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Specificity of the Transport System for Tricarboxylic Acid Cycle Intermediates in Renal Brush Borders

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Summary. Uptake studies employing renal brush border membranes were used to examine the structural specificity of the TCA cycle intermediate transport system. The kinetics of reciprocal inhibition between succinate and citrate revealed these compounds to be transported by a common mechanism. The Michaelis constant for succinate (0.11 mM) was significantly lower than that of citrate (0.28 mM), indicating that the system has a higher affinity for succinate than for citrate. The specificity of the transport system was determined from the relative inhibitory constants of 40 organic acids on the transport of succinate. The results established that the system is highly specific for 4-carbon, terminal dicarboxylic acids in the *trans*-configuration, including the major intermediates of the TCA cycle. The system is comparatively insensitive to monocarboxylates. Substitution of one of several polar, noncharged residues on the α-carbon of succinate permitted interaction of the substrate with the transport system, but substitutions on both the α and β-carbons did not. The structural specificity of the system is fundamentally different from that of the dicarboxylate and tricarboxylate exchange systems of mitochondria. The role of this transport system in the reabsorption of TCA cycle intermediates from the proximal tubule is discussed.

It has been demonstrated that the rabbit renal brush border possesses a Na^+-dependent transport system for citrate and α-ketoglutarate (Kippen et al., 1979*a*). The transport of these compounds is relatively insensitive to the presence of glucose and alanine and a number of other compounds, but is markedly inhibited by the presence of intermediates of the TCA cycle, leading to the suggestion that the system is specific for the transport of TCA cycle intermediates. The present report examines the structural specificity of this transport system. The findings confirm that the system is highly specific, with interactions between substrate and the transport receptor limited to succinate and several structural analogs, including the major intermediates of the the TCA cycle.

Materials and Methods

Purified brush border membrane vesicles were prepared using a modification of our previously described procedure (Kippen et al., 1979*b*), which employs a calcium precipitation step similar to that developed by Schmitz et al. (1973). Briefly, New Zealand White rabbits (2–3 kg) were sacrificed with sodium pentobarbitol (50 mg/kg). The kidneys were perfused *in situ* with a 37 °C solution of 320 mM sorbitol, buffered to pH 7.5 with 1 mM Tris/Hepes[1]. The cortices were then dissected free of medulla with a razor blade and passed through a tissue press of 1.5 mm pore size. All subsequent steps were carried out at 0–4 °C. The minced cortical tissue was homogenized in 320 mM buffered sorbitol (1 g tissue/8 ml buffer) using a Brinkmann Polytron homogenizer equipped with the PT-10 probe. Homogenization was for 20 sec at maximim power. To this initial homogenate was added enough 100 mM $CaCl_2$, 1 mM Tris/Hepes, pH 7.5, to produce a final concentration of Ca^{++} of 10 mM. The solution was stirred for 15 min and then centrifuged at 1,500 × *g* for 5 min. The supernatant was collected and brush border membranes were pelleted by centrifugation at 50,000 × *g* for 30 min. This membrane preparation, while developed independently, is very similar to methods developed recently by other groups (Kessler et al., 1978; Hilden & Sacktor, 1979; Malathi et al., 1979). Membranes were resuspended in 320 mM sorbitol-Tris/Hepes using a glass-Teflon homogenizer. In those experiments that required the pre-equilibration of the purified membranes in a solution of osmotic concentration other than 320 mOsm, the final membrane pellet was washed in the new solution, repelleted at 50,000 × *g* for 30 min, and resuspended in the new buffer to a final concentration of 4–8 mg protein/ml and stored at 0–4 °C until use. Transport studies were performed on the day that the membranes were prepared.

Purity of the renal brush border membranes was routinely determined by assay of trehalase, a marker for brush border membranes, ($Na^+ - K^+$)ATPase, a marker for basal-lateral membranes,

[1] *Abbreviations:* Tris, Tris(hydroxymethyl)aminomethane; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

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and succinate dehydrogenase, a marker for mitochondria (Kippen et al., 1979*b*). Protein was determined by the BioRad protein assay (BioRad Laboratories). Brush border membranes were consistently enriched 10-fold and were essentially free of basal-lateral and mitochondrial enzyme markers.

