Am J Physiol Renal Physiol 286: F152–F160, 2004.
First published September 9, 2003; 10.1152/ajprenal.00264.2003.

Two-photon in vivo microscopy of sulfonefluorescein secretion in normal and cystic rat kidneys

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Submitted 24 July 2003; accepted in final form 3 September 2003

Tanner, George A., Ruben M. Sandoval, and Kenneth W. Dunn. Two-photon in vivo microscopy of sulfonefluorescein secretion in normal and cystic rat kidneys. *Am J Physiol Renal Physiol* 286: F152–F160, 2004. First published September 9, 2003; 10.1152/ajprenal.00264.2003.—Sulfonefluorescein (SF) is a fluorescent organic anion secreted by kidney proximal tubules. The purposes of this study were *1*) to quantify accumulation of SF in normal and cystic rat kidneys in vivo and *2*) to test whether SF accumulation could be used as a marker for cysts derived from proximal tubules. Male Munich-Wistar rats, normal Han:SPRD rats, and heterozygous Han:SPRD rats with autosomal-dominant polycystic kidney disease were anesthetized with Inactin and solutions containing SF were administered by constant intravenous infusion. In Munich-Wistar rats, SF fluorescence in the urinary space of Bowman's capsule averaged 0.15 ± 0.04 (n = 17) times that of glomerular capillary plasma, consistent with extensive plasma protein binding of SF. In normal Han:SPRD rats, steady-state cell cytoplasm SF fluorescence in proximal tubule and distal tubule cells averaged, respectively, 2.7 ± 1.4 (n = 99 tubules) and 0.2 ± 0.2 (n = 17) times that of peritubular capillary plasma. No punctate SF fluorescence was seen in proximal tubule cell cytoplasm. Probenecid reduced proximal tubule cell SF fluorescence to 0.64 ± 0.40 (n = 64) times that of plasma. Ureteral obstruction decreased the proximal tubule cell-to-lumen SF fluorescence gradient, suggesting that tubule fluid flow normally sweeps away secreted SF. In cystic kidneys, cysts derived from proximal tubules could be identified by their uptake of SF, but cell uptake was patchy. We conclude that in vivo two-photon microscopy is a powerful tool for quantifying glomerular and tubular handling of SF, and SF can be used to identify proximal tubule-derived cysts.

organic anion secretion; glomerulus; polycystic kidney disease; two-photon microscopy

SULFONEFLUORESCEIN (SF) is a fluorescent organic molecule that differs from fluorescein by having a sulfonic acid group instead of a carboxylic acid group. It is secreted by kidney proximal tubules by the same system that transports PAH, fluorescein, and many other organic anions (1, 23). In the rat kidney, SF is secreted more vigorously than is fluorescein; the related compound fluorescein-5(6)-sulfonate is apparently not secreted at all (1). Previous in vivo studies on SF transport have used conventional epifluorescence methods (1, 23, 25), and the ability to distinguish cell from lumen accumulation was poor. Boyde et al. (2) recently reported observations on the accumulation of fluorescein, after intravenous bolus injections, using confocal fluorescence microscopy of the rat kidney.

The goals of the present study were twofold. First, we wanted to determine whether the two-photon microscope could be used to study quantitatively the renal handling of SF in vivo. Therefore, we measured, in the superficial kidney cortex of anesthetized rats, steady-state levels of SF in blood plasma, urinary space of Bowman's capsule, tubule cells, and tubule lumens. We measured the glomerular filterability of SF. We also tested whether proximal tubule cell SF levels were affected by probenecid, a competitive transport inhibitor, and by acute ureteral occlusion. Second, we tested whether renal cysts of rats with autosomal-dominant polycystic kidney disease (ADPKD) transported SF. We hypothesized that cysts of proximal tubule origin could be identified by the uptake of SF, because only proximal tubules secrete this compound. We also considered that SF accumulation might be deficient if cyst cells had lost the ability to transport SF.

METHODS

Imaging studies were done in three male Munich-Wistar rats with superficial cortical glomeruli, eight normal (+/+) male Han:SPRD rats, and eight heterozygote (cy/+) male Han:SPRD rats with ADPKD. Before surgery, the rats were fasted overnight with ad libitum access to water. They were anesthetized with the long-lasting thiobarbiturate Inactin (130 mg/kg body wt ip; Byk-Gulden, Konstanz, Germany). Surgical procedures included *1*) a tracheostomy, *2*) cannulation of the right femoral artery and vein, *3*) cannulation of the left ureter for urine collection, and *4*) a small flank incision for exposing the left kidney. During preparation, the rats were given intravenously 1 ml of 6 g/dl bovine serum albumin in isotonic saline. A blood sample (0.2 ml) was collected for measurement of the plasma SF blank; this averaged 0.7 ± 0.4 μM (n = 6) in normal rats and 1.2 ± 0.6 μM (n = 6) in rats with PKD [P = not significant (NS)]. For imaging, the rat was placed on the stage of a Nikon Diaphot inverted microscope (Fryer, Huntley, IL). The rat was kept warm by covering it with a water-jacketed heating blanket, and body temperature was monitored with a rectal probe. The exposed kidney rested in a dish, containing 0.9% NaCl, with a coverglass bottom (9).

The rats were infused intravenously with a solution containing 0.26–0.72 mM SF and 3% polyfructosan (PFS; Laevosan, Linz, Austria), a synthetic inulin, in 0.9% NaCl at 3.6 ml/h. In three experiments, dialyzed (1,000 molecular mass cut-off membrane) neutral dextran 3,000, labeled with Texas red (Molecular Probes, Eugene, OR), was also included in the infusion solution at a concentration of 0.2–0.4 mg/ml, and in one experiment we gave rhodamine B-labeled neutral dextran 10,000 (Molecular Probes) by bolus injection. In one experiment, we infused a 0.14 mM sodium fluorescein solution at 3.6 ml/h. To identify cell nuclei, in 12 of 16 experiments we injected intravenously 0.1 ml per 100 g body wt of a 10 mg/ml solution of Hoechst dye 33342 in isotonic saline. Urine was collected under oil for timed periods, and 0.25-ml arterial blood samples were obtained periodically for measurements of plasma SF and PFS concentrations. Urine and plasma SF concentrations were measured using a Turner

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fluorometer, using fluorescein standards, after alkalinizing the samples (1). PFS was measured using an anthrone method (6). Renal clearances of SF and PFS (a measure of glomerular filtration rate or GFR) were calculated using standard formulas.

In two experiments in normal Han:SPRD rats, the competitive transport inhibitor probenecid (200 mg/kg body wt) was given intravenously after control measurements had been made, and then proximal tubule levels of SF were recorded more than 20 min later. Two Munich-Wistar rats were infused intravenously with 1.0 M mannitol, 30 mM NaCl, 10 mM KCl, 1.5% PFS, and 0.2 mM SF solution at 5.8 ml/h. In these rats, we recorded plasma, proximal tubule cell, and lumen SF fluorescence before and 20–40 min after occluding the left ureter.

