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Biotin uptake mechanisms in brush-border and basolateral membrane vesicles isolated from rabbit kidney cortex

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Biotin transport was studied using brush-border and basolateral membrane vesicles isolated from rabbit kidney cortex. An inwardly directed Na^+ gradient stimulated biotin uptake into brush-border membrane vesicles and a transient accumulation of the anion against its concentration gradient was observed. In contrast, uptake of biotin by basolateral membrane vesicles was found to be Na^+-gradient insensitive. Generation of a negative intravesicular potential by valinomycin-induced K^+ diffusion potentials or by the presence of Na^+ salts of anions of different permeabilities enhanced biotin uptake by brush-border membrane vesicles, suggesting an electrogenic mechanism. The Na^+ gradient-dependent uptake of biotin into brush-border membrane vesicles was saturable with an apparent K_m of 28 μM. The Na^+-dependent uptake of tracer biotin was significantly inhibited by 50 μM biotin, and thioctic acid but not by 50 μM L-lactate, D-glucose, or succinate. Finally, the existence in both types of membrane vesicles of a H^+/biotin$^-$ cotransport system could not be demonstrated. These results are consistent with a model for biotin reabsorption in which the Na^+/biotin$^-$ cotransporter in luminal membranes provides the driving force for uphill transport of this vitamin.

Introduction

The mechanisms by which the kidney handles biotin (vitamin H) in man and experimental animals has not yet been established. Most previous studies have been devoted to the intestinal absorption of this vitamin (for reviews, see Refs. 1 and 2). Recent evidence suggests that intestinal transport of biotin occurs via a specific carrier-mediated process in certain mammalian species such as hamsters and mouse [3,4]. However, intestinal absorption of biotin has been assumed to occur via a passive mechanism in both rabbit and rat, two species in which biotin is a vitamin [4].

Recently, Na^+ gradient-dependent transports of ascorbic acid (vitamin C) and nicotinic acid (vitamin PP) have been described in renal brush-border membrane vesicles from rat and rabbit, respectively [5,6]. Such coupled processes may contribute importantly to conservation of vitamins in the whole animal. In this paper, brush-border and basolateral membrane vesicles isolated from rabbit kidney cortex have been used to investigate the mechanisms of biotin uptake. Our study demonstrates the existence in renal plasma membranes of an electrogenic Na^+/biotin$^-$ cotransport mechanism which is confined to the brush border membranes. A simple model is proposed to illustrate the role of the luminal Na^+/biotin$^-$ cotransporter in mediating reabsorption of this vitamin in the proximal tubule.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.

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Material and Methods

Preparation of membrane vesicles. Basolateral and brush-border membrane vesicles were isolated simultaneously from the rabbit renal cortex with the use of a self-orienting Percoll gradient, as recently described in detail [7]. The quality of the membrane preparations, evaluated by measuring the enrichment of enzyme markers, was the same as recently reported [7]. Basolateral and brush-border membranes showed a 16–25 and a 10–12-fold enrichment in $(Na^+ + K^+)$-ATPase and trehalase above the homogenate, respectively. Absolute activities of $(Na^+ + K +)$-ATPase in basolateral membranes and of trehalase in brush-border membranes were 0.91 ± 0.08 and 2.27 ± 0.40 μmol $\cdot$ min^{-1} $\cdot$ (mg protein)$^{-1}$, respectively. Protein concentrations were determined by the method of Lowry et al. [8]. Studies to ascertain membrane sidedness of basolateral membrane vesicles revealed that the majority (85–90%) of the vesicles were oriented as in the intact cell (right-side-out) [7]. In preparation for transport studies, the membranes were diluted 20-fold into appropriate media, centrifuged at $200\,000 \times g$ for 20 min, resuspended at a protein concentration of 3–10 mg/ml, and equilibrated for 90 min at 25°C until use.

Transport studies. The uptake of [^{3}H]biotin by renal brush-border and basolateral membrane vesicles was assayed at 25°C by a rapid filtration technique. Incubations were started by the addition of 10 μl of the membrane suspension (30–100 μg of protein) to 100 μl of incubation medium preequilibrated to 25°C. Incubations were terminated by the addition of 1 ml of an ice-cold solution containing 150 mM NaCl, 10 mM Tris-Hepes (pH 7.4) and 0.1 mM biotin (wash buffer). The membranes were rapidly collected on the center of a prewetted Sartorius filter (SM 113, pore size 0.6 μm) and washed three times with 2 ml of cold wash buffer. The vesicles were in contact with the wash buffer for approx 6 s. Identical results were obtained when the filtration procedure was delayed an additional 10 s, indicating absence of loss of biotin during the stopping and washing procedure (data not shown). In some experiments, the uptakes of 17 nM [^{3}H]biotin and 8 μM L-[^{14}C]glucose were measured simultaneously. Uptake of D-[^{14}C]glucose was measured using the above-mentioned procedure except that the wash buffer was modified by replacing biotin by 0.1 mM phloridzin. The relative changes of transmembrane potential across the membranes was determined by measuring the accumulation of [^{3}H]triphenulmethylphosphonium by filtration, as described [6]. In preliminary experiments not shown, it was found that the uptake of 17 nM or 80 μM biotin by brush-border and basolateral membrane vesicles, measured in the presence of an inwardly directed Na^+ gradient, was a linear function of time for 1 min of incubation. Thus, the uptake at 30 s was used to estimate the initial rate of biotin uptake. In the present study, we found difficult to obtain quantitative estimates of rates for shorter incubations because biotin is a low V_{max} system and also because of the relatively low specific activity of the tritiated biotin. All incubation solutions were filtered through 0.45 μm Millipore filters prior to use. The composition of solutions used to incubate the membranes is given in the figure legends.

