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Sulphate-Ion/Sodium-Ion Co-Transport by Brush-Border Membrane Vesicles Isolated from Rat Kidney Cortex

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Uptake of SO_4^{2-} into brush-border membrane vesicles isolated from rat kindey cortex by a Ca^{2+}-precipitation method was investigated by using a rapid-filtration technique. Uptake of SO_4^{2-} by the vesicles was osmotically sensitive and represented transport into an intravesicular space. Transport of SO_4^{2-} by brush-border membranes was stimulated in the presence of Na^+, compared with the presence of K^+ or other univalent cations. A typical 'overshoot' phenomenon was observed in the presence of an NaCl gradient (100mM-Na^+ outside/zero mM-Na^+ inside). Radioactive-SO_4^{2-} exchange was faster in the presence of Na^+ than in the presence of K^+. Addition of gramicidin-D, an ionophore for univalent cations, decreased the Na^+-gradient-driven SO_4^{2-} uptake. SO_4^{2-} uptake was only saturable in the presence of Na^+. Counter-transport of Na^+-dependent SO_4^{2-} transport was shown with MoO_4^{2-} and $S_2O_3^{2-}$, but not with PO_4^{2-}. Changing the electrical potential difference across the vesicle membrane by establishing different diffusion potentials (anion replacement; K^+ gradient ± valinomycin) was not able to alter Na^+-dependent SO_4^{2-} uptake. The experiments indicate the presence of an electroneutral Na^+/SO_4^{2-}-co-transport system in brush-border membrane vesicles isolated from rat kidney cortex.

Since in mammals only small amounts of SO_4^{2-} appear in the urine (urine/plasma concentration ratios as low as 0.1 have been reported), it was proposed that SO_4^{2-} was reabsorbed actively by the renal tubules (for review see Mudge *et al.*, 1973). As suggested by stopped-flow and micropuncture experiments, as well as by experiments with kidney slices, SO_4^{2-} reabsorption takes place in the region of the proximal tubule (Deyrup & Ussing, 1955; Hierholzer *et al.*, 1960; Lechene *et al.*, 1974).

Little is known about the mechanisms involved in mammalian transepithelial transport of SO_4^{2-}. In the experiments on SO_4^{2-} transport in ileum Na^+ dependence has been shown (Anast *et al.*, 1965). Similarly recent micropuncture experiments performed by Ullrich *et al.* (1979) have also demonstrated Na^+ dependence of renal SO_4^{2-} reabsorption. On the basis of these observations we can postulate that proximal-tubular SO_4^{2-} reabsorption might be coupled to the primary active transport of Na^+ via a Na^+/SO_4^{2-}-co-transport system located in the brush-border membrane, similar to the Na^+-dependent reabsorption of sugars, amino acids and P_i (for review see Murer & Kinne, 1977).

In the present study we describe experiments on SO_4^{2-} transport with brush-border membrane vesicles isolated from rat kidney cortex. The results obtained are consistent with the existence of a Na^+/SO_4^{2-}-co-transport system located in the brush-border membrane of the proximal-tubular epithelial cell.

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Methods

Brush-border membranes of rat kidney cortex were prepared from male Wistar rats (180–220g) by the method of Evers *et al.* (1978). Briefly, thin slices of the renal cortex were homogenized in a hypoosmotic medium; after addition of $CaCl_2$ (final concentration 10mM) the brush-border membranes were purified by differential centrifugation.

Uptake of radioactively labelled compounds by isolated brush-border membrane vesicles was measured by a rapid-filtration technique as described previously (Berner *et al.*, 1976; Evers *et al.*, 1976). The exact compositions of the incubation media are given in the Figure legends. All experiments were performed at least in duplicate and were repeated at least 3 times with similar results. Error variation among duplicate values was around 5%. Within the same experiment with different experimental conditions the equilibrium value varied by about 10–15%.

Protein determination was carried out by the method of Lowry *et al.* (1951) with bovine serum albumin (Behringwerke, Marburg, Germany) as standard. All enzyme assays were performed with a model 8600 LKB reaction-rate analyser at 37°C. Alkaline phosphatase (EC 3.1.3.1) was used as marker enzyme for the brush-border membrane and the activity was determined by using a test kit with *p*-nitrophenyl phosphate as substrate (Berner & Kinne, 1976).