Two-photon microscopy was performed using a Bio-Rad MRC 1024 confocal/2-photon system (Bio-Rad, Hercules, CA). Illumination was provided by a Spectra-Physics (Mountain View, CA) Tsunami Lite Titanium-Sapphire laser, usually tuned to a wavelength of 800 nm. The kidney cortex was viewed using a ×60 numerical aperture (NA) 1.2 water immersion objective, and images were simultaneously recorded on three photodetector channels in a nondescanned mode. The "red" photodetector (for detection of rhodamine or Texas red-labeled dextrans) was preceded by a 560- to 650-nm band pass filter; the "green" photodetector (for SF) was preceded by a 500- to 550-nm band pass filter; and the "blue" photodetector (for the Hoechst dye) was preceded by a 440- to 470-nm band pass filter. Images were initially recorded in the absence of any infused fluorescent molecules at different levels of laser output. These measurements provided background levels of fluorescence for *1*) the cell or plasma and *2*) tubule lumen, cyst lumen, and Bowman's capsule lumen measurements.

The tubular or cyst structures chosen for measurements were usually selected because of a clearly visible capillary blood vessel in the same field and the ability to simultaneously image the tubule or cyst lumen. Measurements of plasma SF fluorescence were usually done on the thin, cell-free layer just inside the blood vessel wall. We tried to avoid bias of our measurements toward renal epithelial cells with intense accumulation of SF by also making measurements on weakly fluorescent cells in the same field. Image collections were usually started 20–30 min after beginning SF administration and were continued for up to 2 h. Plasma SF levels were constant and a steady state was present.

Only superficial structures could be studied, because the fluorescence signal is attenuated with depth of focus into the kidney substance at constant levels of illumination. The signal from peritubular capillary blood vessels often was not detectable more than roughly 10 μm below the kidney surface at the laser power levels employed. Sometimes it was not possible to see a blood vessel and the tubule lumen simultaneously. In that case, we recorded an image from the superficial cortex (with blood vessels and tubule epithelial cells) and then imaged the same tubule deeper in the kidney. The measurements in cells from the same tubule at two different focus levels were used to correct the lumen value for signal attenuation and permitted us to calculate the lumen/plasma SF fluorescence intensity ratio. In some of the cystic kidneys, we collected through-focus images of the cysts, recording images at 1-μm intervals for a total depth of ~40 μm. The thickness of each optical section, at a wavelength of 800 nm and with the ×60 NA 1.2 objective, is slightly less than 1 μm.

To determine whether we could quantify SF concentration using the two-photon microscope, a series of SF solutions in pH 7.4 phosphate-buffered saline, with SF concentrations from 0 to 32 μM, was prepared in cover glass chambers, and images were recorded in the green channel. To determine pH dependency of SF fluorescence, a series of solutions, all containing 15 μM SF, with a pH between 5.3 and 7.8, was studied similarly at an excitation wavelength of 800 nm. Fluorescence intensity, on a scale of 0–255, was measured using the Metamorph image analysis system (Universal Imaging, West Chester, PA).

Equilibrium dialysis experiments, to measure the plasma protein binding of SF, were done on heparinized plasma obtained by cardiac puncture from six anesthetized Han:SPRD rats (3 normals and 3 rats with ADPKD). The plasma was kept frozen at −80°C until use. One-milliliter aliquots were encased in sacs of 3,500 molecular mass cutoff dialysis tubing (Spectra/Por, Thomas Scientific, Swedesboro, NJ). The sacs were immersed in 125-ml stoppered flasks containing 5% CO_2 and 40 ml Ringer solution (125 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, bubbled with 5% CO_2) with SF or without SF (for plasma blank determination) and were shaken for 20 h in a water bath at 37°C in the dark. Aliquots of the final medium and plasma were analyzed for SF using a Turner fluorometer. At equilibrium, the plasma SF averaged 5.5 ± 1.4 μM ($n = 6$) in these experiments.

All values are means ± SD. If variances were homogeneous, comparisons were made by simple *t*-tests. If homogeneity of variances could not be assumed, comparisons were made using the Welch-Satterthwaite *t*'-test. A P value < 0.05 is considered significant.

Experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and protocols were approved by the Indiana University School of Medicine Animal Care and Use Committee.

RESULTS

Figure 1 shows SF fluorescence intensity as a function of SF concentration. A linear relationship was observed over a wide range of concentrations, but the fluorescence intensity leveled off at high-SF concentrations. In our in vivo experiments, we stayed within the linear range, except for measurements in distal tubule lumens. SF fluorescence intensity was pH sensitive at an excitation wave length of 800 nm (Fig. 2). In additional experiments (data not shown), we varied the excitation wave length over the range from 750 to 900 nm (in 25-nm increments) but did not find an excitation wave length with a lower pH sensitivity.

In measurements done on superficial glomeruli of three normal Munich-Wistar rats, SF fluorescence intensity in the

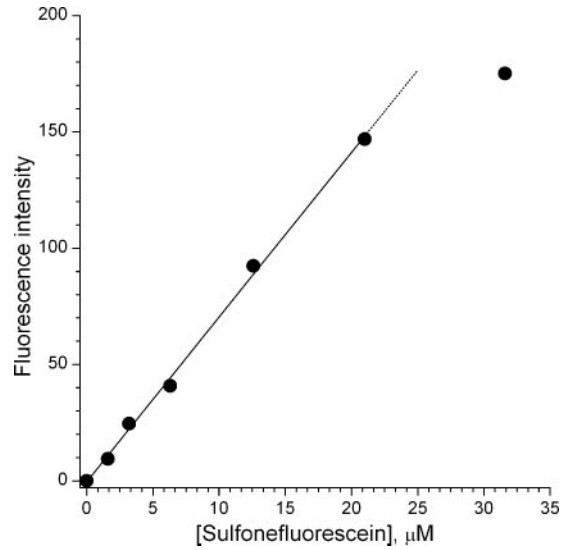

Fig. 1. Sulfonefluorescein (SF) fluorescence intensity as a function of SF concentration. SF was dissolved in phosphate-buffered saline (pH 7.4) and the SF was excited at a wavelength of 800 nm, laser output 40%, using the 2-photon microscope. Fluorescence emission was collected in the "green" channel and quantified using the Metamorph program. At a high-SF concentration, the fluorescence intensity levels off due to saturation of the system.