Measurement of uptake of ^{14}C-labeled substrates was performed using a Millipore filtration procedure. Uptake was initiated by addition of 50 μl of the brush border membrane suspension to 100 μl of uptake buffer. The uptake buffer used in experiments examining the time course of uptake or the kinetics of transport consisted of 150 mM NaCl (resulting in a final extravesicular Na^+ concentration of 100 mM), 1 mM Tris/Hepes, pH 7.5, 20 mM sorbitol, 0.125 or 0.25 μCi/ml of ^{14}C-labeled substrate, and sufficient unlabeled substrate to produce the desired final concentration. Uptake was terminated by addition of 850 μl of ice-cold stop buffer consisting of 320 mM sorbitol, 1 mM Tris/Hepes, pH 7.5. The suspension was then filtered through a 0.45 μm HAWP Millipore filter and washed with 4 ml of ice-cold stop buffer. The filter was placed in 10 ml of scintillation cocktail (Formula 963; New England Nuclear, Boston, Mass.) and radioactivity determined with a liquid scintillation counter. The stopping, filtering, and washing procedure took less than 10 sec. A correction for nonspecific binding of radioactive label to the membranes and filter was made by subtracting from all samples the level of radioactivity measured in blank samples to which 850 μl of ice-cold stop buffer was added to the uptake buffer immediately prior to addition of membranes. In those studies examining the inhibitory effect of various organic acids on succinate transport, uptake buffer consisted of 150 mM NaCl, 0.125 μCi/ml ^{14}C-succinate, 57 mM Tris (the concentration required to buffer the highest concentration of organic acid used in the study), a concentration of Hepes required to achieve pH 7.5, and sufficient sorbitol to produce a final osmotic concentration of 460 mOsm. The stop buffer consisted of 459 mM sorbitol and 1 mM Tris/Hepes, pH 7.5. All uptake experiments were carried out at 22 °C.

All ^{14}C-labeled substrates were obtained from New England Nuclear (Boston, Mass.). All other chemicals were of the highest commercial grade available.

Results and Discussion

Kinetics of Transport of Citrate and Succinate

The time course of uptake of succinic acid and citric acid under conditions of an inwardly directed Na^+ gradient (Fig. 1) displayed the "overshoot" phenomenon typically associated with Na^+-dependent transport processes for a number of organic substrates in membrane vesicular preparations (Sacktor, 1977). Accumulation of radioactivity was a linear function of time for at least 15 sec, reached a maximum value at 2 min, and then gradually declined toward an "equilibrium" value within 120 min.[2] In the absence of extravesicular Na^+ (replaced isosmotically with sorbitol), uptake at 15 sec of both substrates was reduced 40-fold and no overshoot was observed. In all subsequent experiments, 15-sec incubations under conditions of a 100 mM inwardly directed Na^+ gradient were used to estimate the initial rate of unidirectional influx (J^i) of substrate.

