Tissue Concentration and Urinary Excretion Pattern of Sulfofluorescein by the Rat Kidney

U. Ammer, Yu Natochin, and K.J. Ullrich

ABSTRACT
In order to find a fluorescein analog that is excreted in a way similar to p-aminohippurate (PAH) and is suitable to register excretion into the urine and also to monitor continuously the concentration within cortical tissue, the interference of fluorescein, sulfofluorescein (SF), and fluorescein-5(6)-sulfonate with the contraluminal transport systems of PAH, succinate, and sulfate and with the luminal transport system of sulfate and lactate was evaluated. All three substances exerted a strong inhibitory potency against contraluminal PAH uptake (apparent $K_i$, 0.06 to 0.1 mmol/L) and also showed a moderate to small inhibitory potency against contraluminal sulfate transport (apparent $K_i$, 0.7 to 5.3 mmol/L). None of the three substrates interacted with the contraluminal dicarboxylate transport. Luminally, fluorescein and SF interacted with the lactate transporter (apparent $K_i$, 3.0 mmol/L), whereas SF and fluorescein-5(6)-sulfonate had a very weak inhibitory potency against luminal sulfate transport (apparent $K_i$, 30 to 40 mmol/L). Because of its relatively low interference with the contraluminal sulfate transport, we preferred SF in the following study (protein binding, 88%) over fluorescein-5(6)-sulfonate (protein binding, 43.3%) and fluorescein (protein binding, 77.3%). A bolus injection of SF was given together with (14C)inulin into the jugular vein to rats in mannitol diuresis. Excretion of both substances in the urine was measured in 5-mm samples. Fluorescence of the kidney surface was monitored at the exposed kidney with a photocell (excitation light, 470 nm; emission light, 530 nm). The effect of interfering substances was evaluated by their application 1 min before, simultaneously, or 1 min after SF bolus injection. PAH < probenecid < apalillin inhibited SF excretion in the urine and, to a similar degree, SF fluorescence in cortical tissue. Inhibition was strongest when the substances were given simultaneously with SF. With the injection of α-ketoglutarate, glutarate, and succinate, an increase of SF excretion in the urine and, partially also, of SF content in tissue was seen. Tetrafluorosuccinate and mercaptosuccinate inhibited urinary SF excretion and tissue fluorescence strongly. Therefore, SF secretion is completely inhibited whereas filtration remained unaffected. The injection of acetate, lactate, and pyruvate as well as of 2-chloropropionate and thiosulfate did not change urinary excretion and tissue fluorescence of SF significantly. In SF-preloaded animals, the injection of α-ketoglutarate, glutarate, and succinate caused an immediate decrease of cellular SF content, whereas the injection of glutarate caused an increase. SF fluorescence in tissue was linearly related to urinary excretion of SF, whereas urinary excretion of inulin was barely affected. Thus, measurement of SF excretion into the urine together with SF fluorescence in cortical tissue is a useful tool with which to study the interaction of substrates with the PAH transport system.

Key Words: p-Aminohippurate, dicarboxylate transport, fluorescein, protein binding, intermediates of cellular metabolism

One of the most important functions of the kidney is the excretion of xenobiotics, i.e., chemicals and drugs (1–5). Disposal of these substances by the urine is due to glomerular filtration and to secretory or reabsorptive transport by the organic anion and cation transport systems in proximal tubular cells. In recent years, special interest was paid to the relationship between chemical structure and interaction of the transported substances with the contraluminal transport systems for organic anions (p-aminohippurate [PAH], succinate, sulfate/oxalate) and organic cations (tetraethylammonium, N'-methylnicotinamide, TEA) (6). This approach helps us to
understand the first and possibly rate-determining step of organic anion and cation secretion, i.e., the uptake into proximal tubular cells. Because of overlapping specificities of the transport systems and involvement of the intracellular compartment, it is yet impossible to understand the complex interactions in overall transcellular secretary or reabsorptive transport. The main interacting transport systems are those of PAH and of dicarboxylates (7). The best approach would be to measure the overall transport rate of PAH and \( \alpha \)-ketoglutarate in relation to their intracellular concentrations. Because the methods for such an approach are not available at the moment, we looked for fluorescent substances, which show transport parameters similar to those of PAH and dicarboxylates. In this article, we report on fluorescein analogs that fulfill the first requirement and are able to show the interaction with inhibitors of the PAH transport system and with dicarboxylates.

**METHODS**

**Chemicals Used**

Apalclillin (Lumota\(^\text{\textregistered}\)) was obtained from Thomae (Biberach, Germany). Probenecid, PAH, glutarate, \( \alpha \)-ketoglutarate, mannitol, and BSA were purchased from Sigma (Deisenhofen, Germany). Tetrafluoresuccinate (TFS) was a gift of the Hoechst AG (Frankfurt, Germany). Acetate, thiosulfate, and succinate were obtained from Merck (Darmstadt, Germany). Mercaptosuccinate and 2-chloropropionate were purchased from EGA Chemie (Steinheim, Germany). Pyruvate was purchased from Boehringer (Mannheim, Germany); lactate was purchased from Serva (Heidelberg, Germany). Sulfofluorescein (SF) was a gift from Prof. M. Steinhausen (Heidelberg, Germany) (prepared as described in reference 8). \( [^{14}\text{C}] \text{Inulin} \) was purchased from Du Pont (NEN) (Dreieich, Germany). Fluorescein-5(6)-sulfonate was obtained from Molecular Probes (Eugene, OR).