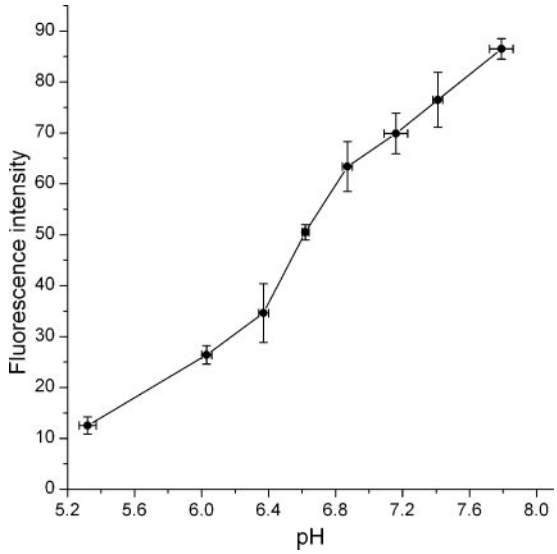

Fig. 2. pH dependency of SF fluorescence intensity. Solutions containing 15 μM SF, with pHs between 5.3 and 7.8, were excited at 800 nm, and emission was collected in the "green" channel. SF fluorescence decreases as the pH falls.

urinary space of Bowman's capsule averaged 15 ± 4% (n = 17) of glomerular capillary plasma SF fluorescence intensity (Fig. 3). Equilibrium dialysis experiments on plasma collected from normal and heterozygous cystic Han:SPRD rats revealed that only 12 ± 2% of plasma SF was freely diffusible. Both of these results are consistent with extensive binding of SF to plasma proteins in the rat. In vitro binding of SF was identical in plasma from normal Han:SPRD rats and Han:SPRD rats with ADPKD.

Whole kidney clearance measurements in Han:SPRD rats are summarized in Table 1. The rats ranged in age from 3.5 to 8.5 mo old. The GFR (C_{PFS}) in the rats with ADPKD was significantly decreased ($P < 0.01$) and averaged 38% of the GFR in normal rats. The C_{SF}/GFR ratio averaged 0.59 ± 0.13 in normal rats and 0.50 ± 0.04 in rats with ADPKD (P = NS). The renal clearance of SF is below the GFR, despite tubular secretion of SF, because of the extensive binding of SF by plasma proteins and consequent low glomerular filterability of SF. Assuming a glomerular filterability of SF of 15% (0.15), and a clearance ratio of 0.59 in the normal kidney, we calculated that ~75% of the SF excreted in the urine is due to tubular secretion of this molecule.

Table 1. *Clearance measurements in Han:SPRD normal rats (+/+) and rats with ADPKD (cy/+)*

	Normal Rats	Rats with ADPKD	P Value
Age, days	132±22 (6)	159±49 (6)	NS
BW, g	442±24 (6)	446±58 (6)	NS
Plasma SF, μM	4.3±1.8 (6)	5.0±1.0 (5)	NS
C_{PFS}, μl·min^{-1}·100 g BW^{-1}	366±102 (5)	139±111 (5)	<0.01
C_{SF}/C_{PFS}	0.59±0.13 (5)	0.50±0.04 (4)	NS

Values are means ± SD (number of animals). Clearance (C) data are for the left kidney. PFS, polyfructosan; SF, sulfonefluorescein; BW, body weight; ADPKD, autosomal-dominant polycystic kidney disease; NS, not significant.

Figure 4*A* shows a low-power view of the kidney surface of a normal rat infused intravenously with SF. As first described by Steinhausen et al. (23), SF intensities in proximal tubules are not uniform but are higher in late proximal tubules than in early proximal tubules. Figure 4*B* shows a representative high-power view from a normal kidney. Proximal tubule cell SF fluorescence intensity clearly exceeds that of both the plasma and the tubule lumen. For 50 normal proximal tubules, there was a highly significant correlation (r = +0.88, $P <$ 0.001) between cell cytoplasm SF and lumen SF, as would be expected from the known difference between early and late proximal tubule segments. SF fluorescence is extremely low in distal tubule cells but is high in distal tubule lumens (Fig. 4*B*). Figure 4*C* shows that the background fluorescence level recorded before administering of SF or Hoechst dye is low; this image was recorded from the same experiment, at the same laser power output, as in Fig. 4*B*.

SF fluorescence intensity in nuclei exceeded that in the surrounding proximal tubule cell cytoplasm; the nucleus/cytoplasm fluorescence ratio averaged 1.34 ± 0.17, n = 11. This ratio is higher than unity probably mainly because of a pH effect (see DISCUSSION). These measurements in nuclei were done in the absence of Hoechst dye to avoid errors caused by spillover of fluorescence from the "blue" into the "green" channel. SF fluorescence intensity was slightly elevated in the brush border region of proximal tubule cells. No other cell organelle accumulation of SF was seen, even when the magnification was increased 5- to 10-fold, using the microscope's zoom function. Figure 5*A* shows results of confocal imaging from an experiment in which SF and neutral dextran 3000-Texas red were infused. The intracellular accumulation of dextran in particulate structures is clearly visible, but no

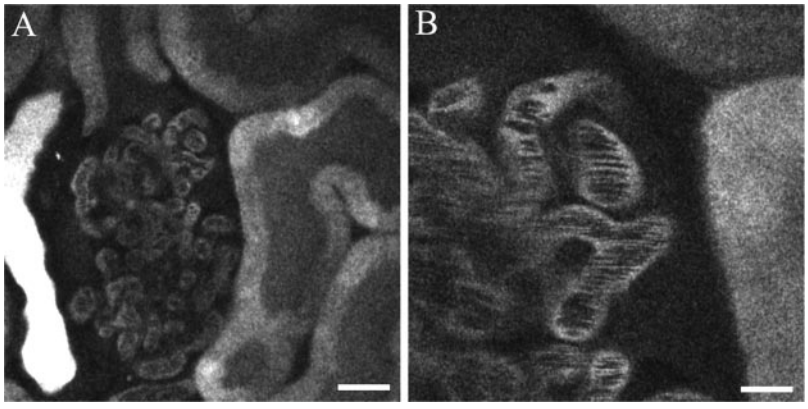

Fig. 3. *A*: superficial glomerulus from a Munich-Wistar rat. The SF fluorescence intensity in the urinary space of Bowman's capsule is 19% of that in the glomerular capillary plasma. SF is highly concentrated in the lumen of the distal tubule on the *left*. Scale = 30 μm. *B*: part of the same glomerulus taken with the ×3 zoom function. Red cells appear as black streaks in the glomerular capillaries. Scale = 10 μm.

