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Riboflavin transport by rabbit renal brush border membrane vesicles

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Abstract

The present study examined riboflavin (RF) uptake by isolated rabbit renal brush border membrane (BBM). RF uptake was linear for up to 30 s and leveled off thereafter reaching an equilibrium with longer incubation. Studies on RF uptake as a function of incubation medium osmolarity indicated that the uptake was the results of transport (61.4%) into the intravesicular space as well as binding (38.6%) to membrane surfaces. The process of RF uptake was saturable as a function of substrate concentration with an apparent K_m of 25.7 ± 7.6 μM and V_{max} of 75.6 ± 14.7 pmol/mg protein/10 s. *cis*-Addition of unlabeled RF and its structural analogues, lumiflavin and lumichrome, inhibited the uptake of [^{3}H]RF significantly, indicating the involvement of a carrier-mediated process in RF uptake by renal BBM. RF uptake by renal BBM was partly Na^+-dependent so that when Na^+ was replaced by potassium, choline, lithium or tetramethylammonium, the RF uptake was reduced to ca. 60% of the control. This Na^+-dependency was unlikely to be due to Na^+-cotransport mechanism because RF uptake occurred without the characteristic 'overshoot' phenomenon as for other Na^+-cotransport systems and the elimination of transmembrane Na^+-gradient by preloading Na^+ to the intravesicular space did not affect RF uptake. In contrast, removal of Na^+ eliminated the binding component of RF uptake, suggesting the requirement of Na^+ for RF binding to BBM. The RF uptake was not affected when extravesicular pH was varied within the physiological pH range of 6.5 to 8.5. No effect on BBM [^{3}H]RF uptake was found when the transmembrane electrical potential was altered by either the presence of anions with different membrane permeability ($Cl^- = NO_3^- > SO_4^- >$ gluconate$^-$) or by using nigericin (10 μg/mg protein) with an outwardly or inwardly directed transmembrane K^+ gradient. The uptake of RF by BBM vesicles was, however, inhibited by probenecid and organic anion transport inhibitors, 4,4-diiso-thiocyanatostilbene-2,2-disulfonic acid (DIDS, 1 mM) and 4-acetamido-4-isothiocyanatostilbene-2,2-disulfonic acid (SITS, 1 mM). In summary, these results demonstrate the existence of a membrane-associated, and organic anion inhibitor-sensitive, carrier system for RF uptake by renal BBM. © 1997 Elsevier Science B.V.

Keywords: Riboflavin; Brush border membrane; Organic anion transport

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1. Introduction

Riboflavin (RF) is a water-soluble vitamin essential for normal cellular functions, growth and development. RF in its coenzyme forms, riboflavin-5′-phosphate (FMN) and flavin adenine dinucleotide (FAD), serves in key metabolic reactions in the body, which include carbohydrate, amino acid and lipid metabolism and the conversion of folic acid and pyridoxine into their coenzyme forms [1–3]. RF deficiency thus leads to a series of clinical abnormalities including growth retardation, degenerative changes in the nervous system, anemia and skin lesions [1,4].

Mammals cannot synthesize RF but obtain the vitamin from the diet through absorption in the small intestine. Elimination of RF from the body takes place only in the kidneys, where in addition to intact RF, some other metabolites have been recently identified in the urine [3,5]. RF has been shown to be transported bi-directionally by the renal tubules, i.e., reabsorbed and secreted depending on its prevailing plasma concentration [6,7]. At low RF plasma concentrations, the vitamin that has filtered through the glomeruli is reabsorbed by renal tubules through a saturable process [6,7]. This tubular reabsorption process ensures efficient extraction of this essential nutrient from the lumen to minimize its loss in the urine. To further understand the renal tubular handling of RF, Spector [8] used rabbit kidney slices to study RF uptake and found evidence for the involvement of a saturable, energy-dependent system. The existence of a saturable system for RF uptake was also confirmed by Browers-Kormo and McCormick [9] who used isolated kidney cells in suspension and further showed that Na^+ removal from the incubation medium inhibits RF uptake.