**Preparation of the Animals**

The fluorescein experiments were performed on male Wistar rats (Winkelmann, Kirchborchern, Germany) of 180 to 200 g body wt fed on Altromin (Lage, Germany) standard diet and tap water. The rats were anesthetized by the injection of Inactin (Byk Gulden, Konstanz, Germany) (120 to 150 mg/kg body wt ip) and by being mounted on a heated operating table with thermostatic control set at 37°C. An incision was made to cannulate the urinary bladder with a polyethylene catheter for the collection of urine samples. A second incision was made to cannulate the jugular vein to infuse mannitol and test solutions. Afterwards, the left kidney was prepared and the surrounding fascia was removed. The exposed kidney was immobilized in a plastic cup, resting on cotton wool. The surface was kept moist by superfusion with saline solution.

**Determination of the Apparent \( K_i \) Values of the Used Test Substances**

In stop-flow peritubular capillary perfusion experiments, the transport of organic anions (\( [^{3}\text{H}] \text{PAH} \), \( [^{14}\text{C}] \text{succinate} \), \( [^{35}\text{S}] \text{sulfate} \), and \( [^{14}\text{C}] \text{lactate} \) and organic cations (\( [^{3}\text{H}] \text{N}^{+}-\text{methyl nicotinamide} \) into proximal tubular cells was measured (6). The kidney of an anesthetized rat was separated from the surrounding fascia, and the capsule was removed. Then, the kidney was immobilized and covered with paraffin oil heated to 37°C. The renal artery and vein were clamped, and the tubules collapsed because the luminal fluid was reabsorbed and glomerular filtration ceased. Immediately thereafter, a thick superficial capillary was impaled by an oil-filled sampling pipette for sample collection. At a distance of 100 to 140 \( \mu \text{m} \) from this glass capillary, another blood vessel was punctured with the filling pipette. Through the filling pipette, a rapid injection of an isotonic solution containing different concentrations of radiolabeled substances and \( [^{14}\text{C}] \text{Inulin} \) as extracellular space marker was made. After 1 to 4 s, the test solution was withdrawn into the sampling pipette and the disappearance of the labeled substance could be determined. The apparent \( K_i \) values were evaluated, assuming competitive inhibition as described elsewhere (9).

**Measurement of Tissue Fluorescence**

The fluorescence equipment was developed by Mr. V. Rohlicek in our institute and consists of three units: light source, detection unit, and recording equipment. (1) For the excitation of the SF fluorescence on the kidney surface, a halogen lamp (GBD 6433; Phillips, Kassel, Germany) was used. The light was filtered by four optical filters: a heat absorption filter (Spindler, Göttingen, Germany), a dichroic filter blue (Deutsche Nichimen, Düsseldorf, Germany), a dichroic filter magenta (Deutsche Nichimen), a condenser (Spindler), and a BG 1 filter (Deutsche Nichimen). The resulting light has a wave length of 470 nm and could be guided through fiber optics on the kidney surface. (2) The emission was detected at 530 nm with a photocell (Siliconide; Hamamatsu Photonics, Herrsching, Germany) with a BG 18 filter (Deutsche Nichimen). (3) The detected signal was amplified and plotted on a chart recorder (Abimed, Langenfeld, Germany).

The fluorescence equipment was fixed 1 cm above the surface of the kidney. Changes in kidney tissue fluorescence intensity (excitation at 470 nm; emiss-
sion measured at 530 nm) could thus be continuously registered.

**Fluorescence Experiments**

Experiments were started by the induction of an osmotic diuresis with a starting dose of 1 mL of 20% mannitol solution infused within 5 min, followed by a continuous infusion of 10% mannitol solution with 5% BSA at an infusion rate of 3 mL/h. After 10 min, a constant diuresis was reached with a urine flow rate of 0.06 to 0.08 mL/min. Then, 0.125 mL of the test solution containing 0.98 mmol/L of SF and 0.09 mmol/L of [14C]inulin was injected and urine samples were collected at 5-min intervals, while the mannitol infusion was continued. Twenty-five to 30 min after the first, i.e., control, injection, a second iv injection of inulin and SF was made and the following interfering substrates: apalacillin, probenecid, PAH succinate, TFS, glutarate, α-ketoglutarate, mercaptosuccinate, 2-chloropropionate, and thiosulfate (100 mmol/L in a 0.125-mL bolus) were given. 1 min before, simultaneously, and 1 min after the SF/inulin injection.

