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Transport of tricarboxylic acid cycle intermediates by membrane vesicles from renal brush border

(citrate/α-ketoglutarate/proximal tubule)

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ABSTRACT **The uptake of citrate and α-ketoglutarate by membrane vesicles from rabbit renal brush border was studied by a rapid filtration technique. Both compounds exhibited transport characteristics similar to those seen for the sodium-dependent cotransport systems previously described for sugars and amino acids in brush border membranes. The estimated sodium-dependent V_{max} and K_m were 17 nmol per mg of protein per min and 0.18 mM for citrate and 17 nmol per mg of protein per min and 1.0 mM for α-ketoglutarate. The initial rate of citrate transport was 5 times that of sugars and amino acids under comparable conditions. Uptake rates of 0.1 mM citrate and α-ketoglutarate were inhibited by >90% by 10 mM succinate, malate, fumarate, or oxaloacetate, indicating the presence in the brush border membrane of a transport system highly specialized for the renal conservation of intermediates of the tricarboxylic acid cycle.**

A major role of the kidney in its homeostatic regulation of the composition of the body fluids is the conservation of metabolic substrates and intermediates. The study of the renal handling of substances that undergo metabolic transformations (i.e., sugars, amino acids, and tricarboxylic acid cycle intermediates) is greatly complicated by two major difficulties. First, the intact respiring renal cortex will rapidly metabolize such compounds as soon as they enter the tubule cell (1), providing for significant problems in distinguishing between transport and metabolism. In the case of sugars and amino acids this problem has been met to some extent by the use of nonmetabolized analogues such as methyl α-D-glucoside and α-aminoisobutyric acid as models for the transport of sugars (2) and amino acids (3), respectively. Second, renal transport occurs across both the luminal (brush border) and peritubular (basal-lateral) aspects of the epithelium. Consequently, it is often difficult to estimate the extent to which transport of a substance occurs across each membrane and, additionally, to clearly define the characteristic transport properties of each membrane. In the past several years, an approach to this problem has been developed that is based upon the isolation and purification of the membranes from both aspects of the renal proximal tubular epithelium (4). These membranes are essentially devoid of the cellular metabolic apparatus, allowing for the study of transport mechanisms in the absence of the complications produced by metabolic processes. Vesicles prepared from renal brush border membranes rapidly transport sugars and amino acids by a characteristic sodium gradient-dependent process (5, 6). We report here the use of membrane vesicles of the renal brush border to establish the presence in the proximal tubule luminal membrane of a sodium-dependent transport system highly specific for intermediates of the tricarboxylic acid cycle.

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MATERIALS AND METHODS

Preparation of Brush Border Membrane Vesicles. New Zealand White rabbits weighing 2–3 kg were killed with sodium pentobarbital (50 mg/kg). The kidneys were perfused via the renal arteries with buffer C (250 mM sorbitol/12.5 mM NaCl/0.5 mM Na_2EDTA, in 5 mM histidine/imidazole buffer, pH 7.5) warmed to 37°C. All subsequent steps were carried out at 0–4°C. The kidneys were removed, decapsulated, and sliced longitudinally through the pole into anterior and posterior halves. The cortices were dissected free with a razor blade and put through a tissue press with a 1.5-mm pore size. The tissue was placed in buffer C (1 g of tissue per 8 ml of buffer) and homogenized with a Brinkmann Polytron homogenizer (20 sec at maximum power). Subsequent membrane purification utilized differential and density gradient centrifugation as previously developed in this laboratory (7). The final brush border membranes were washed by suspending in 300 mM mannitol in 1 mM Tris/Hepes buffer (1 mM Hepes brought to pH 7.5 with Tris) and pelleted by centrifugation at 50,000 × *g* for 45 min. The membranes were resuspended in the mannitol/Tris/Hepes buffer to a concentration of 8–10 mg of protein per ml and stored at 0–4°C until use. Transport studies were performed either the same day or on the morning after preparation of the membranes. Previous work (7) has indicated that brush border membranes prepared by our methods retain their capacity to transport substrates for at least 7 days.

Purity of the renal brush border membranes was routinely determined by assay of alkaline phosphatase (8) and trehalase (9), markers for the brush border membrane, Na^+,K^+-ATPase (10), a marker for basal-lateral membranes, and succinate dehydrogenase (11), a marker for mitochondria. Protein was determined by the Bio-Rad protein assay (Bio-Rad Laboratories). Brush border membranes were consistently enriched 10-fold and they were essentially free of basal-lateral membranes, mitochondria, nuclei, and endoplasmic reticulum.