While the above described studies have provided important information about the renal tubular handling of RF, little is currently known about the mechanisms of the individual *trans*-epithelial transport processes. In particular, because studies with kidney slices and isolated kidney epithelial cells in suspension cannot differentiate between uptake across the apical brush border membrane (BBM) and the peritubular basolateral membrane (BLM), the membrane domain where the reported saturable uptake system(s) of RF is located remains unknown,. The aim of the present study is to examine the uptake process of RF across the rabbit renal BBM.

2. Materials and methods

2.1. Animals

New Zealand White male rabbits, weighing 1.5–2.0 kg, were used in these studies. The animals were maintained on an ad lib diet of standard rabbit chow with free access to tap water for drinking.

2.2. BBM vesicle preparation

Purified BBM vesicles were prepared from rabbit renal cortex by the conventional magnesium-precipitation method [10]. Purification of BBM preparation, as assessed by the enrichment of BBM enzyme marker, was monitored routinely as reported previously [11]. Thus, the enrichment of BBM enzyme marker, alkaline phosphatase, averaged 13.8 ± 0.8 fold ($n = 24$) from the renal cortex homogenate, while other non-BBM enzymes such as Na^+–K^+–ATPase for BLM, succinic dehydrogenase for mitochondria, and cytochrome-c reductase for endoplasmic reticulum were 0.82 ± 0.01, 0.34 ± 0.03, and 0.53 ± 0.06 fold ($n = 15$) lower than the cortex homogenate, respectively.

2.3. BBM [^{3}H]RF uptake measurement

Final BBM vesicles were suspended in a medium that comprised of (in mM): 300 mannitol, 10 $MgSO_4$, 10 tris(hydroxymethyl)aminomethane (Tris), and 16 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4. [^{3}H]RF uptake was measured by a Millipore rapid-filtration procedure at 24°C, and was initiated by mixing 10 μl BBM vesicle suspension with 90 μl of uptake medium comprised of (in mM): 100 NaCl, 80 mannitol, 20 HEPES–Tris, pH 7.4, containing labeled and unlabeled RF. Incubation was terminated at the indicated time by adding 2 ml of ice-cold stop medium comprised of (in mM): 100 NaCl, 100 mannitol, 20 K_2HPO_4, pH 7.4. The suspension was filtered and washed twice with 2 ml of stop solution. The filter membrane was then dis-

solved in 5 ml of scintillation fluid (UltimaGold, Packard) and counted for radioactivity in a liquid scintillation counter (1600 TR, Packard). All measurements were carried out in triplicate and expressed as fmol/mg protein/unit time. The protein concentration was assayed using Coomassie Brilliant blue G250 with bovine serum albumin as the reference protein [12]. The purity of the stock [^{3}H]RF and the metabolic form of the radioactivity taken up by BBM vesicles following incubation with [^{3}H]RF were determined using silica-gel precoated TLC plates and a solvent system of ethanol and water (9:1 v/v) [13].

2.4. Statistical analysis

Data are means ± SE. Statistical significance was assessed by one-way analysis of variance (ANOVA).

3. Results

3.1. General characteristics of BBM RF uptake

Fig. 1 depicts the uptake of 0.1 μM [^{3}H]RF as a function of time. The uptake of RF increased linearly for up to 30 s of incubation and leveled off thereafter. The uptake of RF may involve binding of RF to BBM, as evidenced from separate studies where RF uptake at equilibrium (30 min) was examined as a function of uptake medium osmolarity. As shown in Fig. 2, with uptake medium osmolarity varied from 200 to 650 mOsm by altering the concentration of mannitol (0 to 450 mM), the BBM uptake of [^{3}H]RF showed an inverse and linear ($r = 0.99$) relationship with uptake medium osmolarity. Extrapolating the uptake line to infinite osmolarity intersected the y-axis at ca. 58. From this finding, we calculated that the degree of binding of RF to BBM at equilibrium was ca. 38.6% and the rest of the uptake was due to transport into an active intravesicular space. The metabolic form of the radioactivity taken up by BBM following 30 min incubation with 0.33 μM [^{3}H]RF was also examined, using the TLC system described in Section 2. The results showed that the majority of the radioactivity (86.3 ± 0.3%, $n = 3$) taken up by the vesicles to be in the form of RF.