**Measurement of SF and [14C]Inulin in Urine Samples and Calculation of Normalized Excretion Rates**

Urinary SF measurements were carried out on a spectrofluorometer (Shimadzu RF-510; Gamma Analysetechnik, Bremerhaven, Germany). Excitation and emission wavelengths were 470 and 530 nm, respectively. The samples were prepared from 0.1 mL of the original urine sample and 0.1 mL of 0.1 N NaOH and were filled up to a volume of 3 mL with bidistilled water. Fluorescence intensities of the samples were calibrated with SF-containing standards. Urinary [14C]inulin concentration was measured in a Kontron Betamatik scintillation counter (Düsseldorf, Germany). Ten microliters of the original urine sample was diluted in 100 µL of bidistilled water and 5 mL of Picofluor 15 (Packard, Frankfurt, Germany) as scintillation fluid. The excretion of [14C]inulin and SF beginning from the time of the first bolus injection to minute 25 (= five urine collection periods) was used as control (= 100%) (Figure 1). In all experiments, 25 min after the first injection, a second injection of [14C]inulin/SF with additional inhibitors was made. Inhibitors were injected either 1 min before, simultaneously with, or 1 min after SF. For the second bolus injection, the measured and calculated urinary excretion had to be corrected for the amount of [14C]inulin and SF that was still being excreted because of the first injection of SF and inulin. This was done by extrapolating the urinary excretion rates and subtracting them from the following excretion rates. After correction of the excretion rates of the second bolus injection, it could be related to that of the first injection. In each 5-min urine sample, the amounts of [14C]inulin and SF were calculated and the values of the five samples after iv injection were summed up and related to the amount of [14C]inulin and SF injected (normalized excretion rates for [14C]-inulin, 54.2 ± 0.6%; for SF, 21.9 ± 0.5% under control conditions; Figure 2A).

**Calculation of Excretion Fraction (SF Excretion:Inulin Excretion)**

The calculation of the ratio was done by using the normalized 25-min excretion rates of SF and inulin. In Figure 2B, the calculated ratios of the control condition are shown (mean value, 0.4 ± 0.001). The same ratio is shown in Figure 2C, including the correction for plasma protein binding of SF. The mean value of 3.4 ± 0.06 indicates that the amount of secreted SF is 3.4 times the amount of filtered SF.

Because the peak of SF excretion in urine comes later than the peak of [14C]inulin, we correlated the normalized SF excretion rates of samples 3 to 5 with that of [14C]inulin in the preceding urine samples 2 to 4. These rates showed for one injection only a very small scatter. The excretion fraction of SF calculated in this way was within the mean of 0.38 ± 0.005 (Figure 2B). In Figure 2C, the data were corrected for the plasma protein binding of SF, i.e., mean value of 3.2 ± 0.04. Because the values for the excretion fraction gained from both methods deviate only very little from each other, we give in Tables 1 and 2 only that of method 1.

![Figure 1. Bolus injection of 122.5 nmol of SF plus 12.1 nmol of (14C)inulin into the jugular vein. The time curve of fluorescence intensity of renal tissue and the urinary excretion of both substances is shown: tissue fluorescence in arbitrary (arb) units and urinary excretion as ratio of excreted amount in each 5-min urine sample divided by the injected amount. SF values are not corrected for protein binding. t, time.](image-url)
Figure 2. (A) Urinary excretion of inulin and SF, after injection in the jugular vein, summed up for over 25 min, and related to the injected (inj) amount of inulin and SF. SF values are uncorrected for plasma protein binding. (B) Excretion fraction of SF (i.e., SF excretion/inulin [In] excretion) as calculated from the values shown in panel A, i.e., summed up for 25 min or over 10 to 25 min, corrected for the delay of SF excretion compared with that of inulin (see Methods) on the right side of panel B. (C) Excretion fraction of SF with correction for protein binding (values shown in panel B).

TABLE 1. Urinary excretion of bolus-injected fluorescein analogs

<table>
<thead>
<tr>
<th>Condition</th>
<th>Inulin Excreted Over 25 min (% of Injected)</th>
<th>Test (a,b) versus Control (c) (x100)</th>
<th>Dye Excreted Over 25 min (% of Injected)</th>
<th>Test (a,b) versus Control (c) (x100)</th>
<th>Excretion Fraction: (Dye Excreted) F (Inulin Excreted)</th>
<th>Test (a,b) versus Control (c) (x100)</th>
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<tbody>
<tr>
<td>Animal a</td>
<td>58.1</td>
<td>100</td>
<td>23.6</td>
<td>85</td>
<td>1.79</td>
<td>44.3</td>
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<tr>
<td></td>
<td>b</td>
<td>58.6</td>
<td>100.9</td>
<td>28.5</td>
<td>102.6</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>57.3</td>
<td>98.6</td>
<td>27.8</td>
<td>100</td>
<td>4.04</td>
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<td>Animal b</td>
<td>53.4</td>
<td>100</td>
<td>27.2</td>
<td>72.4</td>
<td>0.89</td>
<td>20.9</td>
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<tr>
<td></td>
<td>a</td>
<td>60.8</td>
<td>113.8</td>
<td>34.0</td>
<td>90.6</td>
<td>2.47</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>73.5</td>
<td>137.6</td>
<td>37.5</td>
<td>100</td>
<td>4.25</td>
</tr>
</tbody>
</table>

* a, 122.5 nmol of fluorescein; b, 122.5 nmol of fluorescein-5(8)-sulfonate; c, 122.5 nmol of SF (= control). To each injection, 12.1 nmol of (14C)inulin was added. 1, 2, and 3, time course of the injections. F, factor to correct the data for protein binding: a, 4.41; b, 1.76; c, 8.3.

Evaluation of the Tissue Concentration of SF

The content of SF in tissue was related to the maximal fluorescence intensity (H) in tissue. The maximal fluorescence content was normally reached 1 min after the injection of SF and is given in arbitrary units, i.e., millimeters of excursion on the ink writer. Furthermore, the area under the fluorescence curve was used to quantify the tissue content of SF. Therefore, the area was integrated for 5 min, beginning from the time of increased fluorescence intensity to the intensity 5 min later. The area under the curve was given in arbitrary units (millimeter per minute). In addition, the half time ($t_{1/2}$) of SF intensity decay in renal tissue was determined.