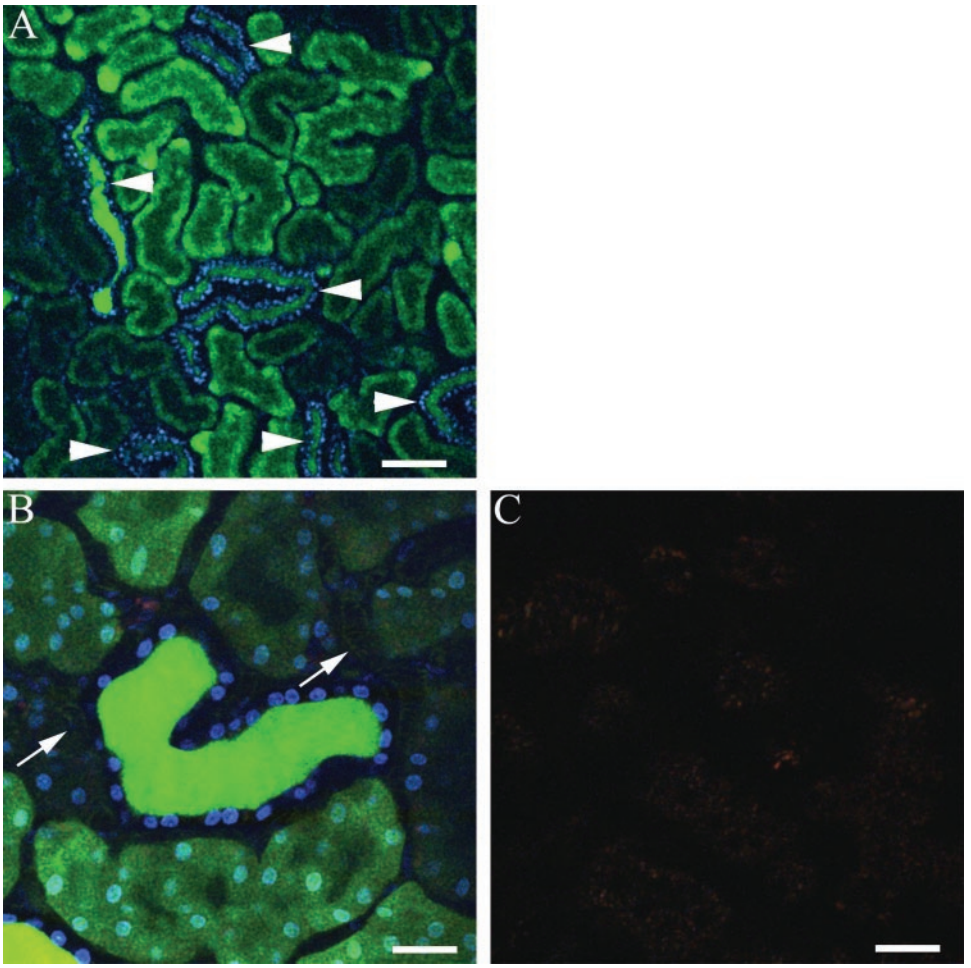

Fig. 4. *A*: low-power (×20) view of the surface of a normal rat kidney during constant intravenous infusion of SF solution. Surface proximal tubules show different degrees of SF fluorescence intensity, mainly due to greater cell accumulation in late as opposed to early proximal convoluted tubule segments. Distal tubules (arrowheads) are identified by the high concentration of SF in their lumens and the absence of SF within their cells. Scale = 90 μm. *B*: higher power (×60) view of normal kidney. The distal tubule cell nuclei stain especially brightly with the Hoechst dye. SF is concentrated in the distal tubule lumen due to filtration of SF, secretion by upstream proximal tubule segments, and water reabsorption along the nephron. SF fluorescence intensity in proximal tubule cells may be several times higher than in blood plasma (fine arrows). Scale = 30 μm. *C*: background fluorescence, recorded using the same laser power output as in *B*, from a comparable field. Scale = 30 μm.

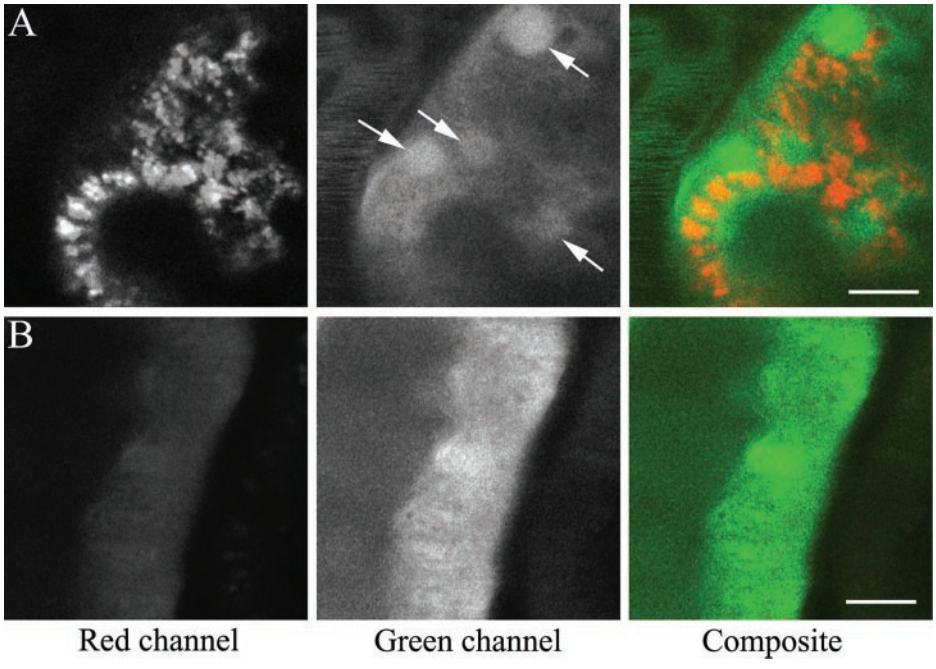

Fig. 5. *A*: high-power view (×60, ×5 zoom) of a proximal tubule from a normal rat kidney, taken by confocal (single photon) microscopy (488-nm excitation wavelength). In this experiment, we infused dextran 3,000-Texas Red, but no Hoechst dye was administered. The fluorescent dextran is visible in particulate structures. The nuclei (arrows) show a higher SF fluorescence intensity than the cytoplasm. No punctate cytoplasmic SF fluorescence is evident. *B*: high-power view (×60, ×5 zoom) of a proximal tubule, taken by 2-photon microscopy. Fluorescein, instead of SF, but no dextran or Hoechst dye, was infused intravenously. Fluorescein was present diffusely in the cell, and there was no indication of uptake into vesicles (punctate fluorescence). Scales = 10 μm.