Activity of $(Na^+ + K^+)$-stimulated ATPase (EC 3.6.1.3), a marker of the basolateral plasma membrane, was assayed as reported by Berner & Kinne (1976). The alkaline phosphatase activity of the brush-border membrane vesicles isolated from rat kidney cortex was enriched about 12-fold compared with the starting homogenate, whereas $(Na^+ + K^+)$-stimulated ATPase was not enriched in the final membrane fraction.

Materials

$H_2{}^{35}SO_4$ (sp. radioactivity 43 Ci/mg, theoretical maximum) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer was obtained from Serva (Heidelberg, Germany). The reagents needed for $(Na^+ + K^+)$-stimulated ATPase assays were obtained from Boehringer (Mannheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany). All chemicals were of the highest purity available.

Results

When brush-border membrane vesicles isolated from rat kidney cortex were prepared in an NaCl-free medium and incubated in a 100mM-NaCl-containing buffer, the uptake of SO_4^{2-} showed an 'overshoot' phenomenon during the first 2min and reached equilibrium after about 60min (Fig. 1, upper curve). This 'overshoot' indicates an intravesicular accumulation of SO_4^{2-} above the equilibrium concentration and occurs because of the persistence of an Na^+ gradient when the intravesicular SO_4^{2-} has already reached the concentration in the incubation medium (Evers *et al.*, 1976; Murer & Kinne, 1977). When the Na^+ gradient was replaced by a K^+ gradient, the initial uptake (i.e. after 20s) of SO_4^{2-} was 25-fold lower than with NaCl and the 'overshoot' did not occur (Fig. 1, lower curve).

Uptake of a substrate by an isolated membrane fraction can be explained by binding to the membrane surface and/or by transport into a vesicle space. To discriminate between these two possibilities, binding of SO_4^{2-} to the membranes was analysed at equilibrium distribution of SO_4^{2-} (prolonged incubation). Fig. 2 demonstrates the influence of osmolarity of the

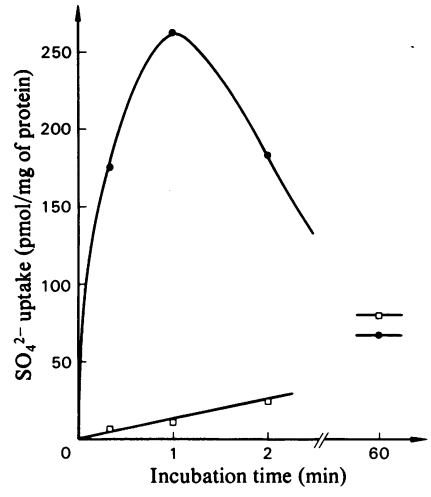

Fig. 1. *Effect of Na^+ and K^+ gradients on SO_4^{2-} uptake by brush-border membrane vesicles*
Membrane vesicles were prepared in 100mM-mannitol/ 20mM-Hepes/Tris (pH 7.4) and incubated at 25°C in the same medium containing also 0.075mM-$Na_2{}^{35}SO_4$ and 100mM-NaCl (●), or 0.075mM-$K_2{}^{35}SO_4$ and 100mM-KCl (□).

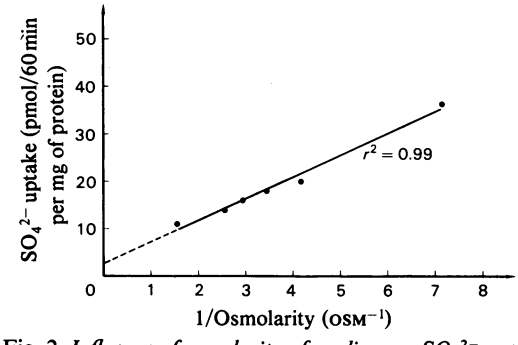

Fig. 2. *Influence of osmolarity of medium on SO_4^{2-} uptake by isolated brush-border membrane vesicles*
The uptake of 0.075mM-$Na_2{}^{35}SO_4$ was determined in the presence of 100mM-mannitol, 20mM-Hepes/Tris (pH 7.4), 10mM-NaCl and sufficient cellobiose to give the indicated osmolarity. The values given represent equilibrium values obtained after 60min of incubation at 25°C.