Radioactivity of the filters was determined in 5 ml Aquasol using a SL 4000 Intertechnique liquid scintillation counter. Values for the non-specific retention of radioactivity (0.01% of the total radioactivity for [^{3}H]biotin) on the filters were substracted from the values of the incubated samples. All incubations were carried out at least in triplicate with freshly prepared membrane vesicles on three separate occasions (three rabbits). Although absolute biotin uptake varied from one preparation to another, comparable results were obtained with the different preparations, and, therefore, the results of representative experiments are presented.

To determine the identity of the accumulated compound, brush-border and basolateral membrane vesicles were incubated for 80 min at 25°C in a medium containing 17 nM [^{3}H]biotin, 100 mM NaCl, 100 mM sucrose and 10 mM Tris-Hepes (pH 7.4). These membranes were then collected by filtration by the standard procedure. The accumulated radioactivity on 8 filters was extracted with 300 μl of distilled water containing 0.1 mM biotin and 6 mM mercaptoethanol. Aliquots of the extracts were spotted on silica-gel plates with wedged-tip divisions [9]. The tritiated biotin from Amersham served as a standard and was spotted

on the same plates. The following solvent systems were used: (1) ethanol/water (1 : 9 v/v) (R_F biotin, 0.87); and (2) acetic acid/acetone/methanol/benzene (1 : 1 : 4 : 14, v/v) (R_F biotin, 0.6). After separation, the distribution of radioactivity was analyzed by scintillation counting of sections of the chromatogram.

Chemicals. The (+)-[8,9-^{3}H]biotin (33 Ci/mmol) was obtained from Amersham International, Amersham. D-[U-^{14}C]glucose (250 mCi/mmol), L-[^{14}C]glucose (40 mCi/mmol) and [^{3}H]triphenylmethylphosphonium (3.5 Ci/mmol) were from New England Nuclear. Valinomycin, biotin, thioctic acid and biocytin were purchased from Sigma Chemical Co., St. Louis. Other chemicals were of the highest purity available from commercial sources.

Results

Influence of cation gradients on uptake of biotin

The effect of cation gradients on uptake of 17 nM [^{3}H]biotin by brush-border and basolateral membrane vesicles as a function of time of incubation is illustrated in Fig. 1. The presence of an inwardly directed NaCl gradient markedly enhanced the initial rate of biotin uptake by brush-border vesicles (left panel), with a maximal accumulation occurring at about 4 to 6 min. Thereafter the amount of biotin slowly declined as biotin effluxed from the vesicles reaching equilibrium by 80 min. At the peak of the overshoot, biotin uptake was about 50% higher ($P < 0.01$) than the final equilibrium value. This finding suggested that the imposition of a Na^+ gradient provided the driving force to affect the transient movement of biotin against its concentration gradient. In contrast, in the presence of an inwardly directed KCl gradient the initial rate of uptake was approximately 5-fold slower and no overshoot was detected. Under Na^+-equilibrated conditions, the initial rate of biotin uptake was much slower than under Na^+ gradient conditions, and the overshoot was abolished. These data indicated that it was not the concentration of Na^+ per se but the Na^+ gradient that was required for uphill transport of biotin into the brush-border membrane vesicles. However, the rate of biotin uptake in Na^+-equilibrated vesicles was stimulated relative to the

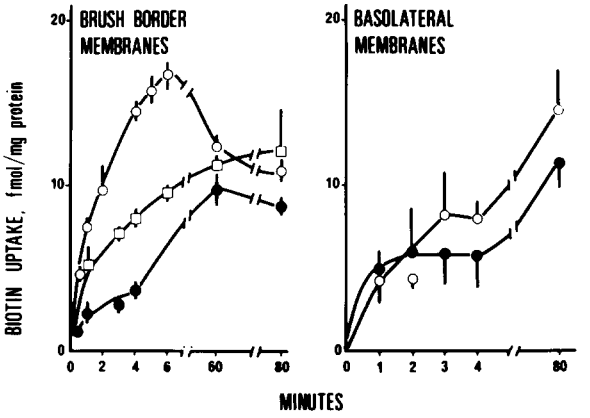

Fig. 1. Effect of a Na^+ gradient (extravesicular > intravesicular) on biotin uptake in brush-border and basolateral membrane vesicles from rabbit kidney. When uptake was measured in presence of an inward Na^+ (○) or K^+ (●) gradient, vesicles were preloaded for 90 min at 25°C in (mM) 300 sucrose, 20 Tris-Hepes pH 7.4, and samples (10 μl) of vesicles were added to 100 μl of medium containing 19 nM [^{3}H]biotin, 20 mM Tris-Hepes (pH 7.4), and 100 mM Na^+ or K^+ as the chloride salts replacing sucrose iso-osmotically. When uptake was measured in presence of 100 mM Na^+ but in absence of a gradient (□), vesicles were preincubated for 90 min in (mM) 100 sucrose, 100 NaCl, and 20 Tris-Hepes (pH 7.4) and 10 μl of vesicles were incubated in 100 μl of the same medium containing 19 nM labeled biotin. Means ±S.D., $n = 4$.

uptake in the presence of KCl, indicating facilitation of transport by Na^+ even in the absence of the Na^+ gradient. Under all conditions, the final level of uptake of biotin was the same, indicating that equilibrium was established. In other experiments not illustrated, we found that the Na^+ gradient-dependent uptake of biotin in brush-border membrane vesicles was unaffected by the presence of an outwardly directed K^+ gradient (in the absence of valinomycin). Thus, the K^+ electrochemical gradient, that exists in vivo, could not provide an additional driving force for biotin transport in a manner analogous to that observed for Na^+-dependent transport of L-glutamate in renal vesicles [10,11].