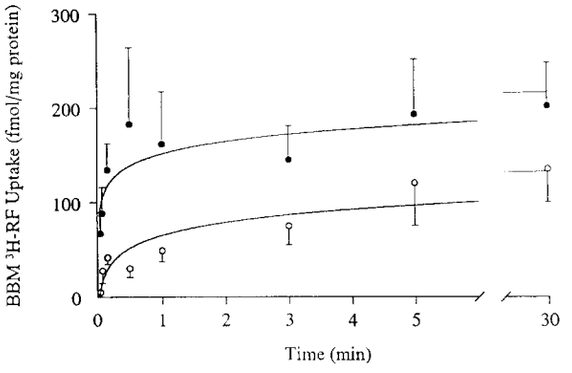

Fig. 1. Uptake of RF by renal BBM as a function of time. BBM vesicles were suspended in a medium of 300 mM mannitol, 10 mM $MgSO_4$, 26 mM Tris–HEPES, pH 7.4. [^{3}H]RF uptake was measured by incubation in an uptake medium of 100 mM NaCl (closed circles) or 100 mM choline chloride (open circles), 80 mM mannitol, 20 mM HEPES–Tris, pH 7.4, containing labeled and unlabeled RF (0.1 μM). Incubation was terminated at the indicated time by a stop medium of 100 mM NaCl, 100 mM mannitol, 20 mM K_2HPO_4, pH 7.4. Each data point represents means ± SE of 12 determinations from 4 separate BBM preparations.

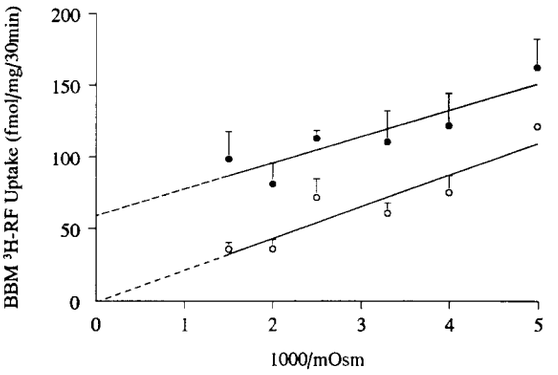

Fig. 2. Effect of incubation medium osmolarity on RF uptake by renal BBM. BBM vesicles were suspended in a medium of 300 mM mannitol, 10 mM $MgSO_4$, 26 mM Tris–HEPES, pH 7.4. Incubation was performed for 30 min in an uptake medium consisting of 100 mM NaCl (closed circles) or tetramethylammonium (open circles), 20 mM HEPES–Tris, pH 7.4 containing labeled and unlabeled RF (0.1 μM) and different amounts of mannitol (0–450 mM). Each data point represents means ± SE of 12 determinations from 4 separate BBM preparations.

3.2. Involvement of a membrane-associated carrier system

To test the involvement of a membrane-associated carrier system in the uptake of RF by renal BBM, we