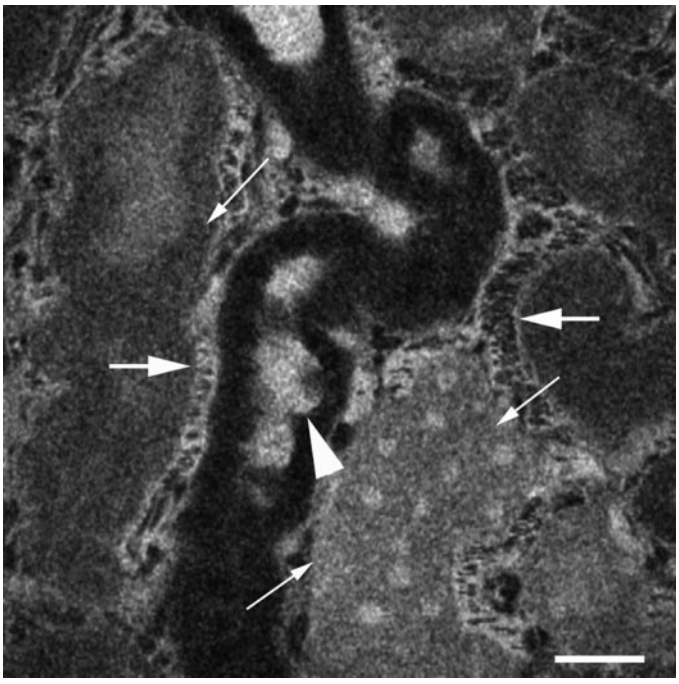

Fig. 6. Effect of probenecid on accumulation of SF in the normal rat kidney. A distal tubule is seen coursing down the center of the field; part of its lumen is visible (arrowhead). Blood vessels are indicated by thick arrows. SF fluorescence intensity, expressed as a percentage of the plasma SF fluorescence intensity, is 8, 35, and 57%, respectively, for the distal tubule cells and proximal tubule cells on the *left* and *right* (thin arrows). Proximal tubule cell SF intensities after probenecid were reduced compared with values seen in the same animal before administration of this transport inhibitor. Scale = 30 μm.

punctate accumulation of SF was detected. When fluorescein, instead of SF, was administered, this organic anion accumulated in proximal tubule cells, but no punctate fluorescein fluorescence was observed (Fig. 5*B*).

Probenecid decreased the accumulation of SF in normal proximal tubule cells (Fig. 6). The proximal tubule cell cytoplasm/plasma SF ratio averaged 2.67 ± 1.11 (n = 21) before and 0.64 ± 0.40 (n = 64) after probenecid administration ($P <$ 0.001). Ureteral occlusion for 20–40 min in two Munich-Wistar rats resulted in increases in proximal tubule cell cytoplasm/plasma and lumen/plasma SF intensity ratios (Table 2). The lumen SF fluorescence remained below the cytoplasm SF fluorescence, but the lumen/cell ratio was significantly increased with ureteral occlusion.

The outer diameter of proximal tubules in normal kidneys averaged 54 ± 6 μm (n = 92). In the rats with cystic kidneys, we defined a cyst as an epithelial structure having an outer diameter more than ×1.5 that of an average normal proximal tubule, i.e., >81 μm.

In cystic kidneys, most cysts accumulated SF, supporting their proximal tubule origin. As we previously reported (25), surface cysts in the Han:SPRD rat are almost invariably connected to functioning glomeruli. This is seen in Fig. 7, where rhodamine-dextran 10,000, a compound that is filtered but not secreted, is seen in the cyst lumen. Uptake of SF in cyst cells was uneven; some cells or groups of cells showed greatly diminished uptake (Fig. 8*A*). A few cysts (Fig. 8*B*) failed to accumulate SF in the cell cytoplasm; these "nontransporting cysts" were probably of distal tubule origin (see DISCUSSION). The uneven nature of SF uptake by proximal cysts was confirmed by through-focus imaging of cysts; this revealed that cells with impaired SF accumulation sometimes formed patches next to cells with more normal SF uptake (Fig. 9).

Peritubular capillary plasma SF fluorescence averaged 30 ± 13 (n = 190; range 9–63) intensity units in eight normal rats under control conditions. Duplicate measurements differed, on average, by 4.6 ± 4.4 (n = 44) fluorescence intensity units. We did not attempt to determine absolute plasma SF concentrations because the fluorescence intensity depends on *1*) the laser power output and *2*) depth of focus in the tissue. These two factors should not affect the cell cytoplasm/plasma or lumen/plasma SF fluorescence ratio in a given image, however, and so our measurements (Table 3) are expressed as a ratio.

Table 3 summarizes our quantitative measurements of SF fluorescence intensity in normal and cystic kidneys of Han:SPRD rats. In normal kidneys, we found that steady-state SF fluorescence in the cell cytoplasm of proximal tubules was usually higher than in the surrounding peritubular capillary blood plasma (average cytoplasm/plasma fluorescence ratio = 2.7 ± 1.4, n = 99, with a range from 0.4 to 6.5). Low ratios probably are from measurements made in the early proximal convoluted tubule segments. The proximal tubule lumen/plasma fluorescence ratio averaged 1.5 ± 1.3, n = 50, with a range from 0.01 to 6.9. Cell cytoplasm SF fluorescence intensity exceeded lumen values in all but one of 50 tubules.

SF was virtually absent from distal tubule cells but highly concentrated in the tubule lumen due to secretion by more proximal segments and water reabsorption. The distal cell/plasma SF fluorescence ratio averaged 0.2 ± 0.2 (n = 17) in normal kidneys, with a range from 0.0 to 0.5 (Table 3). These low values are consistent with the absence of SF transport by distal tubule cells. The distal cell lumen/plasma SF fluorescence ratio averaged 5.7 ± 2.4 (n = 21), with a range from 2.6 to 10.8. These data underestimate the extent of SF concentration in distal tubule lumens, because we excluded many measurements where the system was saturated by intense fluorescence.

DISCUSSION

The two-photon microscope, with its elimination of out-of-plane fluorescence, permits much more clear localization of fluorescent molecules in biological samples than conventional epifluorescence microscopy. Compared with confocal micros-

Table 2. *SF fluorescence intensity ratios before and 20–40 min after ureteral occlusion*

	Before Ureteral Occlusion (n=15)	After Ureteral Occlusion (n=29)	P Value
Proximal tubule cell cytoplasm/plasma	1.57±0.64	2.54±1.11	<0.005
Proximal tubule lumen/plasma	0.79±0.40	1.86±1.13	<0.001
Proximal tubule lumen/proximal tubule cell cytoplasm	0.50±0.10	0.70±0.15	<0.001

Values are means ± SD (number of measurements) from 2 Munich-Wistar rats.