In contrast to these observations, the uptake of biotin by basolateral membrane vesicles was not significantly affected by the imposition of an inwardly directed Na^+ gradient and consequently no overshoot was observed (Fig. 1, right panel). In three individual experiments, the 4-min uptake of biotin, measured in the presence of an inward

Na^+ gradient, was $40 \pm 7.3\%$ lower than corresponding uptake at equilibrium. In contrast, in three independent experiments employing brush-border membrane vesicles, the 4-min uptake of biotin in presence of an inward Na^+ gradient averaged $94 \pm 7.0\%$ of the maximal intravesicular content, corresponding to a level of uptake $54.2 \pm 0.6\%$ higher than the equilibrium value. Nevertheless, we found that, in the presence of a 100 mM NaCl gradient (out-to-in) the 1-min uptake of 20 μM L-[^{3}H]glutamate by basolateral membrane vesicles (15.26 ± 1.50 pmol/mg protein) was significantly greater than that measured in the presence of a 100 mM KCl gradient (5.19 ± 2.29 pmol/mg protein, means $\pm$SD, $n = 4$), indicating functional activity of these membranes. Altogether, these data provided strong evidence that the Na^+/biotin$^-$ cotransport system was located in the luminal membrane and absent from the basolateral membrane.

Transport of biotin into vesicles

To ascertain that the uptake of biotin by brush-border and basolateral membrane vesicles represented transport into membrane vesicles rather than binding, uptake at equilibrium was measured when intravesicular space was decreased by increasing the medium osmolality with sucrose. As shown in Table I, the equilibrium uptake of biotin in both types of membrane vesicles decreased as the sucrose concentration increased,

TABLE I

EFFECT OF MEDIUM SUCROSE ON BIOTIN UPTAKE BY BRUSH-BORDER AND BASOLATERAL MEMBRANE VESICLES

Vesicles were preloaded with 150 mM sucrose buffered with 20 mM Tris-Hepes (pH 7.4). Uptake was measured after 80 min in a medium containing 17 nM [^{3}H]biotin, 100 mM NaCl and various concentration of sucrose. Means $\pm$S.D., $n = 4$

Sucrose (mM)	Biotin uptake (fmol/mg protein)	
	Brush-border vesicles	Basolateral vesicles
150	6.35 ± 0.53	6.73 ± 0.63
320	5.20 ± 0.25	4.81 ± 0.49
490	3.98 ± 0.86	
660	3.14 ± 0.46	3.28 ± 1.12
1000	2.60 ± 0.31	2.16 ± 0.29

TABLE II

EFFECT OF VARIOUS MONOVALENT CATIONS ON BIOTIN UPTAKE BY BRUSH-BORDER MEMBRANE VESICLES

Brush-border membranes were preincubated for 90 min in (mM) 300 sucrose, 20 Tris-Hepes (pH 7.4). Initial rates (30 s) of 17 nM [^{3}H]biotin uptake were measured after 1 : 11 dilution of vesicles into a medium containing 100 mM sucrose, 20 mM Tris-hepes (pH 7.4) and 100 mM of the chloride salt of each cation listed in the table. Values are means $\pm$S.D. of four to seven experiments

Cation	Biotin uptake (fmol/mg protein)
Na^+	4.71 ± 0.83
K^+	1.33 ± 0.27
Li^+	1.19 ± 0.31
Choline$^+$	0.80 ± 0.24

indicating transort of biotin into an intravesicular space. However, extrapolation of these data to infinite osmolarity suggested a binding component of 2 fmol/mg protein for both types of membrane vesicles. This binding component would account for only 15% of the total uptake of biotin measured under standard conditions, i.e. with 100 mM sucrose and 100 mM NaCl (see also Table VII). The radioactivity taken up by brush-border and basolateral membrane vesicles incubated for 80 min with [^{3}H]biotin appeared in a single spot on silica-gel plates, using two solvent systems, and cochromatographed for about 95% with authentic biotin. These findings indicated that biotin was accumulated by renal membrane vesicles and not metabolized.

The specificity of the Na^+ gradient in stimulating the initial rate of biotin uptake is demonstrated in the experiments summarized in Table II. The rate was 3–6-times greater in the presence of a 100 mM Na^+ gradient than with gradients of K^+, Li^+ or choline$^+$. In contrast, we found that the 30-s uptakes of 5 μM L-[^{14}C]glucose, used as a marker for intravesicular volume, were identical after imposition of a Na^+ or K^+ gradient (0.64 ± 0.30 vs. 0.70 ± 0.26 pmol/mg, means $\pm$S.D., $n = 4$). These findings argued against alteration of the intravesicular volume as a cause for the increased rate of biotin uptake seen with an inward Na^+ gradient.