Measurement of Plasma Protein Binding

Plasma protein binding of SF, fluorescein, and fluorescein-5(6)-sulfonate was determined by the method described by De Lange et al. (10). Rats were prepared and set under osmotic diuresis similar to the experiments. Five minutes after the injection of 122.5 nmol of fluorescent dye into the jugular vein, the blood of the animals was collected and the plasma was separated. Then, the plasma was centrifuged.
with ultra centrifugation units (Centricon-Tm30-units; Amicon, Witten, Germany). The concentrations of the fluorescent dyes in plasma and ultrafiltrate were determined by the method described above. The experiment was repeated after the animal was preloaded with 0.125 mL of 100 mmol/L apaceillin to study the effect of apaceillin on SF plasma protein binding. Plasma protein binding (P) was calculated according to the formula: P = (C - Cf)/C with C being the total dye concentration in plasma and Cf being the free dye concentration in the ultrafiltrate. Each test of protein binding, as described in the next paragraph, was performed on two rats. The two determinations deviated from each other by between 0.3 and 1.6 “absolute” %).

RESULTS

Plasma Protein Binding of the Tested Fluorescent Dyes

An intravenous injection of 122.5 nmol of SF resulted 3 to 4 min later in a total concentration in plasma of 2.8 nmol/L and in plasma ultrafiltrate of 0.3 nmol/L. The resulting plasma protein binding of SF was calculated to be 88.5%. After the iv injection of 122.5 nmol of fluorescein, the concentration of fluorescein in plasma was 1.65 nmol/L and the concentration in plasma ultrafiltrate was 0.38 nmol/L. The plasma protein binding was, in this case, 77.3%. The procedure was repeated by the injection of fluorescein-5(6)-sulfonate into the jugular vein. The concentration of that dye in plasma was 2.43 nmol/L and in the ultrafiltrate was 1.38 nmol/L. The calculated plasma protein binding for the fluorescein-5(6)-sulfonate was 43.4%. Preloading the animal with apaceillin (1.23 nmol) and the subsequent injection of SF into the jugular vein resulted in reduced plasma protein binding of 66%.

Urinary Excretion Patterns on the Tested Fluorescent Dyes

The determination of the excretion pattern of fluorescein, fluorescein-5(6)-sulfonate, and SF is shown in Figure 3 and Table 1. After the injection of the first bolus, which contained 122.5 nmol of fluorescein and 12.1 nmol of [14C]inulin, the increase of tissue fluorescence was seen within 1 min. The maximal content of fluorescence (H) was determined to be 20 arbitrary units and decreased with a half value time of 4.8 min. Five-minute integration of the area under the fluorescence curve in tissue resulted in an area of 78.1 arbitrary units × min. After 30 min, a second bolus injection was given containing 122.5 nmol of fluorescein-5(6)-sulfonate and 12.1 nmol of [14C]inulin. Again, the maximal fluorescence (H) in tissue was reached within 1 min, was determined to be 17 arbitrary units, and decreased with a t1/2 of 3.5 min. The integrated 5-min area of the tissue curve was 59.5 arbitrary units × min. Sixty minutes after the start of the experiment, the third bolus injection containing 122.5 nmol of SF and 12.1 nmol of [14C]inulin was injected into the jugular vein. The measured maximal tissue fluorescence (H) was 22 arbitrary units. The area under the fluorescence curve in tissue was measured to be 87.5 arbitrary units × min. The determination of the urinary excretion of the injected dyes is shown in the middle part of Figure 3. For all three dye injections, the excretion rate of inulin integrated for 25 min was in the same range (Table 1) and the excretion of each dye expressed as the percentage of injected dye was not very different. Calculation of the excretion fraction, when the protein binding is considered, gave quite different results. It turned out that the excretion fraction of fluorescein-5(6)-sulfonate is 0.9, i.e., this dye is excreted by

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**TABLE 2. Tissue content of the bolus-injected fluorescein analogs**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Tissue Fluorescence Maximal Height (mm)</th>
<th>Test (a,b) versus Control (c) (×100)</th>
<th>Decay of Tissue Fluorescence (t5) (min)</th>
<th>Test (a,b) versus Control (c) (×100)</th>
<th>Tissue Fluorescence Area (5 min) (mm/mm)</th>
<th>Test (a,b) versus Control (c) (×100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>a</td>
<td>20</td>
<td>90.9</td>
<td>4.8</td>
<td>78.1</td>
<td>89.3</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>17</td>
<td>77.3</td>
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<td>68.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>22</td>
<td>100.0</td>
<td>11.5</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td>100.0</td>
<td>10.2</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* a, 122.5 nmol of fluorescein; b, 122.5 nmol of fluorescein-5(6)-sulfonate; c, 122.5 nmol of SF (= control). 1, 2, and 3, time course of bolus injections.
given together with (14C)inulin. Tissue fluorescence intensity, nmol of fluorescein-5(6)-sulfonate, and 122.5 nmol of SF are shown. Arb, arbitrary; t, time.

Figure 3. Bolus injection of 122.5 nmol of fluorescein, 122.5 nmol of fluorescein-5(6)-sulfonate, and 122.5 nmol of SF given together with (14C)inulin. Tissue fluorescence intensity, urinary excretion pattern, and diuresis (urine volume/S mm) are shown. Arb, arbitrary; t, time.