examined the substrate uptake as a function of increasing substrate concentration in the uptake medium (0.1–50 μM). As shown in Fig. 3, saturation was observed in RF uptake as a function of RF concentration, suggesting the involvement of a specialized membrane associated carrier system. Kinetic parameters of the observed saturable component of RF uptake were calculated using a computerized model of the Michaelis–Menten equation as described by Wilkinson [14]. The apparent K_m and V_{max} of the saturable uptake process were 25.7 ± 7.6 μM and 75.6 ± 14.7 pmol/mg protein/10 s, respectively (n = 5). To further examine the involvement of a membrane-associated carrier system for RF uptake, we examined the effect of *cis*-addition of 50 μM unlabeled RF and the structural analogues, lumiflavin and lumichrome, on the uptake of 0.1 μM [^{3}H]RF. Results from these studies showed that all the three compounds tested caused a significant ($p < 0.05$) inhibition on [^{3}H]RF uptake (from the control value of 192 ± 23 fmol/mg protein/10 s to 81 ± 17 fmol/mg protein/10 s with unlabeled RF, 79 ± 14 fmol/mg protein/10 s with lumiflavin and 102 ± 18 fmol/mg protein/10 s with lumichrome, mean ± SE of 12 determinations from 4 separate BBM preparations.).

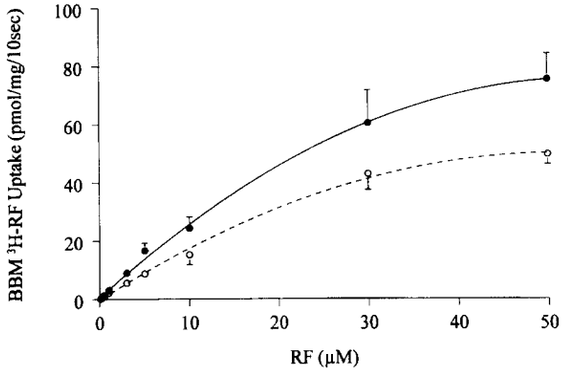

Fig. 3. Uptake of RF by renal BBM as a function of concentration. BBM vesicles were suspended in a medium of 300 mM mannitol, 10 mM $MgSO_4$, 26 mM Tris–HEPES, pH 7.4. Incubation was performed for 10 s in an uptake medium of 20 mM HEPES–Tris, pH 7.4 with 100 mM NaCl (closed circles) or tetramethylammonium (open circles). Different concentrations of RF were added to the incubation medium at the onset of incubation. Each data point represents means ± SE of 5 determinations from 3 separate BBM preparations.

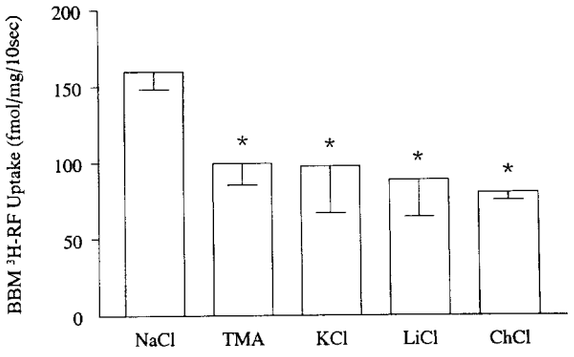

Fig. 4. Effect of Na^+ replacement on RF uptake by renal BBM. BBM vesicles were suspended in a medium of 300 mM mannitol, 10 mM $MgSO_4$, 26 mM Tris–HEPES, pH 7.4. [^{3}H]RF uptake was measured by incubation for 10 s in an uptake medium of 80 mM mannitol, 20 mM HEPES–Tris, pH 7.4, 0.1 μM labeled and unlabeled RF, containing either 100 mM NaCl, or with NaCl replaced by 100 mM tetramethylammonium (TMA), KCl, LiCl, or choline chloride (ChCl). Each data bar represents means ± SE of 9 determinations from 3 separate BBM preparations (* $p <$ 0.05 vs. NaCl).