TABLE III

INFLUENCE OF AN INSIDE-NEGATIVE POTASSIUM DIFFUSION POTENTIAL ON Na^+-DEPENDENT BIOTIN AND D-GLUCOSE TRANSPORT IN BRUSH-BORDER MEMBRANE VESICLES

Vesicles were preincubated for 90 min in (mM) 150 sucrose, 50 K_2SO_4, 20 Tris-Hepes (pH 7.4); treated with valinomycin in ethanol (60 μg/mg of membrane protein), or ethanol (1.6%) for 10 min before transport reaction was started. Uptake was assayed after 1:11 dilution of vesicles into a medium containing (in mM) 150 sucrose, 50 Na_2SO_4, 20 Tris-Hepes (pH 7.4) and either 17 nM [^{3}H]biotin or 5 μM D-[^{14}C]glucose. Means ± S.D., $n = 4$. * $P < 0.05$ vs. respective control values measured without valinomycin.

Valinomycin	Biotin uptake (fmol/mg protein)		D-Glucose uptake (pmol/mg protein)	
	30 s	1 min	30 s	1 min
(−)	3.5±1.44	5.7±1.14	2.5±0.55	4.3±0.16
(+)	8.2±2.47*	12.3±0.45*	11.0±0.66*	18.9±0.75*

Influence of electrical potential

The effect of membrane potential on Na^+-dependent [^{3}H]biotin uptake by brush-border membrane vesicles was examined in two experimental approaches. First, Na^+-coupled biotin uptake was determined under conditions in which an inside-negative K^+ ion diffusion potential was created by addition of valinomycin in the presence of an outwardly directed K^+ gradient. As shown in Table III, valinomycin markedly enhanced the Na^+-dependent glucose accumulation, a known electrogenic process [12,13], indicating that an inside negative potential had indeed been generated by the ionophore. Under identical conditions, valinomycin caused a doubling of the initial rate of Na^+ gradient-dependent biotin uptake. In these experiments, we found that valinomycin had no effect on the equilibrium volume of distribution for [^{3}H]biotin (data not shown). These observations suggested that the cotransport of Na^+ and biotin in luminal membranes was an electrogenic process, i.e., the cotransport was associated with the net transfer of a positive charge.

The role of the membrane potential on Na^+ gradient-dependent biotin transport was further studied by examining the influence of sodium salts with anions of different conductances. Table IV shows that the 30-s uptake of biotin under Na_2SO_4 gradient conditions was inhibited 30 and 40%

TABLE IV

INFLUENCE OF ANION SUBSTITUTION ON BIOTIN AND L-GLUCOSE UPTAKE BY BRUSH-BORDER MEMBRANE VESICLES

Vesicles preloaded in 300 mM sucrose buffered with 20 mM Tris-Hepes (pH 7.4) were incubated in media containing 17 nM [^{3}H]biotin, 8 μM L-[^{14}C]glucose, 100 mM sucrose, 20 mM Tris-Hepes and either 100 mM NaCl, 100 mM $NaNO_3$ or 50 mM Na_2SO_4. Values are means ± S.D. of three to four experiments. * Sulfate vs. chloride and nitrate, $p < 0.005$; the other changes are not significant

Anion	Biotin uptake (fmol/mg protein)		L-Glucose uptake (pmol/mg protein)	
	30 s	80 min	30 s	80 min
Chloride	4.9±0.33	9.4±1.32	1.7±0.21	3.9±0.32
Nitrate	5.7±0.91	8.5±0.80	1.7±0.18	4.1±0.34
Sulfate	3.4±0.40*	9.9±0.67	1.5±0.22	4.4±0.14

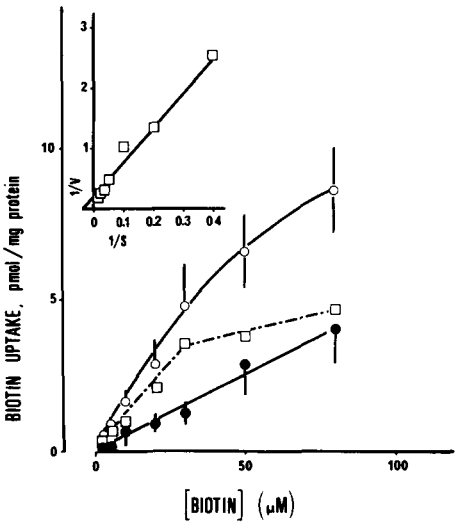

Fig. 2. Kinetics of biotin uptake. Brush-border membrane vesicles were pre-equilibrated with 300 mM sucrose and 20 mM Tris-Hepes (pH 7.4) for 90 min at 25°C. Vesicles were incubated for 30 s in a medium containing 20 mM Tris-Hepes (pH 7.4), 100 mM sucrose with [^{3}H]biotin in the concentrations indicated and either 100 mM NaCl (○) or 100 mM KCl (●). Means ± S.D., $n = 4$. The dashed line is the Na^+-dependent uptake obtained by substracting uptake in presence of KCl from the uptake in the presence of a NaCl gradient. Inset: Lineweaver-Burk plot of the data. The lines describing the Lineweaver-Burk plot and the results obtained in the presence of KCl were drawn by linear regression analysis according to the least-squares method.

relative to those obtained with NaCl and $NaNO_3$ gradients, respectively. In the same experiment, the uptakes of L-glucose at 30-s and at 80 min were similar in the presence of all salt gradients, thus arguing against alteration in the internal volume as the cause for the decreased rate of biotin uptake seen with Na_2SO_4. The conductance of brush-border membrane for Cl^- or NO_3^- is probably higher than for SO_4^{2-} [12,13]. Recent studies, using the lipophilic cation triphenylmethylphosphonium as an indicator of membrane potential, have shown that replacing SO_4^{2-} with Cl^- hyperpolarized the brush-border membrane [6]. Therefore, these results provided further evidence that Na^+-dependent biotin transport was an electrogenic process.