incubation medium on SO_4^{2-} uptake by isolated brush-border membrane vesicles. The amount of SO_4^{2-} taken up by the vesicles at equilibrium was in inverse proportion to the osmolarity in the incubation medium varied by addition of different concentrations of cellobiose. These findings indicate the presence of osmotically reactive membrane

Table 1. *Effect of gramicidin and nigericin on SO_4^{2-} transport*

The amount of SO_4^{2-} taken up during the first 20s and at equilibrium after 60min is presented. The experiments were carried out in an incubation medium as described in Fig. 1. Gramidicin-D or nigericin concentrations added as ethanolic solutions to the vesicles 15min before their incubation with SO_4^{2-} were 50μg/mg of protein. The final ethanol concentration in the assays was 1% (v/v).

Conditions in incubation medium	SO_4^{2-} uptake (pmol/mg of protein) Time period ... 20s	60min
NaCl gradient	97	90
NaCl gradient plus gramicidin	48	73
NaCl gradient plus nigericin	18	92

Table 2. *Inhibition of Na^+-dependent SO_4^{2-} uptake by different $HgCl_2$ concentrations*

The amount of SO_4^{2-} taken up was measured during the first 20s, at 1min and at equilibrium after 60min. The experiments were carried out in an incubation medium containing 100mM-mannitol, 20mM-Hepes/Tris (pH7.4), 100mM-NaCl or -KCl, and 0.075mM-$^{35}SO_4^{2-}$. $HgCl_2$ was added to the incubation media as indicated in the Table. Incubation temperature was 25°C.

$[HgCl_2]$ (M)	Salt gradient	SO_4^{2-} uptake (pmol/mg of protein) Time period ... 20s	1min	60min
0	NaCl	53	100	64
0	KCl	5	11	60
10^{-8}	NaCl	59	96	62
10^{-7}	NaCl	64	78	73
10^{-6}	NaCl	36	46	68
10^{-5}	NaCl	3	10	68

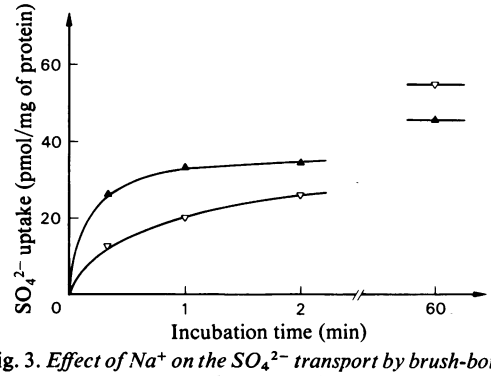

Fig. 3. *Effect of Na^+ on the SO_4^{2-} transport by brush-border membrane vesicles*

Membrane vesicles prepared in 100mM-mannitol, 20mM-Hepes/Tris (pH7.4), 0.075mM-Na_2SO_4 (unlabelled) and 100mM-NaCl (▲) or 0.075mM-K_2SO_4 (unlabelled) and 100mM-KCl (▽) were incubated at 25°C in the same medium containing $^{35}SO_4^{2-}$.

vesicles and of SO_4^{2-} transport into an intravesicular space rather than binding to or incorporation of substrates into the membrane.

Stimulation of anion flux by an Na^+ gradient does not necessarily mean a flux coupling via a co-transport system. In general coupling between cation and anion flux can also be due to an electrical coupling, following the principle of overall electroneutrality of transmembrane ion fluxes (Murer & Kinne, 1977). Addition of gramicidin D, an ionophore for univalent cations (Henderson *et al.*, 1969) decreased the Na^+-gradient-driven SO_4^{2-} uptake in the presence of an NaCl gradient (Table 1). If the Na^+-gradient-dependent movement of SO_4^{2-} across the membrane is caused primarily by the diffusion potential and not by direct coupling, an increased uptake rate of SO_4^{2-} in the presence of the ionophore should be observed, because the Na^+-gradient-dependent diffusion potential should be increased initially by the addition of gramicidin D. The decreased uptake of SO_4^{2-} in the presence of the ionophore as shown in Table 1 therefore provides evidence for a direct coupling between Na^+ flux and SO_4^{2-} flux.