3.3. Na^+-dependency of RF uptake

We found that BBM [^{3}H]RF uptake was sensitive to Na^+. As shown in Figs. 1 and 4, the RF uptake was reduced by ca. 40% when Na^+ in the uptake medium was replaced by other cations including tetramethylammonium, potassium, lithium or choline as compared to that in the presence of Na^+. However, unlike other Na^+-cotransport systems, such as Na^+-coupled glucose, phosphate or amino acid cotransport [15], the RF uptake occurred without an apparent initial rapid *trans*-accumulation into the intravesicular space, i.e., the 'overshoot' phenomenon, and the Na^+-dependency persisted for up to 30 min incubation. The BBM [^{3}H]RF uptake was also not significantly affected when the transmembrane Na^+-gradient was eliminated by preloading an equal concentration (100 mM) of Na^+ to the intravesicular space; i.e., from 163 ± 8.3 fmol/mg protein/10 s when Na^+_{in}/Na^+_{out} = 0/100 mM to 132 ± 15.5 fmol/mg protein/10 s when Na^+_{in}/Na^+_{out} = 100/100 mM (n = 7, $p > 0.1$), as compared to 101 ± 6.3 fmol/mg protein/10 s when Na^+_{in}/Na^+_{out} = 0/0 mM (n = 7, $p < 0.01$ vs. Na^+_{in}/Na^+_{out} = 0/100). These results indicate that the Na^+-dependency of renal BBM

RF uptake may not be due to a direct Na^+-cotransport mechanism. In contrast, as shown in Fig. 2, removal of Na^+ eliminated the binding component of the RF uptake, suggesting that the Na^+-dependency of renal BBM RF uptake may be due to the Na^+-dependent RF binding to BBM. When the kinetic parameters of RF uptake were reevaluated in the absence of Na^+, and therefore without nonspecific RF binding, we obtained a K_m value of 23.2 ± 3.0 μM and a V_{max} of 49.0 ± 9.3 pmol/mg protein/10 s ($n = 5$) (Fig. 3).

3.4. Effect of extravesicular pH

In another study, the effect of varying the uptake medium pH on BBM RF uptake was examined. In these studies, the intravesicular pH was kept at pH 7.4 and the uptake medium pH varied with buffering system of either HEPES–Tris or HEPES–Mes. As shown in Fig. 5, changing extravesicular pH from 7.5 to either 6.5 or 8.5 did not significantly affect BBM RF uptake.

3.5. Effect of transmembrane electrical potential

In other studies, we also examined the effect of altering transmembrane electrical potential by using

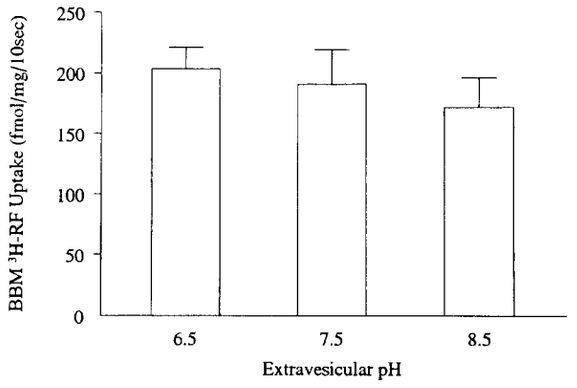

Fig. 5. Effect of extravesicular pH on RF uptake by renal BBM. BBM vesicles were suspended in a medium of 300 mM mannitol, 10 mM $MgSO_4$, 26 mM Tris–HEPES, pH 7.4. Incubation was performed for 10 s in an uptake medium of 100 mM NaCl, 80 mM mannitol, 0.1 μM labeled and unlabeled RF, and pH varied from 5.5 to 9.5 with buffering system of either HEPES–Tris or HEPES–Mes. Each data bar represents means ± SE of 12 determinations from 4 separate BBM preparations.