In preliminary experiments not illustrated, we were unable to detect saturation of the Na^+ gradient-dependent uptake of biotin with external biotin concentrations ranging from 17 nM, the concentration used in the present study, to 2 μM. The effect of biotin was examined further using higher external biotin concentrations (from 2.5 to 80 μM) (Fig. 2). In the presence of an inwardly directed K^+ gradient, the initial rate (30 s) of biotin increased linearly with medium biotin concentrations ranging from 2.5 to 80 μM, consistent with passive diffusion of the anion into the vesicles. In contrast, in the presence of an inwardly directed Na^+ gradient the relationship between external biotin concentration and uptake of biotin was curvilinear. The Na^+ gradient-dependent uptake of biotin obtained by substraction the component of uptake in presence of KCl from uptake in the presence of NaCl gradient was a saturable function of the external biotin concentration. A double reciprocal plot of the Na^+-dependent component as a function of external biotin concentration yields a straight-line relationship. An apparent K_m of 28 μM and a maximum velocity of 4.9 pmol/mg protein per 30 s could be calculated from these data.

Inhibition of biotin uptake by analogues

Table V shows the effects of structural analogues, glucose and various organic anions on the Na^+ gradient-dependent uptake of 17 nM [^{3}H]biotin. It was found that 50 μM unlabelled biotin inhibited the Na^+ gradient-dependent uptake of tracer biotin (17 nM) by $83 \pm 9\%$. A comparable inhibition was observed with thioctic acid, which bears structural resemblance to biotin and is also a monocarboxylic acid. Biocytin, however, the other biotin derivative tested, was not an inhibitor, suggesting that the presence of amide or amino groups precluded recognition by the biotin carrier. Table V also shows that D-glucose, L-lactate and the dicarboxylic acid succinate had no effect. None of the compounds tested inhibited the Na^+-independent uptake of [^{3}H]biotin (data not shown).

TABLE V

INHIBITION OF BIOTIN UPTAKE INTO BRUSH-BORDER MEMBRANE VESICLES

Vesicles were preloaded with 300 mM sucrose buffered with 20 mM Tris-Hepes (pH 7.4). Initial rates (30 s) of 17 nM [^{3}H]biotin uptake were measured after 1:11 dilution of vesicles into a medium containing 100 mM sucrose, 20 mM Tris-Hepes (pH 7.4) and either 100 mM NaCl or 100 mM KCl. The relative rates of biotin uptake were obtained by substracting the uptakes in the presence of the KCl gradient from the corresponding uptakes measured in the presence of the NaCl gradient. The Na^+-dependent uptake measured in the absence of test compounds is designated as having a relative rate of uptake of 100%. Concentration of test substances was 50 μM. Means $\pm$ S.D. of four to eight experiments. * $P < 0.005$; the other changes are not significant

Compounds	Relative rate of biotin uptake (%)
None, control	100
Biotin	16.6 ± 9.04*
Thioctic acid	32.2 ± 1.09*
Biocytin	85.8 ± 21.6
L-Lactic acid	99.8 ± 7.59
Succinic acid	102.0 ± 26.55
D-Glucose	102.6 ± 16.63

Role of pH and potential differences on Na^+-independent biotin uptake

We investigated the possibility that an inwardly directed H^+ gradient provided a driving force for biotin uptake in renal brush-border and basolateral membrane vesicles. These experiments were performed in the presence of valinomycin and equimolar K^+ concentrations on both sides of the vesicles to short-circuit proton diffusion potentials. As illustrated in Table VI, in the presence of

an inwardly directed H^+ gradient ($pH_{in} = 7.5$, $pH_{out} = 6.0$) biotin uptake into brush border and basolateral membrane vesicles was markedly stimulated relative to that measured under non-pH gradient conditions ($pH_{in} = pH_{out} = 7.5$) but not overshoot was observed. However, incubation a pH 6.0, under non-pH gradient conditions ($pH_{in} = pH_{out} = 6.0$), resulted in stimulation of biotin uptake in both types of membrane vesicles that was not significantly different from that given in experiments performed with an imposed pH gradient (pH_{in} 7.5, pH_{out} 6.0). Accordingly, these results indicated that a pH gradient cannot drive biotin uphill and that it is the pH rather than the presence of a pH gradient per se that increased the uptake of biotin. However, the failure to demonstrate a stimulatory effect of the inward H^+ gradient could be due to its rapid collapse by valinomycin. Such an effect of the ionophore is possible because a conductive pathway for H^+ has been demonstrated in brush-border [14,15] and basolateral membranes [15] so that the H^+ gradient would have been dissipated by the movement of compensating charge, i.e., K^+ efflux [16].