It is evident that under gradient conditions osmotic differences across the vesicular membrane exist. Placing the vesicles in a medium with a higher osmolarity (due to the salt gradient) will lead to a shrinkage of the vesicles. The rate at which the

Table 3. *Effect of cation replacement on SO_4^{2-} uptake into brush-border membrane vesicles*
The amount of SO_4^{2-} taken up during the first 20s, and at equilibrium after 60min is presented. The experiments were carried out in an incubation medium as described in Fig. 1.

Salt in incubation medium	Time period ...	SO_4^{2-} uptake (pmol/mg of protein) 20s	60min
0.1M-Choline chloride		6	61
0.1M-LiCl		10	102
0.1M-NaCl		97	90
0.1M-KCl		5	94
0.1M-RbCl		6	96
0.1M-CsCl		6	93

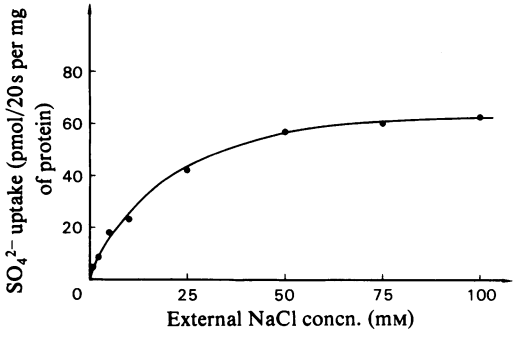

Fig. 4. *Influence of Na^+ concentration of SO_4^{2-} uptake into brush-border membrane vesicles*
Membrane vesicles were prepared in 100mM-mannitol/20mM-Hepes/Tris (pH7.4) and incubated in the same medium containing also 0.075mM-$Na_2{}^{35}SO_4$ and different NaCl concentrations as given in the Figure (1–100mM); KCl was added at appropriate concentrations (99–0mM) to give a constant final salt concentration (100mM).

original vesicular volume is restored will be determined by the rate of the influx of the substance that created the osmotic difference. Increasing the permeability for the cations with the gramicidin ionophore, and especially with the electroneutral cation/proton exchanger nigericin, should increase the rate of re-swelling of the vesicles. Our observation of decreased rather than increased Na^+-gradient-dependent SO_4^{2-}-flux rates tends to rule out the possibility that the observed stimulation is caused by osmotic effects.

A direct effect of Na^+ on the SO_4^{2-} transport across the brush-border membranes is also suggested by the $^{35}SO_4$-exchange experiment shown in Fig. 3. $^{35}SO_4$ influx across the brush-border membrane vesicles measured under exchange conditions in the absence of salt gradients and chemical SO_4^{2-} gradients proceeded more rapidly (about 2 times) in the presence of Na^+ than in the presence of K^+. Furthermore, Na^+-dependent transport of SO_4^{2-} was inhibited by an unspecific inhibitor of protein-mediated processes like $HgCl_2$. As indicated in Table 2, the initial uptake of SO_4^{2-} in the presence of an Na^+ gradient was inhibited by addition of $HgCl_2$ in a concentration of 10^{-6}M to about 30% and in a concentration of 10^{-5}M to about 90%, compared with controls without $HgCl_2$ in the medium. Since it is not likely that $HgCl_2$ affected non-specific binding to or the free diffusion of SO_4^{2-} into the membrane vesicles, it must be assumed that the almost complete inhibition of SO_4^{2-} transport by $HgCl_2$ represents an inactivation of a protein-mediated pathway for SO_4^{2-}. The results presented thus far are therefore strongly in favour of the existence of a Na^+/SO_4^{2-} co-transport mechanism in brush-border membranes isolated from rat kidney cortex.