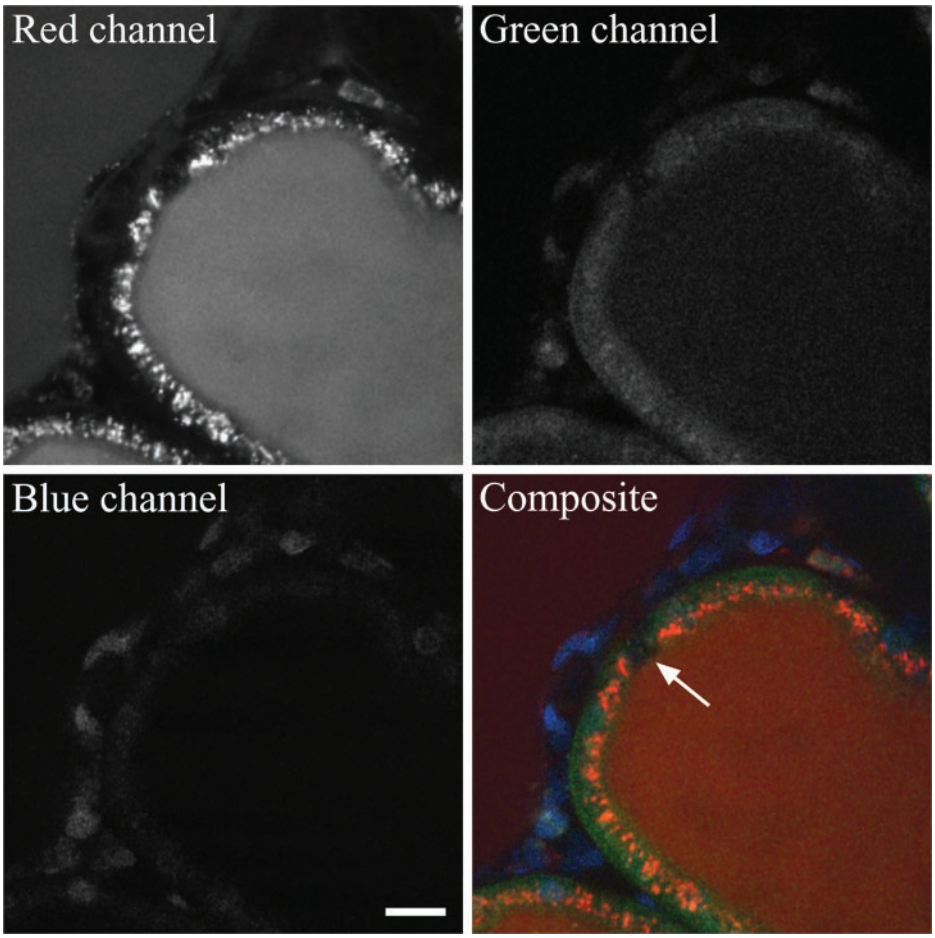

Fig. 7. A superficial cyst from a 6-mo-old Han:SPRD rat, taken with the ×60 objective, zoom function ×2. In this early experiment, bolus injections of Hoechst dye + dextran 10,000-rhodamine and SF were given 49 and 14 min, respectively, before these images were recorded. The cyst lumen contains concentrated dextran solution, suggesting that this cyst was connected to a functioning glomerulus. The dextran is found in endosomes in the cell cytoplasm. No punctate (or vesicular) fluorescence for SF (green channel) is apparent. One cell (arrow) that failed to take up SF also shows no evidence of dextran endocytosis. Scale = 15 μm.

copy, two-photon microscopy has the advantages of reduced photobleaching and improved observation of structures that may be many microns below the kidney surface (9, 22, 27). Previous attempts to localize SF accumulation in rat kidneys to cells or lumen using conventional epifluorescence microscopy had a low resolution, and it was not evident whether the SF was primarily in the cells or the tubule lumen (1, 23, 25). With two-photon microscopy, we can clearly distinguish accumulation in the cell and lumen and even within intracellular compartments.

Our in vitro measurements demonstrate that SF fluorescence intensity is linearly related to SF concentration. They also reveal a shortcoming, however, in that SF fluorescence is pH dependent at an excitation wavelength of 800 nm. We were unable to identify an excitation wavelength in the near-infrared range at which SF fluorescence was less pH sensitive.

In the normal proximal tubule, the effect of pH on SF fluorescence is apparently modest, compared with the steady-state concentration differences that develop. The pH of efferent arteriolar blood on the surface of the rat kidney is 7.27, lower

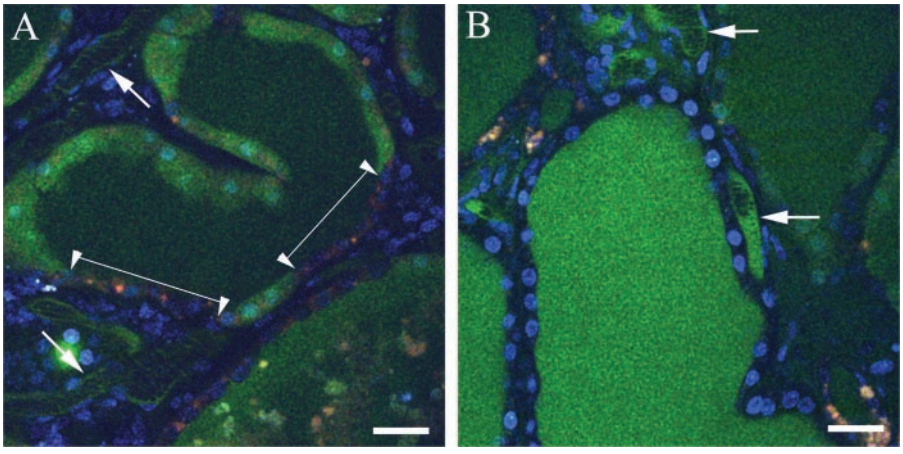

Fig. 8. *A*: superficial cyst from a 5-mo-old Han:SPRD rat, taken with the ×60 objective. Most cyst epithelial cells accumulated SF, but there are noticeable gaps in uptake (arrowheads). The cells that fail to accumulate SF appear to have a reduced cell height. Two blood vessels are indicated by arrows. *B*: "nontransporting cyst" from a 7.5-mo-old Han:SPRD rat. Such cysts were encountered much less frequently than cysts that accumulated SF intracellularly. The cell SF intensity is extremely low, the nuclei are especially bright (Hoechst dye), and there is little intrinsic fluorescence, all of which suggest that this cyst is derived from a distal tubule segment. The luminal SF intensity is equal to that of plasma SF. Arrows point to capillaries. Scales = 30 μm.