In order to find a suitable concentration of SF for all further experiments, SF was given in different amounts into the jugular vein and the excretion pattern was determined. In Figure 4, the measured tissue fluorescence is shown. The determined maximal fluorescence intensities (H) were linearly related to the injected amount of SF. The half times of tissue fluorescence decay were the same for all three injections. The normalized urinary excretion of SF, i.e., related to the injected amount of SF, was almost identical among the three SF doses, which means that the excreted amount is linearly related to the injected amount. Plasma protein binding was, in all three injected SF doses, 88%. For all further experiments, we used an iv dose of 122.5 nmol of SF.

Determination of the Excretion Pattern of SF With Application of Interfering Substances

After a first bolus injection of SF together with inulin (i.e., control), the test injection followed. Hereby, the interfering substances were given iv 1 min before, simultaneously with, or 1 min after SF injection. The results calculated from the second injection were compared with those of the individual controls. In Figure 5, all tested interfering substances given under the three experimental conditions are summarized. Applied inhibitors were: PAH, probenecid, apalcilin (with specificity versus the PAH transport system, apparent K, values of these substances are given in Table 3), α-Ketoglutarate, glutarate, succinate, tetrafuorosuccinate, and mercaptosuccinate were given because of their affinity for both dicarboxylate and the PAH transporter. Furthermore, intracellular metabolites such as acetate, lactate, and pyruvate were tested. Finally, thiosulfate and 2-chloropropionate, having a specificity versus the transport systems of sulfate and lactate, were tested.

Inhibitors of the PAH Transport System. As shown in Figure 5, the inhibitors of the PAH transport system—PAH, probenecid and apalcilin—interact with the excretion of SF to a different degree: PAH (apparent K,0.08 mmol/L; apparent K,0.04 mmol/L) inhibited both urinary excretion and uptake of SF significantly when it was given simultaneously with SF. Urinary excretion of SF decreased by 32.4%. Urinary inulin excretion, however, was unchanged. Thus, the decrease of SF excretion is due to diminished SF secretion by the tubular cells. Tissue fluorescence (integrated area under the curve) fell by 31%, indicating an inhibition of SF uptake. The inhibitory potency of PAH on SF secretion was more or less not detectable when PAH was applied 1 min before or after SF.

Probenecid (apparent K,0.04 mmol/L) inhibited both urinary excretion and uptake of SF in tissue when given 1 min before or simultaneously with SF. In Figure 6, the effect of probenecid on the excretion pattern of SF is demonstrated when it was given 1 min before SF. Urinary inulin excretion was unchanged, whereas the excretion of SF decreased by 31% and the excretion fraction of SF decreased by 35%. Probenecid also reduced maximal tissue fluorescence by 36% and the integrated area of tissue fluorescence by 28%. When probenecid was given in SF-preloaded kidney tissue (Figure 6D; chasing ex-
 experiment), the tissue content of SF decreased instantaneously.

Apalicillin (apparent $K_{ICl}$, $0.02 \text{ mmol/L}$) exerted the strongest inhibition of urinary SF excretion and SF uptake in renal tissue, which was seen in all three tested conditions (Figure 5). In Figure 7, the injection of apalicillin 1 min before SF injection is shown in one experiment in which two control injections were given before the test injection. Urinary excretion of inulin and SF as well as tissue fluorescence was almost the same in the two controls. Subsequent injection of apalicillin caused a reduction of tissue fluorescence by 52%, whereas SF excretion in urine was reduced by 61% and the excretion fraction was reduced by 50%. In experiments in which apalicillin was given 1 min after SF, directly after its injection, a small increase of tissue fluorescence intensity occurred.

Inhibitors of the Dicarboxylate Transport System. As shown in Figure 5, the effect of dicarboxylates on urinary SF excretion and tissue content could be divided into two groups: ones that enhance urinary excretion and tissue uptake of SF, and others that reduce urinary SF excretion and its uptake into tissue.

$\alpha$-Ketoglutarate (apparent $K_{ICl}$, $0.03 \text{ mmol/L}$; apparent $K_{ICl}$, $0.08 \text{ mmol/L}$) (Table 3) given 1 min before or simultaneously with SF resulted in a slight increase in urinary SF excretion by 16 and 27%, whereas it had no effect when given 1 min after SF injection. On the other hand, there was a significant increase of tissue fluorescence seen only under the latter conditions. Under all conditions the excretion of inulin remained constant.

Under all experimental conditions Glutamate (apparent $K_{ICl}$, $0.05 \text{ mmol/L}$; apparent $K_{ICl}$, $0.16 \text{ mmol/L}$) had the strongest effect of enhancing SF excretion in the urine and SF accumulation in renal tissue. In Figure 8, the application of glutamate 1 min before SF injection is shown. The average tissue
TABLE 3. Interaction of the fluorescein analogs and substrates applied in this study with the contraluminal (cl) uptake of (3H)PAH (2-s contact time), (14C)succinate (1s), (14C)methylsuccinate (2s), (35S)sulfate (4 s) (luminal (l) values in brackets) (2s) (3H)N'-methylnicotinamide (NMeN; 4 s) and luminal (1) (14C)lactate (3.5 s) in cortical cells of proximal rat tubulesa