Table 1
Effect of transmembrane potential on RF uptake by renal BBM

$[K^+]$ in/out (mM)	Uptake (fmol/mg protein/10 s)
50/50	259 ± 17
0/50	244 ± 32
50/0	236 ± 10

The effect of inducing a negative or a positive intravesicular potential with the use of nigericin and an outwardly or inwardly directed K^+ gradient, respectively, on BBM RF uptake was examined. BBM vesicles were preloaded with a medium of 200 mM mannitol, 10 mM $MgSO_4$, 26 mM Tris–HEPES, pH 7.4, containing either 50 mM KCl or additional 100 mM mannitol, and were preincubated with nigericin (10 μg/mg protein) for 10 min. Uptake was performed for 10 s incubation in an uptake medium of 100 mM NaCl, 20 mM HEPES–Tris, pH 7.4, 0.1 μM labeled and unlabeled RF, nigericin (10 μg/mg protein), and contained either 50 mM KCl or 100 mM mannitol. Each data point represents means ± SE of 15 determinations from 5 separate BBM preparations.

either anion substitution method or a nigericin-induced K^+-diffusion potential. In the first method, RF uptake was examined in the presence of anions of different membrane permeability ($Cl^- = NO_3^- > SO_4^-$ > gluconate$^-$), where a membrane permeant anion would create a relatively larger transient negative intravesicular electrical potential than a relatively impermeant anion [16]. The results show that BBM RF uptake was not significantly different in the presence of Cl^-, NO_3^-, SO_4^- or gluconate$^-$ (221 ± 22, 248 ± 31, 237 ± 23 and 220 ± 24 fmol/mg protein/10 s, respectively, $n = 5$). In the second method, the effect of inducing a negative or a positive intravesicular potential with the use of nigericin (10 μg/mg protein) and an outwardly or inwardly directed K^+ gradient, respectively, on BBM RF uptake was examined. The results are shown in Table 1. The uptake of RF was found to be not different whether a negative (K^+_{in}/K^+_{out}: 50/0 mM) or a positive (K^+_{in}/K^+_{out}: 0/50 mM) intravesicular potential was induced compared to voltage clamp control (K^+_{in}/K^+_{out}: 50/50 mM).

3.6. Effect of organic anion transport inhibitors

To test the involvement of organic anion transport system in BBM RF uptake, the effect of probenecid and organic anion transport inhibitors, including 4,4-

diisothiocyanatostilbene-2,2-disulfonic acid (DIDS, 1 mM) and 4-acetamido-4-isothiocyanato-stilbene-2,2-disulfonic acid (SITS, 1 mM), on the uptake of RF was examined. It was found that all these agents caused a significant ($p < 0.05$) inhibition on BBM RF uptake (from the control value of 181 ± 14 fmol/mg protein/10 s to 142 ± 15 fmol/mg protein/10 s with probenecid, 62 ± 20 fmol/mg protein/10 s with DIDS and 92 ± 16 fmol/mg protein/10 s with SITS, mean ± SE of 9 determinations from 3 separate BBM preparations.).

4. Discussion

The present study examined RF uptake by rabbit renal BBM. The preparation of BBM vesicles allows observations to be made on RF transport across the apical membrane of the proximal tubular cell without the interference of uptake processes across other cellular membrane domains. The preparation of BBM vesicles also eliminates the interference of RF metabolism by flavokinase and FAD synthetase. This was confirmed by TLC in these studies that the majority of the radioactivity taken up by BBM vesicles to be in the form of RF. The majority of BBM RF uptake (61.4%) was found to be the result of transport of the substrate into an osmotically active intravesicular space (Fig. 2). The time effect of RF uptake showed an initial linear increase for up to 30 s of incubation and leveled off thereafter (Fig. 1). The initial rate of RF uptake was found to be saturable as a function of concentration (Fig. 3), indicating the involvement of a carrier-mediated system. This notion was further supported by the finding that *cis*-addition of unlabeled RF and its structural analogues, lumiflavin and lumichrome, caused a significant inhibition in the uptake of [^{3}H]RF. These findings are in accordance with our previous studies with rabbit intestinal BBM [17] and rat liver basolateral membrane [18].