To examine this possibility, the above-mentioned experiments were repeated in the absence of valinomycin (Table VI). The table shows that the rate of biotin uptake was significantly stimulated in both types of vesicles in the presence of the H^+ gradient (pH_{in} 7.5, pH_{out} 6.0) compared with nongradient conditions ($pH_{in} = pH_{out} = 6.0$), but no overshoot was observed. This stimulatory effect of the inward H^+ gradient could be due to biotin entry in its protonated form, i.e., by nonionic diffusion or by conductive biotin$^-$ uptake driven by the inside-positive H^+ diffusion potential. Also demonstrated in Table VI, the equilibrium levels of biotin uptake in brush-border and basolateral membrane vesicles were greater at external pH 6.0. that at pH 7.5, regardless of the initial pH values of the intravesicular fluids. Since we recently observed that binding of organic anions such as urate (unpublished experiments) or prostaglandin E_2 [17] was increased in acidic

TABLE VI

INFLUENCE OF pH AND INWARDLY DIRECTED H^+ GRADIENT ON BIOTIN UPTAKE IN THE ABSENCE AND PRESENCE OF VALINOMYCIN WITH $K^+_{in} = K^+_{out}$

Vesicles were preloaded with 100 mM sucrose, 25 mM K_2SO_4 buffered with either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 6.0) as indicated. Membrane suspensions (10 μl) were then added to 100 μl of a medium containing 19 nM [^{3}H]biotin, 100 mM sucrose, 25 mM K_2SO_4 and either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 6.0). Membranes were treated either with ethanol (1.6%) or valinomycin in ethanol (60 μg/mg of membrane protein) 10 min before transport reaction was started. Data shown are from representative experiments performed in triplicate or quadruplicate (means ±S.D.). Val, valinomycin. * $p < 0.05$ with respect to the values at $pH_{in} = pH_{out} = 6.0$

Expt. No.	Internal pH	External pH	Val	Biotin uptake (fmol/mg protein)			
				1 min	2 min	4 min	80 min
A. Brush-border vesicles							
I	7.5	7.5	(+)	0.7±0.01	1.3±0.71	2.0±0.96	7.5±1.95
	6.0	6.0	(+)	3.5±1.14	3.6±1.54	6.6±1.88	12.2±1.25
	7.5	6.0	(+)	4.8±1.36	4.9±0.85	4.8±1.50	13.4±0.98
II	7.5	7.5	(−)	1.5±0.7			8.7±1.8
	6.0	6.0	(−)	6.0±0.59	8.0±1.11	9.8±0.76	18.3±2.7
	7.5	6.0	(−)	10.1±1.87*	13.1±2.42*	14.9±1.33*	21.1±2.7
B. Basolateral vesicles							
III	7.5	7.5	(+)	0.9±0.01	2.6±0.76	2.7±1.32	8.8±3.17
	6.0	6.0	(+)	4.2±0.67	4.6±2.58	4.7±0.67	15.0±0.44
	7.5	6.0	(+)	3.5±1.16	4.6±1.34	7.2±2.86	13.5±1.27
IV	7.5	7.5	(−)	1.7±0.6			10.2±0.74
	6.0	6.0	(−)	5.1±0.75	5.4±0.69	6.6±1.04	21.2±1.5
	7.5	6.0	(−)	6.7±0.90*	10.1±1.58*	8.4±1.69*	18.2±1.1

TABLE VII

INFLUENCE OF pH ON BIOTIN AND L-GLUCOSE UPTAKE

The membrane vesicles were preincubated for 60 min at 25°C in 100 mM sucrose, 25 mM K_2SO_4 buffered with either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 6.0). Membrane suspensions (10 μl) were then added to 100 μl of the respective preincubation medium containing 19 nM [^{3}H]biotin and 8.8 μM L-[^{14}C]glucose. Uptake of radiolabelled solutes was terminated a 80 min either in the standard way, or by adding 3 ml ice-cold distilled water to vesicle prior to their collection by filtration. Means ± S.D., $n = 5$. * $p < 0.05$ vs. respective controls

Conditions	Biotin uptake (fmol/mg protein)		L-Glucose uptake (pmol/mg protein)	
	Standard stop solution	Distilled water stop solution	Standard stop solution	Distilled water stop solution
Brush-border vesicles				
$pH_i = pH_0 = 7.5$	11.5 ± 0.21	1.8 ± 0.35	4.8 ± 0.29	0.8 ± 0.14
$pH_i = pH_0 = 6.0$	15.7 ± 1.05*	4.2 ± 0.44*	4.2 ± 0.22*	0.8 ± 0.20
Basolateral vesicles				
$pH_i = pH_0 = 7.5$	12.6 ± 1.01	1.9 ± 0.90	5.4 ± 0.78	0.9 ± 0.51
$pH_i = pH_0 = 6.0$	19.9 ± 0.76*	5.0 ± 0.30*	4.5 ± 0.33*	0.7 ± 0.21

media, the possibility of pH-dependent binding of biotin was next studied. In these experiments performed without an imposed pH gradient, membrane vesicles were incubated at pH 7.5 or at pH 6.0 with 17 nM [^{3}H]biotin and 8 μM L-[^{14}C]glucose. The 80-min uptakes of [^{3}H]biotin and L-[^{14}C]glucose were measured either in the standard way or immediately after hypotonic lysis of the vesicles. As indicated in Table VII, the equilibrium uptake values for biotin that were obtained with the standard stop solution were significantly increased at low pH by 37 and 58% in brush-border and basolateral membrane vesicles, respectively. In contrast, L-[^{14}C]-glucose uptake was inhibited at pH 6.0 by 12 and 16% in brush-border and basolateral membrane vesicles, respectively.