Table 3 shows the effect of different cation gradients on SO_4^{2-} transport by brush-border membrane vesicles. Among the cations tested only Na^+ exerted a stimulatory effect, compared with the uptake of SO_4^{2-} in the presence of a choline gradient. In Fig. 4 the initial SO_4^{2-} uptake in the presence of 0.075mM-Na_2SO_4 is shown as a function of increasing Na^+ concentration; in this experiment Na^+ was present under gradient conditions (vesicle outside > vesicle inside). Under such conditions half maximal saturation of the stimulatory effect of Na^+ is around 25mM. Since the driving force for the transport system is directly related to the magnitude of the Na^+-concentration difference across the membrane, it is evident that half-saturation constants found under Na^+-gradient conditions do not reflect only properties of a postulated Na^+ site of the transport system. The observed value for half saturation reflects a mixture of different effects including also the properties of the mechanism(s) responsible for dissipative Na^+ fluxes. A better estimate for an apparent affinity constant of the postulated Na^+ site can be obtained under salt pre-equilibrated conditions, since there will be no alterations in the driving forces

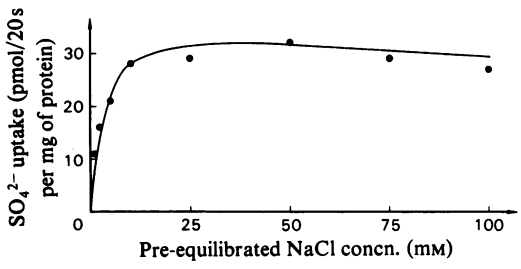

Fig. 5. *Effect of Na^+ concentration on SO_4^{2-} uptake into Na^+-pre-equilibrated membranes*

Membranes were prepared in 100mM-mannitol/20 mM-Hepes/Tris (pH 7.4) and preincubated for 1h with the same salt concentration that was used in the incubation medium and then incubated in the medium as described in the legend to Fig. 4.

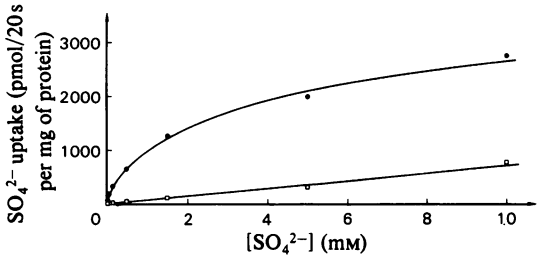

Fig. 6. *Saturation of SO_4^{2-} uptake*

Membrane vesicles were prepared in 100mM-mannitol/20mM-Hepes/Tris (pH 7.4) and incubated in a medium containing 100mM-mannitol, 20mM-Hepes/Tris (pH 7.4) in the presence of 100mM-NaCl (●) or in the presence of 100mM-KCl (□).

for the transport system related to increased Na^+ concentrations. In the experiments shown in Fig. 5 the membrane vesicles were pre-equilibrated with the different Na^+ concentrations. Under these experimental conditions the Na^+ effect on SO_4^{2-} uptake showed saturation at much lower Na^+ concentrations (around 6mM).

The uptake of SO_4^{2-} was saturable in the presence of an Na^+ gradient (Fig. 6, upper curve). The smaller and not saturable uptake in the presence of a K^+ gradient might indicate uptake by simple diffusion (Fig. 6, lower curve). Application of Michaelis–Menten kinetics to the difference between Na^+-dependent SO_4^{2-} uptake and uptake in the presence of K^+ shows an apparent affinity constant of approx. 1mM (from a Michaelis–Menten plot; results not shown).

