of the Na^+-D-glucose cotransporter and the M_r 400,000 polypeptide was increased, iopamidol led to a higher increase in the excretion of these proteins and the excretion of villin was significantly increased (Fig. 11 c,d). These data suggest that iopamidol normally leads to the shedding of small areas of membrane. However, in patients with diabetes iopamidol may induce the shedding of larger areas of membrane or of total microvilli.

To obtain a diagnostic tool for an early differential diagnosis of kidney malfunctions, the excretion of the above-described proteins, of additional proteins from the proximal tubule, and of proteins from other nephron segments has to be investigated during different types of kidney malfunction and typical excretion patterns have to be established. When the properties and origin of the analyzed proteins are known, the observed excretion patterns may contribute to the understanding of pathogenic mechanisms of kidney malfunctions. In addition to the described studies on the effects of iopamidol treatment we are currently using the above-described assay to investigate the urinary excretion of the Na^+-D-glucose cotransporter and other brush border proteins during cyclosporin A treatment. This appeared to be interesting since we recently found that cyclosporin A binds specifically to the Na^+-D-glucose cotransporter

[2] This estimation is based on morphometric data from rat kidney since no data are available from human kidneys.

in the renal brush border membrane without inhibiting D-glucose transport activity but possibly altering the turnover of the Na^+-D-glucose cotransporter [59].

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