When membrane vesicles were subjected to an osmotic shock, 84–85% of the accumulated biotin and L-glucose were lost at pH 7.5, suggesting that binding represented approx. 15–16% of total biotin and L-glucose uptakes. In experiments similar to those shown in Table VII, Warnock and Yee [18] found that 87–90% of L-glucose accumulated in renal brush-border membrane vesicles was lost when the intravesicular space was abolished. A low pH had no effect on the amount of L-glucose remaining bound to brush-border and basolateral membranes after hypotonic lysis, consistent with the findings of others [18,19]. In contrast, the binding of biotin was significantly increased in acidic media, and this component would account for 27 and 25% of the total uptake of biotin by brush-border and basolateral membrane vesicles, respectively. However, this enhanced binding of biotin observed at pH 6.0 cannot explained entirely the increase in total uptake seen in acidic media. Such a discrepancy could be due to some displacement of the biotin bound at pH 6.0 by the osmotic shock procedure.

The next series of experiments were designed to evaluate the possible influence of induced electrical potentials on Na^+-independent biotin uptake into brush-border and basolateral membrane vesicles (Table VIII). In the first series of experiments, a K^+ gradient was established $K^+_{in} > K^+_{out}$ The addition of valinomycin, under these conditions, generated an electronegative interior in both types of membrane vesicles, as evidenced by the increased uptake of triphenylmethylphosphonium, a cation that passively equilibrates with the membrane potential [20]. The ionophore, however, had no measurable effect on biotin uptake suggesting that biotin$^-$ transport is not sensitive to membrane potential in both types of membrane vesicles. In the second series of experiments, vesicles were made inside positive by the application of an inwardly directed K^+ gradient in presence of

TABLE VIII

INFLUENCE OF OUTWARDLY AND INWARDLY DIRECTED K^+ GRADIENTS AND VALINOMYCIN ON BIOTIN AND TRIPHENYLMETHYLPHOSPHONIUM UPTAKE

In $K^+_{in} > K^+_{out}$ experiments membrane vesicles were preloaded in 200 mM sucrose, 10 mM K_2SO_4 and 50 mM Tris-Hepes (pH 7.4). Uptake of either 17 nM [^{3}H]biotin or 10 μM [^{3}H]triphenylmethylphosphonium were measured at 1 min after 1:11 dilution of vesicles into a medium containing 200 mM sucrose and 50 mM Tris-Hepes (pH 7.4). In $K^+_{out} > K^+_{in}$ experiments, experimental conditions and ionic compositions of membrane vesicles and incubation medium were the same as in $K^+_{in} > K^+_{out}$ experiments, but 10 mM K_2SO_4 was present only in incubation media. Vesicles were treated either with valinomycin or ethanol as described in the legend to Table II. Means ± S.D. of five to six experiments. * $P < 0.05$ vs. respective values measured without valinomycin

Conditions	Biotin uptake (fmol/mg)		Triphenylmethylphosphonium uptake (pmol/mg)	
	− Val	+ Val	− Val	+ Val
$K^+_{in} > K^+_{out}$				
Brush-border vesicles	1.22 ± 0.26	1.14 ± 0.23	3.97 ± 0.82	7.00 ± 2.35 *
Basolateral vesicles	1.24 ± 0.54	1.18 ± 0.47	6.86 ± 1.99	11.22 ± 1.14 *
$K^+_{out} > K^+_{in}$				
Brush-border vesicles	2.29 ± 0.62	2.51 ± 0.50		
Basolateral vesicles	2.20 ± 0.84	2.73 ± 0.81		

valinomycin. Under these conditions, biotin uptake was stimulated by 10% in brush-border membrane vesicles and by 24% in basolateral membrane vesicles, but these changes were not statistically significant. These results suggested that a conductive pathway may not play an important role in biotin transport across brush-border and basolateral membranes.

In another series of experiments not illustrated, we found that the 1-min uptake of biotin measured into basolateral membrane vesicles in presence of an inward K_2SO_4 gradient increased linearly ($r = 0.93$, $n = 16$) with increasing medium biotin concentrations throughout the range 0.05–5 mM. No significant effect of valinomycin was measured at any of the concentrations of biotin tested. These findings further supported the view that diffusion of biotin across basolateral membranes was not voltage sensitive.

Discussion

Little is known about the mechanisms by which water-soluble vitamins cross the plasma membrane of renal cell. Direct information about these mechanisms is, to the best of our knowledge, limited to the demonstration that renal microvillus membranes contain Na^+-cotransport pathways for L-ascorbate [5], nicotinate [6] and pantothenate [21]. In this investigation we present the results of experiments that examine the mechanisms of uptake of biotin or vitamin H, a substance involved as coenzyme in a small number of carboxylation and decarboxylation reactions, using plasma membrane vesicles derived from the luminal and contraluminal surface of proximal tubular cells.