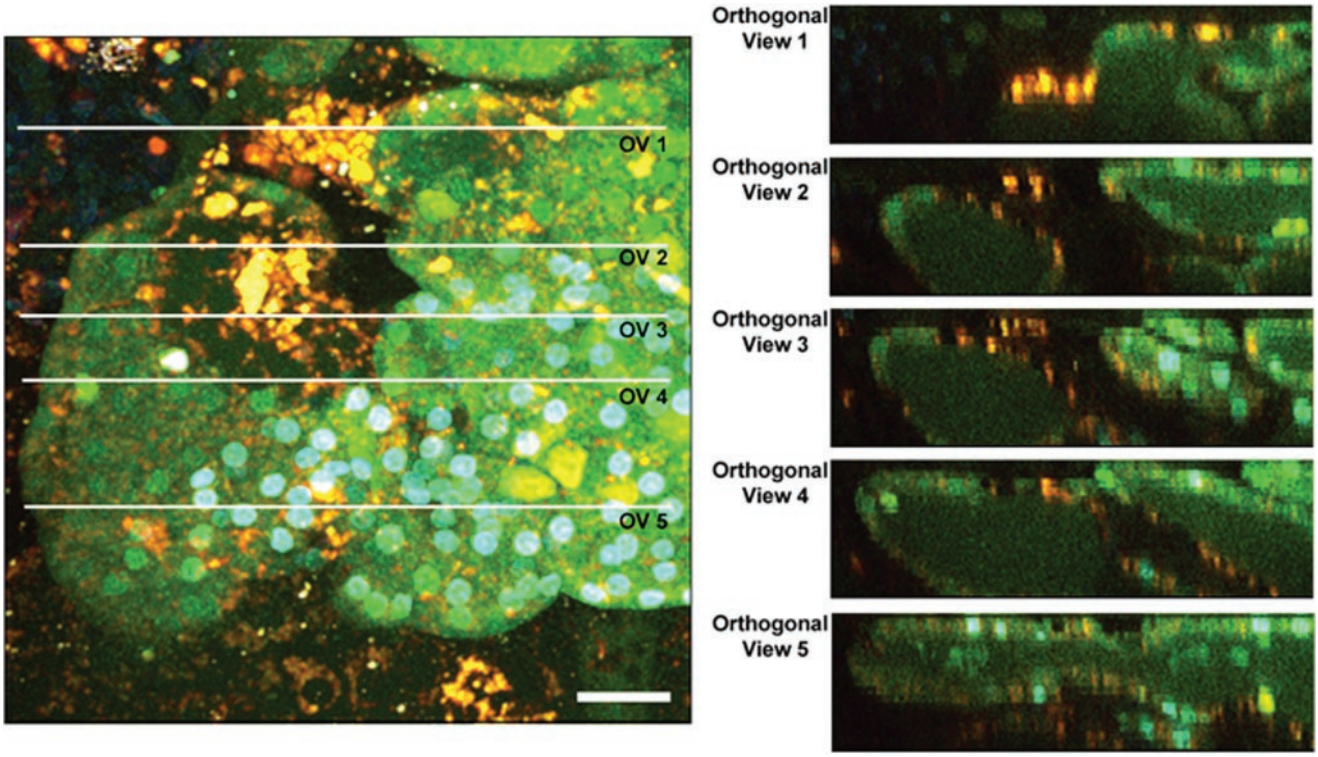

Fig. 9. A superficial cyst from a 5-mo-old Han:SPRD rat. *Right*: orthogonal views (OVs) were obtained by through-sectioning through the cyst in 1-μm steps. *Left, top left*: dark patch of cyst epithelial cells shows greatly diminished uptake of SF, considerable intrinsic (yellow/orange) fluorescence, and diminished accumulation of Hoechst dye in cell nuclei. The OVs show these cells as dark regions adjacent to cells showing the green fluorescence of SF. Continuity of the cyst lumen is seen in *OV 5*. Scale = 30 μm.

than systemic arterial blood, owing to an elevated P_{CO_2}, due largely to metabolic CO_2 production and countercurrent exchange (8). The pH of luminal fluid in the early proximal tubule is 7.06 and 6.70 at the end of the proximal convoluted tubule (8). Pastoriza-Munoz et al. (18) reported average pHs in early, mid, and late proximal tubule cell cytoplasm of 7.1, 7.0, and 6.9. Because we expressed our measurements as a lumen/plasma or cell/plasma SF fluorescence intensity ratio and did not measure pH, the ratios we report probably underestimate SF concentration ratios by ~6% in an early proximal tubule cell with a pH of 7.1 and by ~25% in the late proximal tubule lumen with a pH of 6.7, based on the relationship shown in Fig. 2. These errors are modest compared with the changes caused by uptake of SF by the cells and the concentration of SF in the lumen due to tubular SF secretion and water reabsorption.

Table 3. *Relative SF fluorescence intensity measurements*

	Normal Kidneys	Cystic Kidneys	*P* Value
Proximal tubule, cytoplasm	2.7±1.4 (*n*=99)	1.8±1.5 (*n*=23)	<0.02
Proximal tubule, lumen	1.5±1.3 (*n*=50)	0.8±0.8 (*n*=11)	NS
Distal tubule, cytoplasm	0.2±0.2 (*n*=17)	0.0±0.1 (*n*=4)	NS
Distal tubule, lumen	5.7±2.4 (*n*=21)	6.4±4.3 (*n*=5)	NS
Proximal cyst, cytoplasm		1.2±0.9 (*n*=40)	
Proximal cyst, lumen		0.7±0.3 (*n*=20)	
"Nontransporting" cyst, cytoplasm		0.2±0.1 (*n*=8)	
"Nontransporting" cyst, lumen		1.2±0.4 (*n*=8)	

Values are means ± SD (number of measurements) expressed as the ratio to plasma SF fluorescence in adjacent blood capillaries. Data are from Han:SPRD rats.

Our observations that normal proximal tubule cells accumulate SF and that this accumulation is reduced by probenecid are consistent with the classic model for secretion of organic anions, such as PAH, in the mammalian kidney (3). The active uptake step occurs at the peritubular cell membrane via an anion-α-ketoglutarate exchanger and is competitively inhibited by probenecid. Movement of organic anions from cell to lumen is downhill. The nature of the luminal membrane step in the rat kidney is controversial (3, 26). Ullrich and Rumrich (26) concluded that it is not influenced by electrical gradients and appears to involve an anion exchanger. Passive movement down a concentration gradient is consistent with our observation that proximal tubule cell SF fluorescence intensities were almost invariably higher than luminal SF fluorescence intensities. The flow of fluid through the proximal tubule lumen appears to sweep away secreted SF. This is supported by the finding that when the ureter was blocked, and therefore flow in the proximal tubule was diminished, the cell SF rose and the cell-to-lumen gradient was diminished. The very low levels of SF in distal tubule cells are consistent with the absence of a secretory mechanism for organic anion transport in this part of the nephron.

The status of intracellular organic anions has been the subject of several recent studies. Miller, Pritchard, and coworkers (13, 14, 20), using confocal microscopy, reported that fluorescein is compartmentalized within rabbit and teleost proximal tubule cells, cultured opossum kidney cells, and crab

urinary bladder epithelial cells. They saw punctate fluorescence in structures with an apparent size of ~1–10 μm in the cell cytoplasm and suggested that these structures may be vesicles engaged in transcellular movement of fluorescein. Other in vitro studies (12) in rat kidney tubule cells failed to reveal an accumulation of fluorescein in vesicular structures but reported that fluorescein was taken up by mitochondria. In contrast to these reports, we found no evidence for punctate cytoplasmic fluorescence of SF or fluorescein (Fig. 5*B*) in rat proximal tubules in vivo, despite our ability to detect punctate fluorescence with dextrans (Figs. 5*A* and 7). Low-molecular-mass dextrans are filtered, endocytosed by proximal tubule cells, and are incorporated into endosomes (11).

Recent observations by Dantzler (5) may explain our failure to observe punctate SF fluorescence in the in vivo rat kidney. They observed that in rabbit proximal tubules in vitro, fluorescein only appears in punctate compartments in the cytoplasm when a bicarbonate-free (Tris) buffer is used, but not when a more physiological bicarbonate/CO_2 buffer is used. Most of the earlier studies that reported punctate fluorescein fluorescence were done on renal or urinary bladder epithelial cells incubated in Tris-buffered media (13, 14). Our findings support the idea (5) that accumulation of organic anions in intracellular compartments may not be a factor in secretion by mammalian proximal tubules under physiological conditions.