Transport Studies. All experiments were conducted in the presence of gradients of NaCl or other salts, except where indicated. Uptake was initiated by addition of 50 μl of brush border membrane suspension to 100 μl of uptake buffer. The uptake buffer was 1 mM Tris/Hepes containing either 300 mM mannitol or 100 mM mannitol plus 100 mM salt. The uptake buffer contained 0.1 mM of the transported substrate (including tracer amounts of the corresponding ^{14}C-labeled compound). In the experiments with inhibitors of transport, the inhibitor concentration was always 10 mM. Uptake was terminated by addition of 850 μl of ice-cold stop buffer consisting of 154 mM NaCl in 1 mM Tris/Hepes, pH 7.5. The suspension was then

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filtered through a 0.45-μm HAWP Millipore filter and washed with 4 ml of stop buffer. The filter was placed in 10 ml of Aquasol (New England Nuclear) and radioactivity was measured by liquid scintillation spectrometry. The stopping, filtration, and washing procedures took less than 10 sec. Correction for nonspecific binding to the filters and membranes was made by subtracting from all data the value of a blank obtained routinely by adding membranes to a tube to which stop buffer had already been added. All experiments were conducted at 22°C.

Materials. All ^{14}C-labeled isotopes were obtained from New England Nuclear. All other chemicals were of reagent-grade purity and were purchased from either Sigma or Calbiochem.

RESULTS

Time Course of Transport. The time course of uptake of 0.1 mM citrate into brush border membrane vesicles in the presence of various salts is illustrated in Fig. 1. In confirmation of the recent abstract by Medow *et al.* (12), there was a rapid overshoot characteristic of the electrogenic sodium cotransport process, now well described (13). Citrate uptake by membranes that had been preequilibrated in the Na^+-containing uptake buffer was significantly greater than that in the presence of 300 mM mannitol only (0.4 nmol per mg of protein per min versus 0.03 nmol per mg of protein per min), indicating facilitation of transport by Na^+ in addition to the gradient-dependent phenomena. Citrate uptake in the presence of a gradient of LiCl was significantly greater than in the presence of gradients of KCl or choline Cl, which had essentially no effect. In prelimi-

Table 1. Initial rates of uptake of various compounds by renal brush border membranes

Compound	Uptake, pmol per mg of protein per 15 sec	
	$+Na^+$	$-Na^+$
Citrate	2830 ± 50	<10
α-Ketoglutarate	660 ± 20	<10
Succinate	2260 ± 10	<10
Pyruvate	700 ± 40	<10
Lactate	850 ± 10	<10
D-Glucose	630 ± 90	27 ± 4
Methyl α-D-glucoside	690 ± 10	10 ± 6
L-Alanine	340 ± 30	40 ± 4
α-Aminoisobutyric acid	99 ± 2	75 ± 4

Data show mean values ± SEM obtained from a single experiment done in triplicate on one batch of membranes. Uptake was estimated from 15-sec incubations in the presence or absence of a 100 mM NaCl gradient. All compounds were at a concentration of 0.1 mM.