We have induced uphill biotin uptake into brush-border membrane vesicles by imposition of an inwardly directed Na^+ gradient. This finding is consistent with the existence of a biotin$^-$/Na^+ cotransporter in the luminal membrane. The finding that the Na^+ gradient-dependent uptakes of biotin and D-glucose are stimulated by inside-negative membrane potentials strongly suggests that the Na^+ gradient-dependent uptake of biotin is electrogenic. Thus, the Na^+/biotin$^-$ cotransport system resembles the D-glucose [12,13], citrate [22], L-ascorbate [5], L-proline [23], *myo*-inositol [24], succinate [25], and L-lactate [26] cotransport systems but differs from the electroneutral nicotinate [6] and L-glutamate [27] cotransport pathways. The effect of electrical potential on L-glutamate transport in renal vesicles, however, remains controversial (for reviews, see refs. 28 and 29). Since biotin is a monocarboxylic acid, the simplest stoichiometry would be the cotransport of two

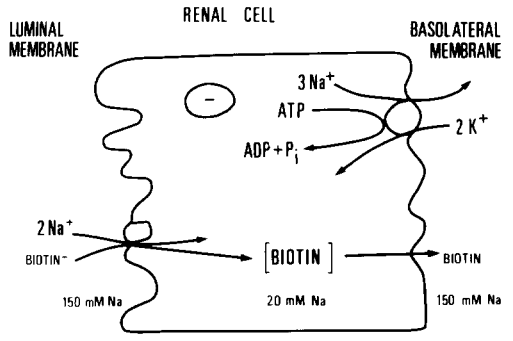

Fig. 3. Model for biotin reabsorption via Na^+/biotin$^-$ cotransport across the brush-border membrane of the proximal tubule. See text for details.

Na^+ ions with one biotin anion across the luminal membrane. However, a more detailed analysis of the Na^+-dependent biotin transport properties and of the possible dependence of this coupled process on pH will be necessary to evaluate this suggestion.

Our studies provide evidence that the Na^+/biotin$^-$ cotransporter detected in brush-border membranes was absent from basolateral membranes. The inability to demonstrate Na^+/biotin$^-$ cotransport cannot be explained by damage of the putative cotransporter in the basolateral membrane during the preparative procedure because both membranes were isolated simultaneously under identical conditions [7]. Moreover, we have shown Na^+ gradient-dependent transport of L-glutamate by rabbit renal basolateral membrane vesicles that was comparable to that previously reported by others [30] in rat renal basolateral membrane vesicles. This finding is consistent with functional activity of the basolateral membranes.

The Na^+ gradient-dependent biotin transport pathway in luminal membranes demonstrated specificity. When tested at a concentration of 50 μM, the uptake of tracer biotin (17 nM) was strongly inhibited by biotin and thioctic acid, is whereas other carboxylic acids which have not structural resemblance to biotin, such as L-lactate and succinate, were not inhibitory. No interaction was detected with D-glucose. Altogether, our observations suggest that the system for transporting biotin, and presumably also thioctic acid, is distinct from those for D-glucose, succinate, and L-lactate, which are also cotransported with Na^+ by an electrogenic mechanism.

Renal brush-border and basolateral membrane vesicles have been shown to possess proton-cotransport or hydroxyl-antiport pathways for various organic inorganic anions [17,31–33]. We found, however, that an inward H^+ gradient, under voltage-clamped conditions, had no effect on biotin uptake, arguing against the presence in both types of vesicles of a biotin$^-$/H^+ cotransporter or its equivalent, i.e., a biotin$^-$/OH^- exchanger. On the other hand, in absence of the valinomycin voltage clamp, we observed that an inward H^+ gradient stimulated biotin uptake, but no overshoot could be detected. There are, at least, two possible explanations for this enhancement. First, the stimulation of biotin uptake could be explained by transport of the biotin anion driven by an inside-positive H^+ diffusion potential. This possibility was precluded, however, because we could not demonstrate the presence of a conductive pathway for biotin$^-$ uptake into these membranes. Second, there could be a small diffusion of biotin in its protonated form. Such a process of nonionic diffusion would be apparent only in absence of valinomycin because of the rapid dissipation of the imposed pH gradient by a voltage clamp with $K^+_{in} = K^+_{out}$ and valinomycin [16]. It should be emphasized, however, that because the pH studies were carried out in the absence of sodium the possible existence of an anion-biotin exchange system in basolateral membrane vesicles cannot be excluded. Indeed, recent studies have demonstrated activation of an anion-OH-exchanger in rat [29] and rabbit [34] renal basolateral membrane vesicles by a sodium gradient.

On the basis of the observed characteristics of biotin transport at both faces of the renal cell we would suggest a simple model whereby biotin could be reabsorbed by the proximal tubule. As illustrated in Fig. 3, it is proposed that the concentrative step is located at the brush-border membrane. Biotin is taken up against its concentration gradient into the renal tubular cell by an electrogenic sodium-biotin coupled process. The driving force for this pathway would be the electrochemical potential difference of Na^+ across the luminal membrane, which is generated by the activity of the basolateral enzyme, ($Na^+ + K^+$)-ATPase. Since we could not demonstrate the presence of a Na^+ or of a H^+ cotransport pathway for

biotin in basolateral membrane vesicles, it is proposed that intracellular biotin diffuses across this membrane into the peritubular fluid, presumably by nonionic diffusion, to complete the process of transepithelial absorption. Clearly, elucidating the metabolism of biotin by renal cells will be necessary to evaluate the model shown in Fig. 3 for uphill biotin reabsorption.

In conclusion, we have demonstrated the existence of an electrogenic Na^+-biotin$^-$ transport system which is confined to the luminal membrane of the rabbit proximal tubule. Such a directly coupled pathway may play an important role in vectorial transport of vitamin H and thus could be an essential mechanism of conservation of this vitamin.

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