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PAH, cl</th>
<th>Succinate, cl</th>
<th>Methylsuccinate, cl</th>
<th>Sulfate, cl (l)</th>
<th>NMeN, cl</th>
<th>Lactate, l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td>0.06 ± 0.01</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>0.66 ± 0.18 (NS)</td>
<td>—</td>
<td>2.6 ± 0.42</td>
</tr>
<tr>
<td>Fluorescein-5(6)-sulfonate</td>
<td>0.1 ± 0.02</td>
<td>—</td>
<td>—</td>
<td>2.1 ± 1.1 (38.9)</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>SF</td>
<td>0.08 ± 0.02</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>5.3 ± 5.9 (27.7)</td>
<td>—</td>
<td>3.5 ± 0.42</td>
</tr>
<tr>
<td>Acetate (32, 37)</td>
<td>&gt;5 (NS)</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>4.1</td>
</tr>
<tr>
<td>PAH (35)</td>
<td>0.08</td>
<td>3.9</td>
<td>—</td>
<td>4.1</td>
<td>4.3</td>
<td>—</td>
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<tr>
<td>Apcillin (6)</td>
<td>0.02</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>&gt;10 (NS)</td>
<td>&gt;5 (NS)</td>
<td>—</td>
</tr>
<tr>
<td>2-Cl-propionate (32)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.04</td>
</tr>
<tr>
<td>Glutarate (33, 36)</td>
<td>0.05</td>
<td>0.24</td>
<td>0.16</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>α-Ketoglutarate (33, 36)</td>
<td>0.03</td>
<td>—</td>
<td>0.08</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lactate (31, 34)</td>
<td>&gt;5 (NS)</td>
<td>—</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>1.1</td>
<td>—</td>
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<tr>
<td>Mercaptosuccinate (32, 33)</td>
<td>0.77</td>
<td>0.28</td>
<td>0.37 (0.28)</td>
<td>0.37 (0.28)</td>
<td>21.6</td>
<td>—</td>
</tr>
<tr>
<td>Probenecid (6)</td>
<td>0.04</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>7.3</td>
<td>&gt;5 (NS)</td>
<td>—</td>
</tr>
<tr>
<td>Pyruvate (32, 34)</td>
<td>&gt;5 (NS)</td>
<td>—</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>0.54</td>
<td>—</td>
</tr>
<tr>
<td>Succinate (36)</td>
<td>1.3</td>
<td>0.04</td>
<td>0.11</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>TFS</td>
<td>1.4 ± 0.5</td>
<td>0.13 ± 0.08</td>
<td>—</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>Thiosulfate (34)</td>
<td>&gt;10 (NS)</td>
<td>—</td>
<td>—</td>
<td>0.37 (0.28)</td>
<td>21.6</td>
<td>—</td>
</tr>
</tbody>
</table>

a When the data are from former publications, the reference number is given in parentheses. The values (apparent K, in millimoles per liter) are means ± SE from 20 measurements in three rats. NS, not significant.

Fluorescence intensity (H) was increased by 23%, whereas the area under the tissue fluorescence curve increased by 28%. Urinary insulin excretion was unchanged. but that of SF was increased by 20%. The excretion fraction increased in parallel by 17%. In experiments when glutarate was given after the tubular cells were preloaded, the detected fluorescence intensity in tissue increased considerably. followed by a slow decay (Figure 8D). Glutarate and α-ketoglutarate acted as enhancers of the urinary excretion of SF. That enhancement could be due to increased intracellular levels of these dicarboxylic acids, which could be used as countertransportees for the contra-luminal PAH uptake (7, 11).

Succinate (apparent K,Cl,PAH, 1.3 mmol/L; apparent K,Cl,succ, 0.05 mmol/L) application 1 min before, simultaneous with, or after SF did not change the urinary SF excretion or its tissue uptake (Figure 5). Succinate therefore was not able to enhance the contraluminal PAH/anion exchanger.

TFS (apparent K,Cl,PAH, 1.4 mmol/L; apparent K,Cl,succ, 0.13 mmol/L) decreased urinary excretion and tissue uptake of SF under all tested conditions (Figure 5). As shown in Figure 9, a bolus injection of TFS 1 min before SF caused a decrease of SF tissue content. The measured area under the tissue fluorescence curve was reduced by 25%. Urinary insulin excretion was constant, whereas the urinary excretion of SF decreased to 50% and the excretion frac-
Interaction of thiosulfate and 2-chloropropionate with the excretion pattern of SF. Thiosulfate, a good inhibitor of contraluminal and luminal sulfate transport (Table 3), as well as 2-chloropropionate, a strong inhibitor of luminal lactate transport (Table 3), did not show any interference with the urinary SF excretion or the SF content in renal tissue.

DISCUSSION
To the Method
Selection of the Test Dye. Considering the chemical structure of the tested fluorescein dyes, we orig-
Finally suggested that fluorescein would interact with the contraluminal PAH and the luminal lactate transport system, that SF would interact with the contraluminal PAH and also with the contraluminal sulfate transport system, and that fluorescein-5(6)-sulfonate would interact with the contraluminal PAH and also with the contraluminal and luminal dicarboxylate transport systems. However, as shown in Table 3, it turned out that these presumptions were wrong. Fluorescein shows a good interaction with the contraluminal PAH and contraluminal sulfate transport systems, but it shows no interaction with the luminal lactate transport system. SF exerted the same interaction as fluorescein with the contraluminal PAH and the luminal lactate transport systems, but a 10 times weaker interaction with the contraluminal sulfate transport system was seen. Fluorescein-5(6)-sulfonate exerted the same inhibitory potency against the contraluminal PAH transport system and contraluminal sulfate transport systems but showed no interaction with the dicarboxylate transport and luminal lactate transport systems. From these interaction data, one could propose that all three fluorescein analogs, especially fluorescein and fluorescein-5(6)-sulfonate, would be suitable to monitor the transport steps involved in the transtubular secretion of PAH. However, the evaluation of the excretion fraction (i.e., the factor by which the amount of secreted dye exceeds that of the filtered dye) led to a different conclusion. In the experiment shown in Tables 1 and 2, the excretion fraction of fluorescein (protein binding, 77.3%) was calculated to be 1.8, that of SF (protein binding, 88.5%) was 4.0, and that of fluorescein-5(6)-sulfonate (protein binding, 43.3%) was 0.9. Thus, only SF behaves similarly to PAH, whereas fluorescein is secreted to a smaller extent and fluorescein-5(6)-sulfonate is only filtered and reabsorbed to a small extent. Thus, despite its high protein binding, SF is the best among the test analogs to monitor the secretion steps of substances with transport behavior similar to those of PAH.