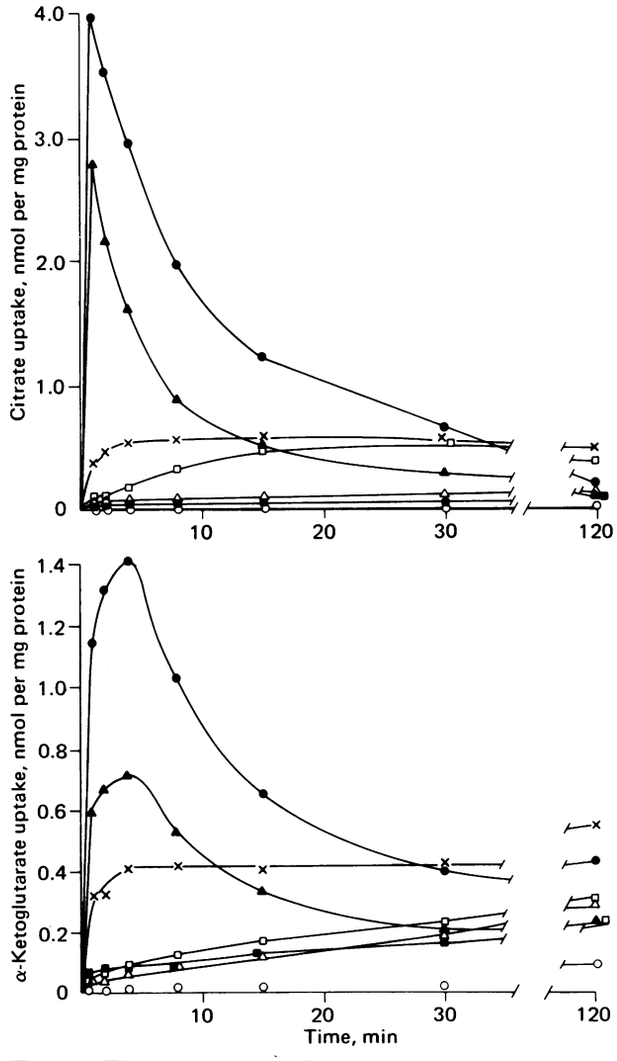

FIG. 1. Time courses of uptake of citrate (*Upper*) and α-ketoglutarate (*Lower*) by renal brush border membranes in the presence of gradients (external to internal) of 100 mM of various salts, in membranes preequilibrated with 100 mM NaCl, or in the absence of salt. ×, NaCl (preequilibrated); ●, NaCl; ▲, NaSCN; △, KCl; □, LiCl; ■, choline Cl; ○, mannitol.

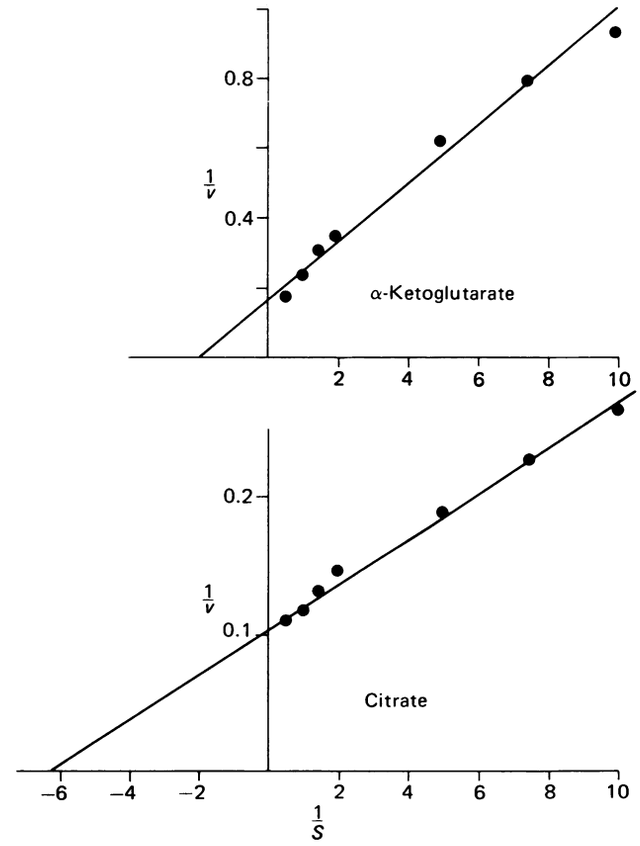

FIG. 2. Lineweaver–Burk plots of sodium-dependent transport of citrate and α-ketoglutarate as a function of their concentrations. Data shown are uptakes at 30 sec from a single typical experiment done in duplicate, Values for $1/S$ are given in mM^{-1}; $1/v$, (nmol per mg of protein per 30 sec)$^{-1}$.

Table 2. Effects of various compounds on the rates of uptake of citrate and α-ketoglutarate by renal brush border membranes

Inhibitor	Citrate uptake		α-Ketoglutarate uptake	
	pmol per mg protein	% inhibition	pmol per mg protein	% inhibition
α-Ketoglutarate	480 ± 150	83	—	—
Citrate	—	—	80 ± 10	90
Succinate	70 ± 20	98	20 ± 10	98
Isocitrate	2580 ± 510	11	590 ± 20	23
Cis-aconitate	1440 ± 380	50	530 ± 30	30
Malate	160 ± 50	94	50 ± 10	93
Fumarate	130 ± 30	96	20 ± 10	98
Oxaloacetate	270 ± 40	90	50 ± 10	94
Pyruvate	2240 ± 650	22	490 ± 96	35
Lactate	2330 ± 410	19	600 ± 30	21
Glutamate	2210 ± 636	23	720 ± 20	5
Glutamine	1500 ± 340	48	430 ± 30	44
Acetate	2410 ± 316	16	600 ± 56	23
Alanine	1560 ± 280	46	450 ± 20	40
D-Glucose	2090 ± 370	28	616 ± 56	21
p-Aminohippurate	2330 ± 580	19	620 ± 50	19

Data are the mean ± SEM of three experiments done in duplicate. Uptake was estimated from 30-sec incubations in the presence of a NaCl gradient. Concentrations of citrate and α-ketoglutarate were 0.1 mM; inhibitor concentrations were 10 mM. The control uptakes of citrate and α-ketoglutarate were 2900 and 760 pmol per mg of protein per 30 sec, respectively.

nary experiments, addition of valinomycin (to increase the K^+ permeability) did not increase the uptake of citrate in the presence of a KCl gradient. This appears to rule out a simple electrical coupling between citrate and cation uptake.