We observed that SF fluorescence intensity in nuclei exceeded that in the cytoplasm of the same cells, on average by 34%. This result could be a consequence of binding of SF to nuclear constituents, but more likely is largely explained by a higher pH in the nucleus. The nucleoplasm is usually more alkaline (e.g., pH 7.6 to 7.8) than the cytoplasm in plant and animal cells (7). If we assume a cell cytoplasm pH of 7.1 and a nuclear pH of 7.7, then this should result in a 23% higher fluorescence intensity in the nucleus, assuming that the SF concentrations in cytoplasm and nucleus are really the same and we can apply the relationship shown in Fig. 2. Surprisingly, Pritchard and Miller (20) observed, by confocal microscopy, that fluorescein is actually excluded from the nuclei of rabbit proximal tubule cells in vitro.

Accumulation of SF in proximal tubule cells might be considered to be modest, because the cell cytoplasm/peritubular capillary plasma fluorescence ratios averaged only about 3 (Table 3). SF, however, is heavily bound to plasma proteins, and so the free plasma SF concentration is only ~12–15% of the total SF concentration. If we assume that intracellular SF is free, then the free SF concentration ratio for cell/plasma is ~20:1. Measurements of SF binding by cytoplasmic proteins have not been reported, but many other secreted organic anions appear to be extensively bound within cells (20). Hence, we can only place an approximate upper limit (20:1) on the concentration gradient developed by SF secretory transport across the peritubular cell membrane of proximal tubule cells. In in vitro studies of isolated rabbit kidney proximal tubules, Sullivan et al. (24) observed much higher accumulation of fluorescein. Steady-state cell concentrations were more than 100× those of the bathing medium, when the medium concentration was ~5 μM. Differences between SF and fluorescein transport, species differences, or the absence of tubule fluid flow in the collapsed isolated tubules may explain why greater accumulation was observed in their studies.

Plant lectins have often been used to identify the site of origin of cysts in PKD kidneys (4, 10, 19, 21) but are not suitable for in vivo use because they cause agglutination of red cells. SF accumulation is a convenient in vivo marker for proximal tubules, because only cells derived from this nephron segment secrete this molecule. In the cystic kidneys, proximal tubules showed a diminished intensity of SF accumulation compared with normal kidney proximal tubules (Table 3). This decrease might reflect, in part, accumulation of competing organic anions in the plasma of rats with impaired renal function. Most cysts accumulated SF, supporting the conclusion from histochemical studies that most cysts are derived from proximal tubules in the Han:SPRD rat (4, 21).

The accumulation of SF in proximal cyst epithelial cells was quite variable. Sometimes there was a single cell without SF fluorescence adjacent to cells with normal SF fluorescence (Fig. 7). Other times, there were stretches or patches of cells where SF fluorescence was greatly diminished or absent, with normal SF fluorescence in adjacent cells (Figs. 8*A* and 9). The cells with low-fluorescence intensity often appeared to have a reduced cell height and sometimes showed an increased density of particles with "orange" (intrinsic) fluorescence. In experiments in which dextran had been injected intravenously, we observed that proximal cyst cells that failed to endocytose dextran also did not accumulate SF, suggesting a broad deficiency of transport functions. Obermüller et al. (16, 17) observed a patchy distribution of enzymes and transporters (alkaline phosphatase, Na-K-ATPase, aquaporin-1, and NaSi-1 cotransporter) in proximal cysts and found that many cyst cells lost the ability to endocytose filtered dextrans in the Han:SPRD rat. They concluded that cyst epithelial cells did not have an altered polarity but seemed to lose completely the markers they studied, indicating a loss of the normal state of cell differentiation. Several electron microscopic studies (4, 21, 25) noted that normal and poorly differentiated epithelial cells are found in the same cyst in the Han:SPRD rat with ADPKD. Nagao et al. (15) recently reported that patches of abnormal epithelial cells, expressing high levels of phosphorylated ERK, are found together with more normal appearing cells in the same cyst. We consider it likely that decreased accumulation of SF in proximal cyst cells reflects loss of this specialized transport function and a more primitive phenotype. Adjacent dedifferentiated cyst cells may be the progeny of a single altered cell or could have resulted from an abnormal local condition that affects several nearby cells.

A few cysts showed luminal SF fluorescence close to that of plasma and very little intracellular SF fluorescence in any of the lining cyst cells. We refer to these as "nontransporting" cysts (Table 3 and Fig. 8*B*). These cysts, conceivably, may have been originally derived from proximal tubules and lost completely their ability to accumulate SF intracellularly. More likely, however, they are derived from distal tubule segments. We base this suspicion on the observations that these cells lack the intrinsic orange fluorescence sometimes found in proximal, but not in distal, tubule cells and that their nuclei stain especially brightly with Hoechst dye, similar to distal cells. The luminal SF fluorescence intensity was similar to that of plasma, suggesting that there was no uphill transport of SF.

The in vivo measurements of glomerular filterability of SF in Munich-Wistar rats suggest that 85% of plasma SF is bound to nonfiltered proteins in plasma. Equilibrium dialysis measure-

ments of SF binding in vitro indicated that 88% of SF is bound in rat plasma. The agreement between these values supports the reliability of these measurements and suggests that two-photon microscopy may be a good method to study glomerular permeability.

In conclusion, this study quantified the renal handling of a fluorescent organic molecule, SF, in the living rat kidney. We determined the glomerular filterability of SF by direct observations in Munich-Wistar rats. We demonstrated that SF accumulates in proximal, but not distal, tubule cells in the normal rat kidney. A transport inhibitor, probenecid, lowered intracellular SF levels, whereas ureteral obstruction resulted in higher intracellular SF levels. In rats with ADPKD, the accumulation of SF by cyst epithelial cells provided an in vivo marker for cysts derived from proximal tubules and confirmed the proximal origin of most cysts. SF accumulation, however, was patchy, suggesting that some cyst cells have lost the specialized ability to secrete SF and may express a dedifferentiated phenotype. The two-photon microscope is a powerful tool for studying both glomerular and tubular functions of the kidney in vivo.

ACKNOWLEDGMENTS

We thank Drs. M. Steinhausen and N. Parekh for the gift of 100 mg SF and Drs. C. Phillips and J. Tanner for critiquing the manuscript.

GRANTS

This research was supported by a National Institutes of Health O'Brien Center award to Dr. B. A. Molitoris, by a National Kidney Foundation of Indiana award to Dr. G. Tanner, and by a grant (Indiana Genomics Initiative) from the Lilly Endowment to the Indiana University School of Medicine. The studies were conducted at the Indiana University Center for Biological Microscopy.

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