The specificity of the transport system for anions was analysed by counter-transport experiments. Table 4 shows that Na^+-stimulated $^{35}SO_4^{2-}$ transport proceeds faster into vesicles preloaded with unlabelled SO_4^{2-}, MoO_4^{2-} and $S_2O_3^{2-}$. This trans-stimulation (counter transport) was not observed with P_i and WO_4^{2-}. The results indicate that MoO_4^{2-} as well as $S_2O_3^{2-}$ and SO_4^{2-} are transported via the same co-transport system. Since the experiment was performed with Na^+-pre-equilibrated vesicles, the transport rates are rather low in these experiments. Inhibition experiments with other anions such as Cl^-, NO_3^-, cyclamate, gluconate and thiocyanate demonstrated that only thiocyanate was able to inhibit Na^+-dependent SO_4^{2-} transport. At a concentration of 100mM under pre-equilibrated conditions thiocyanate inhibited 0.1mM-SO_4^{2-} uptake to an extent of 65% (results not shown). Dipyridamol,

Table 4. *Counter-transport of Na^+-dependent SO_4^{2-} uptake by SO_4^{2-}, $S_2O_3^{2-}$, MoO_4^{2-}, WO_4^{2-} and PO_4^{2-}*

The amount of SO_4^{2-} taken up was measured during the first 20s, at 1min and at equilibrium after 60min. Membrane vesicles loaded with 100mM-mannitol/20mM-Hepes/Tris (pH 7.4) (control) and in addition with unlabelled samples of the given substance to a final concentration of 0.6mM were incubated at 25°C in a medium containing 100mM-mannitol, 20mM-Hepes/Tris (pH 7.4), 100mM-NaCl and 0.06mM-$Na_2{}^{35}SO_4$. In this experiment, every tested substance had its individual control, which contained in the external medium the same concentration of the substance used for counter-transport.

Conditions inside vesicles	Time period ...	SO_4^{2-} uptake (pmol/mg of protein) 20s	1min	60min
No further addition		13	21	27
Plus unlabelled SO_4^{2-} (0.6mM)		37	32	28
No further addition		10	22	30
Plus unlabelled $S_2O_3^{2-}$ (0.6mM)		40	42	31
No further addition		11	21	26
Plus unlabelled MoO_4^{2-} (0.6mM)		34	32	24
No further addition		10	18	24
Plus unlabelled WO_4^{2-} (0.6mM)		12	20	24
No further addition		14	21	27
Plus unlabelled PO_4^{2-} (0.6mM)		17	20	28

Table 5. *Effect of anion replacement on SO_4^{2-} uptake into brush-border membranes*
The amount of SO_4^{2-} taken up was measured during the first 20s, and at equilibrium after 60min. The experiments were carried out in an incubation medium as described in Fig. 1.

Salt in incubation medium	Time period ...	SO_4^{2-} uptake (pmol/mg of protein) 20s	60min
0.1M-$NaNO_3$		108	90
0.1M-NaSCN		70	95
0.1M-NaCl		97	90
0.1M-Sodium cyclamate		81	104
0.1M-Sodium gluconate		99	83

Table 6. *Effect of valinomycin on SO_4^{2-} transport in K^+-preloaded membranes*
The amount of SO_4^{2-} taken up was measured during the first 20s, at 1min, at 2min and at equilibrium after 60min. The membranes were prepared in buffer containing 100mM-mannitol, 20mM-Hepes/Tris (pH7.4) and, in addition, 50mM-potassium gluconate. SO_4^{2-} uptake was initiated by adding 1vol. of K^+-preloaded membranes to 11vol. of incubation medium containing sodium gluconate (50mM), $Na_2{}^{35}SO_4$ (0.075mM), 100mM-mannitol and 20mM-Hepes/Tris (pH7.4). Valinomycin was added as an ethanolic solution at a concentration of 18μg/mg of protein. Incubation temperature was 25°C.

Conditions in incubation medium	Time period ...	SO_4^{2-} uptake (pmol/mg of protein) 20s	1min	2min	60min
Sodium gluconate gradient		60	70	67	46
Sodium gluconate gradient plus valinomycin		59	66	71	46

4,4′-di-isothiocyanatostilbene-2,2′-disulphonic acid, phloretin and furosemide, known inhibitors of anion transport in other biological systems, were without significant effect on Na^+-dependent SO_4^{2-} uptake (results not shown).