In contrast to our previous studies with intestinal BBM where RF uptake was found to be Na^+-independent [17], we found in the present study that part of the renal BBM RF uptake was Na^+-dependent (Figs. 1 and 4). Thus, replacement of Na^+ in the uptake solution by other cations such as tetramethylammonium, potassium, lithium or choline reduced the BBM RF uptake by ca. 40%. These results are in agreement with a previous report where Na^+ removal from the incubation medium suppressed RF uptake by isolated kidney cells [9]. Of note, however, is that, unlike other renal BBM Na^+-cotransport systems, we found in our current study that the uptake of RF did not exhibit the characteristic 'overshoot' phenomenon and the Na^+-dependency persisted for up to 30 min incubation. We also found that elimination of transmembrane Na^+-gradient did not suppress RF uptake. Based on these findings, we consider that the Na^+-dependency of renal BBM RF uptake may be through some yet unidentified indirect interactions with Na^+ rather than a direct Na^+-cotransport mechanism. Alternatively, Na^+ may affect the binding component of RF uptake by renal BBM. We found that both Na^+-dependent RF uptake and RF binding to BBM similarly constitute ca. 40% of the total RF uptake. Furthermore, as shown in Fig. 2, we have indeed found that replacement of Na^+ by tetramethylammonium eliminated the binding component of the RF uptake. A recent study reported a novel Na^+-dependent binding of carnitine to human placental BBM [19]. It was found that the carnitine binding to human placental BBM was Na^+-dependent but without the 'overshoot' phenomenon and was independent of the transmembrane Na^+-gradient. Our current findings of RF uptake by renal BBM thus resemble those findings with carnitine uptake by placental BBM and may suggest the Na^+-dependent binding of RF to renal BBM.

We have examined the effect of extravesicular pH on BBM RF uptake. This is physiologically relevant because the in vivo pH of the proximal tubular fluid (equivalent to the extravesicular pH in the mostly right-side-out BBM vesicles) decreases along the distance of the proximal tubule, from 7.4 in the early proximal convoluted tubule to about 6.5 in the late proximal straight tubule. As shown in Fig. 5, alteration of extravesicular pH between 6.5 and 8.5 had minimal effect on RF uptake.

It has been reported that RF behaves as an anion when it is transported across biological membranes [20,21]. However, conflicting results have been reported previously in regard to the involvement of organic anion transport system in the RF transport by the kidney [8,9]. By using rabbit kidney slices, Spector [8] found that the RF transport was inhibited by

the organic anions *p*-aminohippurate (PAH) and penicillin-G. In contrast, by using isolated kidney cells in suspension, Brower-Kormo and McCormick found no inhibition of RF uptake by these organic anions [9]. In the present study, we found that RF uptake by renal BBM was inhibited by anion transport inhibitors, DIDS, SITS and probenecid, suggesting a possible relationship between this uptake system and the renal organic anion transport mechanism. We also found that BBM RF uptake was not affected by alterations in transmembrane electrical potential, induced by either anion substitution or by nigericin-induced K^+-diffusion potential (Table 1). The transport of RF across the BBM thus appears to be an electroneutral process.

The results of our present study thus indicate that there exists a carrier system for RF transport associated with renal BBM. This carrier system appears to transport RF across the renal BBM through a process which is electroneutral and sensitive to anion transport inhibitors. Since RF has an isoelectric pH of 6, a substantial portion of RF is expected to exist as an anion under the physiological range of pH. If the anionic RF is the preferred species transported, it may involve cotransport with equivalent cations or countertransport with equivalent anions to maintain the electroneutrality. It is not clear from our present study in regard to the other species of ions associated with RF transport. Although we found that the BBM RF uptake is sensitive to Na^+, the involvement of Na^+ is through its effect on RF binding to BBM rather than the cotransport mechanism. Our current findings with renal BBM therefore differ from our previous findings with intestinal BBM. With intestinal BBM, we found that RF transport was Na^+-independent and electroneutral, but was insensitive to anion transport inhibitors [17]. Whether or not these results indicate the involvement of different transport systems between these tissues is not clear. Further studies with eventual cloning and isolation of RF transporters from different tissues will help to clarify this issue.

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