Two other effects deserve to be discussed here:
first, that the protein binding in the concentration ranges that we applied (measured at approximately 4 min after injection of the dye) was linearly related to the dye concentration, i.e., that the fraction of free dye remained constant. Second, that the bound dye is not removed from plasma protein during transit through the kidney, i.e., that the off rate of the bound dye is rather low. All of these effects facilitate the evaluation of the transport parameters.

SF, which contains an SO3H group instead of the COOH group of fluorescein, was proposed in 1975 by Steinhausen et al. (12, 13) as “visible PAH” to investigate organic secretion. The reason for this proposal was that the exchange of COOH for SO3H is analogous to the situation of the SO3H-containing phenolsulfonephthalein (phenol red), which is readily excreted by the PAH transport system, and the COOH-containing phenolphthalein, which apparently did not reach the tubular urine (14). As the study presented here shows, SF and, to some extent, fluorescein could be used as fluorescent substrates to study tubular transport of hydrophobic organic anions (PAH), although both also interact moderately with the luminal lactate transport system. Fluorescein was already applied in a number of respective studies with isolated tubule (15–20).

Evaluation of the Data. In clearance studies, the plasma concentration of the substance is an appropriate reference point. Because of the greater accuracy, clearance studies are performed under steady-state conditions, i.e., at constant plasma concentrations rather than at transient concentrations, i.e., with single injections of the test substance. Unfortunately, we had to apply the single injection method in order to better monitor the dye behavior in the tissue, because we were not able to monitor the plasma concentrations continuously. Thus, we had to compromise and relate urinary excretion and tissue content to the amount of injected test substance. Furthermore, we related the changes of the measured parameters during the injection of interfering substances to the parameters obtained under control conditions. Thus, the method applied proved to be simple and quite accurate to study transport interactions in proximal tubular cells in situ. Nevertheless, the integration of the excretion pattern of SF and inulin in the urine and the integration of the tissue fluorescence signal over 5 min deserves some comments. We evaluated the excretion fraction, i.e., how much of the filtered dye is also secreted, by two methods: first, by summing up the urinary values for 25 min, and second, by averaging the 10- to 25-min values corrected for the SF delay, we obtained almost the same values, i.e., 3.4 and 3.2, respectively. For the sake of simplicity, in this study, we applied only the first method. The tissue signal was also evaluated by two methods: first, integration over 5 min, and second, initial height and half value of decay. Both modes of evaluation gave similar quantitative results. At the end, however, we preferred the method of integrating the tissue fluorescence over 5 min.

An open question remained: how much of the tissue fluorescence is caused by SF within proximal tubular cells and how much by SF located extracellularly, in the tubular urine and in the plasma? By inspecting the photographs published by Steinhausen et al. (13), it became evident that SF is located almost exclusively in the late proximal tubular cells around the welling point of the peritubular capillaries. No fluorescence could be detected in the capillary content or in the lumina of the proximal and distal tubules. To test to what extent SF in tubular urine within proximal tubules, distal tubules, and early collecting ducts might contribute to tissue fluorescence, we compared the tissue fluorescence under strong antidiuresis and in maximal osmotic diuresis in the same animal and found no difference in tissue fluorescence. This indicates that SF in the tubular urine plays no role in overall tissue fluorescence. The precise location of the dye, however, can be revealed in the future by applying confocal microscopy on the proximal tubules in situ (21).

To the Results

Inhibitors of the PAH Transport System (PAH Itself, Probenecid, Apalclllin). When applied 1 min before the injection of SF, the three substrates of the PAH transport system—PAH itself, probenecid, and apalclllin—diminished tissue content and urinary excretion of SF differently, but both to exactly the same extent (Figure 5a). The degree of inhibition they achieve is proportional to their inhibitory potency for the PAH uptake system (K, values: 0.08, 0.04, and 0.02 mmol/L, respectively; Table 3). These data are consistent with the hypothesis that these substances inhibit primarily the uptake of SF from blood into proximal tubular cells without any significant effect on the exit of SF from the tubular cells into the urine. When the three interfering substances were given simultaneously with SF, the inhibitory pattern on both tissue content and urinary excretion of SF was similar, although the reduction of tissue content caused by PAH and probenecid was somewhat larger than the inhibition of urinary excretion. This different behavior, however, could be easily explained by different decay of plasma concentration of SF and the inhibitors.