Rheogenic Na^+-dependent transport of sugars and amino acids was demonstrated in studies with brush-border membrane vesicles isolated from small intestine and kidney cortex (for review see Murer & Kinne, 1977). As shown by Murer & Hopfer (1974), the membrane potential of the brush-border membrane vesicles can be manipulated by imposing artificial diffusion potentials on the membrane. Since diffusion potentials depend on the relative mobility of cations and anions, they can be modified in the presence of cation gradients by means of anion replacement or by the use of valinomycin. As shown in Table 5, neither replacement of Cl^- by the more permeant anion NO_3^- (thiocyanate seems to be an inhibitor, as mentioned above), nor by cyclamate or gluconate (which are probably less-permeant ions), was able to alter Na^+-dependent SO_4^{2-} uptake significantly. In another experimental approach the ionophore valinomycin, which increases the K^+ conductance of membranes (Henderson *et al.*, 1969), was used in the presence of impermeant anions. Membranes were preloaded with potassium gluconate and the SO_4^{2-} uptake in the absence and in the presence of valinomycin was examined under Na^+-gradient conditions from the outside to the inside of the vesicles. Table 6 shows that under these conditions valinomycin did not stimulate the uptake of SO_4^{2-} into the vesicles, although uptake of α-D-glucose was stimulated in the same experiment about 4-fold (results not shown) by valinomycin compared with the uptake without valinomycin. Both experiments lead to the conclusion that the membrane potential does not have an effect on Na^+-dependent SO_4^{2-} uptake. The lack of charge transfer during the transport of the anion SO_4^{2-} can be explained by the assumption that two cations are transported simultaneously with the anion.

Conclusions

The results obtained in the present study correspond to those obtained in the micropuncture experiments of Ullrich *et al.* (1979). Similar to the findings with studies on vesicles Na^+-dependent SO_4^{2-} transport in intact proximal tubules was not influenced by alteration in cellular potential differences, suggesting also electroneutral transport mechanism(s) for SO_4^{2-}; furthermore, SO_4^{2-} transport was inhibited by $S_2O_3^{2-}$ and MoO_4^{2-}, but not by 4,4′-di-isothio-

cyanatostilbene-2,2'-disulphonic acid. The results obtained in the micropuncture experiments together with the results obtained in studies with isolated membrane vesicles lead to the conclusion that SO_4^{2-} transport across rat proximal-tubular brush-border membranes is mediated by an electroneutral Na^+/SO_4^{2-}-co-transport system. Other physiological anions such as P_i or Cl^- do not seem to interfere with the Na^+-dependent transport system for SO_4^{2-}, as suggested by the counter-transport and inhibition experiments.

This finding suggests that the transport of SO_4^{2-} across the proximal-tubular membrane proceeds differently from the SO_4^{2-} transport across other plasma membranes such as erythrocyte membranes or Ehrlich-ascites-tumour-cell membranes, where SO_4^{2-} transfer seems to be governed by an anion-exchange system (Wieth *et al.*, 1973; Lepke *et al.*, 1976; Levinson, 1978). However, such an exchange system could be present in basal lateral plasma membrane of the proximal-tubular epithelial cell, whose transport characteristics might be very similar to the transport characteristics in plasma membranes of non-polarized cells. Such a polarity in the distribution of Na^+-dependent and Na^+-independent transport systems within the cell envelope of the epithelial cell could then explain a vectorial flux of SO_4^{2-} across the epithelium. The presence of an anion-exchange system responsible for the exit of SO_4^{2-} at the contraluminal cell pole could also explain the finding of inhibited SO_4^{2-} transport in the absence of bicarbonate in micropuncture experiments (Ullrich *et al.*, 1979). Since our experiments were performed in the absence of significant amounts of bicarbonate and since the system in the luminal membrane did not show a high pH sensitivity in the range between pH 6.5 and 7.5 (H. Lücke & H. Murer, unpublished work), the inhibited SO_4^{2-} transport in the absence of bicarbonate in the microperfusion experiments seems to be caused by an alteration in the efflux process, rather than by an effect on the transport system in the luminal membrane itself. However, the question of how SO_4^{2-} is transported out of the epithelial cells has to be answered by experiments with isolated basal lateral membranes.

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