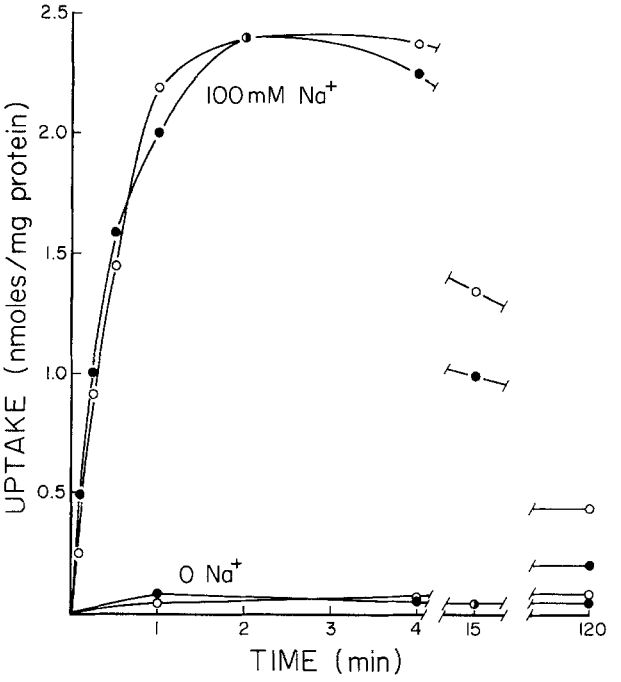


Fig. 1. Time course of uptake of succinate (solid circles) and citrate (open circles) into brush border vesicles in the presence and absence of an inwardly directed 100 mM NaCl gradient. In the condition of 0 Na^+, NaCl was replaced with sorbitol. Data are for a single typical experiment done in triplicate, at a substrate concentration of 0.1 mM

Influxes of succinate and citrate were saturable, reaching a maximum value at high extravesicular substrate concentrations (Fig. 2). The kinetics of transport could be described by the Michaelis-Menten equation. Fitting data to a linear transformation of the Michaelis-Menten equation ($[S]$ *vs.* $[S]/J^i$) permitted calculation of values for the maximal influx (J^i_{max}) and Michaelis constant (K_t) (Table 1).

Kinetics of Inhibition

Succinate and citrate were found to affect the transport of one another in a competitive manner (Fig. 3).

[2] The differences in the 120 min uptakes indicate either that true equilibria of intra- and extravesicular solute concentrations were not achieved in 120 min, or that over that time course changes in vesicular volume occurred. With respect to the first possibility, our experiments have indicated that solute uptake in the absence of salt (particularly NaCl) occurs exceedingly slowly and under some circumstances reaching equilibrium requires 24 hr or more. With respect to the second possibility, detailed theoretical analysis of volume changes requires information not yet available (such as reflection coefficients for each of the solutes.) However, experiments in our laboratory in which vesicular size changes were induced by creating osmotic gradients across the vesicles indicate that the time course of such changes is slow (with a half-time of many minutes). Such phenomena have little relevance to the data presented in this study.