Chasing experiments, i.e., application of the inhibitors 1 min after the injection of SF when the tissue fluorescence was at its peak, were performed with probenecid and apalclllin. In both cases, the tissue content of SF decreased instantaneously, more so after apalclllin than after probenecid, which is again
to be expected from their inhibitory potency against the PAH transport system. Interestingly, after apalculbin, a short increase of tissue fluorescence was seen before the decay. This is probably due to the liberation of SF bound to plasma proteins. Indeed, in a parallel experiment, it was found that preloading of the animal with apalculbin diminished plasma protein binding of SF from 88 to 66%. The striking inhibitory potency of apalculbin towards the contraluminal transport of organic anions encourages us to propose to use this substance in humans, where the uptake of potential nephrotoxic substances by the organic anion transporter into proximal tubular cells should be prevented. Apalculbin, at least, is widely applied to human patients as a long-lasting penicillin analog.

Dicarboxylates That Interact With Both Contraluminal and Luminal Dicarboxylate Transport Systems and the PAH Transport System: α-Ketoglutarate, Glutarate, Succinate, Tetrafluorosuccinate, and Mercaptosuccinate. It was shown by Shimada et al. (7) and also by others (18, 22–24) that the uptake of PAH into the proximal tubular cell occurs in exchange for α-ketoglutarate. Furthermore, it was demonstrated that all of the dicarboxylates interact with the contraluminal PAH transport systems in the sequence (apparent Kp,PAH in millimoles per liter): α-ketoglutarate, 0.03; glutarate, 0.05; mercaptosuccinate, 0.77; succinate, 1.3; and TFS, 1.4 (Table 3). We observed in this study that glutarate, α-ketoglutarate, and succinate augmented the urinary excretion of SF, whereby glutarate, given before or simultaneously with SF, augmented both tissue content and urinary excretion by SF by up to 30%. α-Ketoglutarate and succinate augmented urinary excretion rather than tissue content of SF. In chasing experiments in which glutarate was given 1 min after SF injection, it proved to increase tissue uptake of SF and to delay tissue decay of SF considerably (Figure 8). The glutarate data are in agreement with the hypothesis that this substance augments the urinary excretion of SF by augmenting primarily the contraluminal uptake of SF into proximal tubular cells (17, 20). The mode of action of α-ketoglutarate and succinate to stimulate the urinary excretion of SF rather than uptake in the tissue is not clear.

In contrast to the dicarboxylates, which can be metabolized, TFS and mercaptosuccinate, which cannot be metabolized, diminished both the urinary excretion and the tissue content of SF, whereby the effect on urinary excretion was in all cases larger than the effect on tissue uptake. This indicates an inhibitory effect of these substances on contraluminal uptake but also on the luminal exit step of SF. Chasing experiments (TFS and mercaptosuccinate given 1 min after SF application), however, showed that both substances prevent predominantly the contraluminal uptake of SF (Figures 9D and 10D). In order to find out whether these substances interfere with the Krebs (dicarboxylate) cycle activity, the effect of TFS and mercaptosuccinate on succinate dehydrogenase was tested. It was seen that succinate dehydrogenase was inhibited by mercaptosuccinate but not by TFS (data not shown). Thus, changes in the intracellular concentration of Krebs cycle intermediates, if present at all, are caused by mercaptosuccinate rather than by TFS. Altogether, it seems to us that the inhibitory effect of these substrates on the urinary excretion and tissue content of SF is due to direct interference with SF transport and not because of a change of tissue dicarboxylate, i.e., α-ketoglutarate concentration.

Interaction of the SF Excretion With Intermediates of the Cellular Metabolism. Acetate, lactate, and pyruvate, which quickly enter into the metabolism of proximal tubular cells (25), increase the uptake of PAH and related substrates in renal cortical slices when given to the incubation medium (26–29). Under these conditions, all three substrates apparently augment the intracellular concentration of α-ketoglutarate, which in turn serves as "countertransportee" for PAH uptake. Under our experimental conditions, however, when the kidney is only exposed, but otherwise undisturbed, the intracellular α-ketoglutarate level probably remains high and is barely elevated by acetate, lactate, or pyruvate. Thus, it may not be surprising that we did not see very much change in the urinary excretion and tissue content of SF.

In conclusion, SF turns out to be transported by the proximal tubular cells in a way similar to hydrophobic organic anions (i.e., PAH). By measuring the urinary excretion of shot-injected SF in relation to inulin and by monitoring the intracellular content of SF permanently, it could be determined whether interfering substrates interact with the contraluminal or the luminal transport step of SF. In this study, substances were tested that inhibit predominantly the contraluminal uptake of SF: PAH, probenecid, apalculbin, mercaptosuccinate, and TFS. On the other hand, with glutarate, α-ketoglutarate, and succinate, substances were found that augment overall SF secretion. Taking all of our data together, it could be seen that a linear relation exists between urinary SF secretion and cellular uptake of SF, with little relation to GFR (Figure 11A and B). In a parallel study, we have observed that, in cisplatin-treated rats, the luminal transport step of SF is disturbed, i.e., the urinary excretion of SF is diminished but the tissue content of SF is slightly augmented. Thus, the method described in this article is also suitable to reveal pathologic changes in the transport of hydro-

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1 Determined by Prof. G. v. Jagow and Mr. H. Schecker.
Figure 11. (A) Relationship between urinary excretion of SF and tissue fluorescence of SF of all tested substrates. (B) Relationship between urinary excretion of SF and urinary excretion of inulin of all tested substrates. The solid line is the regression line, and the dashed lines indicate the standard deviation.

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