The uptake of α-ketoglutarate was similar to that of citrate but differed in some details—e.g., the peak overshoot occurred at 4 min rather than earlier as seen for citrate. Studies in our laboratory have indicated that the time of the peak overshoot depends on the affinity of the transport system for the substrate and also that for a given substrate the peak occurs earlier as its concentration is reduced. A significant effect of LiCl was not apparent for α-ketoglutarate uptake.

Uptake at equilibrium (120 min) was approximately the same for citrate and α-ketoglutarate. The estimated intravesicular space, 2–4 μl per mg of protein, was similar to that previously obtained for sugars (7).

Initial Rates of Transport. In order to accurately compare initial transport rates of a variety of compounds under identical conditions, we looked at the 15-sec uptakes of these compounds by the same brush border membrane preparation (Table 1). In the presence of a 100 mM NaCl gradient, the uptake rates of citrate and succinate were 4-fold greater than those for the sugars D-glucose and methyl α-D-glucoside. Initial uptake rates of α-ketoglutarate, pyruvate, and lactate were similar to those of the sugars. In the absence of sodium, the uptakes of citrate, α-ketoglutarate, succinate, pyruvate, and lactate were extremely slow. The initial rate of L-alanine uptake in the presence of a NaCl gradient was half that of D-glucose and that of α-aminoisobutyric acid was somewhat slower.

Concentration Dependence. Variations in rates of sodium-dependent uptake with substrate concentration are shown in Fig. 2 for both citrate and α-ketoglutarate. The uptake of both compounds was clearly saturable and showed a good fit to a simple Michaelis–Menten model. As estimated from Lineweaver–Burk plots from three experiments, the K_m and V_{max} values were 0.18 ± 0.01 mM and 17.1 ± 2.3 nmol per mg of protein per min for citrate and 1.00 ± 0.05 mM and 16.6 ± 2.5 nmol per mg of protein per min for α-ketoglutarate. The V_{max} values for both citrate and α-ketoglutarate are more than 10-fold higher than the value reported for D-glucose in a similar preparation (5), whereas the values for K_m are of the same relative magnitude as those given for D-glucose.

Competition Studies. We examined the rates of uptake of 0.1 mM citrate and α-ketoglutarate in the presence of 10 mM concentrations of various other substances (Table 2). Citrate and α-ketoglutarate were strongly mutually inhibitory. Uptake of both compounds was inhibited by >90% by succinate, malate, fumarate, or oxaloacetate. *Cis*-aconitate showed moderate inhibition. Thus there appears to be a highly specific transport system in the renal brush border membrane for tricarboxylic acid cycle intermediates. The data shown in Table 1 indicate that succinate is transported in a manner identical to that of citrate and α-ketoglutarate, and we infer from the competition studies that fumarate, malate, and oxaloacetate share the same sodium-dependent transport system. Of further interest is the fact that we obtained 40–50% inhibition by glutamine and alanine, and D-glucose also was slightly inhibitory.

DISCUSSION

Previous studies have indicated that certain metabolic substrates, including intermediates of the tricarboxylic acid cycle, are reabsorbed from the glomerular filtrate by the proximal tubule and that transport interactions occur among these compounds (1). However, the mechanism of the reabsorption has remained unclear, at least in part because of the methodological problems already discussed. The present study has utilized highly purified renal brush border membranes to investigate in detail the transport of the tricarboxylic acid cycle intermediates.

We have clearly established that citrate and α-ketoglutarate are transported by a sodium-dependent transport system analogous to the systems for sugars and amino acids. Similar V_{max} values for citrate and α-ketoglutarate and the mutual competitive effects suggest that these compounds share a transport system. In addition, competition experiments suggest that this system is also shared by succinate, fumarate, malate, and oxaloacetate. Whether the other metabolic intermediates that significantly inhibited citrate and α-ketoglutarate transport also share the same system remains to be determined. The inhibitory effects of glucose and alanine (Table 2) recall similar

interactions among sugars and amino acids, and this might be explained by competition of the various substrates for energy supplied by the sodium gradient (14). Information is not yet available to indicate whether the renal brush border membrane contains many separate sodium-dependent transporters or whether transport of different substrates is facilitated by one or more polyfunctional transporters. However, detailed kinetic studies may be able to provide some clarification of this point in the near future.

Finally, the fact that the V_{max} values obtained for citrate and α-ketoglutarate are high relative to the value for D-glucose suggests that the transport of tricarboxylic acid cycle intermediates by the renal brush border is a major function of this membrane. Thus the renal proximal tubule has an effective mechanism for the conservation of many substrates, which then may enter directly into the renal metabolic pool.

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