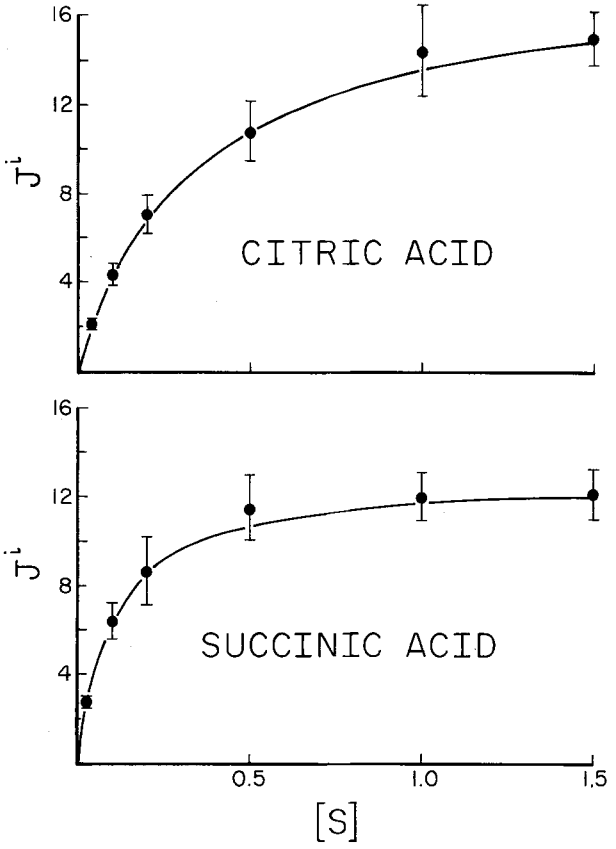


Fig. 2. Effect of increasing concentration of substrate on the influx of citrate and succinate into brush border vesicles. Initial rates of influx J^i are expressed as nmol/(mg protein·min) and substrate concentrations [S] as millimoles per liter. Results are expressed as the means ±1 SEM of data from 2 paired experiments that measured uptake of substrate into vesicles from the same animals; at each point, $n=6$. Curves were calculated from the Michaelis-Menten equation using kinetic constants derived from a linear transformation of the Michaelis-Menten equation ($[S]/J^i$ *vs.* [S]). In these experiments, the mean values for J^i_{max} and K_t, respectively, were: citrate, 18.12 nmol/(mg protein-min), and 0.338 mM; succinate, 12.94 nmol/(mg-min), and 0.105 mM

In four experiments, 0.2 mM succinate increased ($P<0.05$) the Michaelis constant for citrate transport from 0.28±0.05 (SEM) mM to 0.67±0.11 mM. The values for J^i_{max} under these conditions were not significantly different: 19.0±1.5 nmol/(mg protein-min) in the absence of succinate *vs.* 20.1±3.2 nmol/(mg-min) in the presence of 0.2 mM succinate. Three reciprocal experiments with succinate gave similar results: the values for J^i_{max} and K_t in the absence of citrate were 11.7± 2.0 nmol/(mg-min) and 0.12±0.03 mM, respectively; in the presence of 0.2 mM citrate, the J^i_{max} (11.7± 2.8 nmol/(mg-min) was not changed significantly, but the apparent Michaelis constant (K_a) (0.32±0.02 mM) was significantly increased ($P<0.05$). The inhibitory constant (K_i) for citrate as an inhibitor of succinate transport was calculated from the K_t for succinate and the K_a for succinate in the presence of citrate of concentration [i], according to the following equation (Neame & Richards, 1972):

$$K_i = \frac{K_t[i]}{K_a - K_t}.$$

For the experiments described above, the K_i for citrate was 0.15±0.05 mM. The K_i for succinate as an inhibitor of citrate transport was 0.22±0.10 mM. In neither case were these values significantly different from the respective K_t's of these substrates, consistent with the hypothesis that succinate and citrate compete for the same transport pathway.

The transport system shared by citrate and succinate also accommodates α-ketoglutarate. It has been shown (Kippen et al., 1979*a*) that renal brush border membrane vesicles accumulate α-ketoglutarate via a sodium dependent pathway. This transport was inhibited by >90% by both succinate and citrate. Moreover, the transport of succinate (Table 2) and citrate (Kippen et al., 1979*a*) can be inhibited by α-ketoglutarate. Table 1 presents a summary of the kinetic data for succinate, citrate, and α-ketoglutarate. The maximal rates of uptake of these substrates are similar, consistent with their transport *via* a common pathway. It should be noted, however, that the 30% difference in J^i_{max} for succinate and citrate found in the present study is real, and was consistently observed in paired experiments on vesicles prepared from the same animal ($P<0.05$). This difference in J^i_{max} does not imply that these compounds are transported by different pathways. Rather, the difference is probably due to the different net charges of the substrates at pH 7.5 (succinate, −2; citrate, −3), which would be expected to result in different mobility characteristics of the loaded transport mechanism. Of greater relevance to the present study are the values of K_t for the substrates. They vary 10-fold, falling in the order: succinate (0.1 mM) < citrate (0.2 mM) < α-ketoglutarate (1 mM). Michaelis constants provide a rough

Table 1. Kinetic constants for succinate, citrate and α-ketoglutarate transport in renal brush border membrane vesicles

Compound	n	J^i_{max} (nmol/mg protein-min)	K_t (mM)
Succinate	10	13.9±1.0	0.11±0.02
Citrate	4	19.0±1.5	0.28±0.10
Citrate[b]	3	17.1±2.3	0.18±0.01
α-Ketoglutarate[b]	3	16.6±2.5	1.00±0.05

[a] Initial rates of influx were estimated from 15-sec incubations, under conditions of a 100-mM inwardly directed Na^+ gradient. Values are means±SEM.

[b] Data from Kippen et al. (1979*a*).

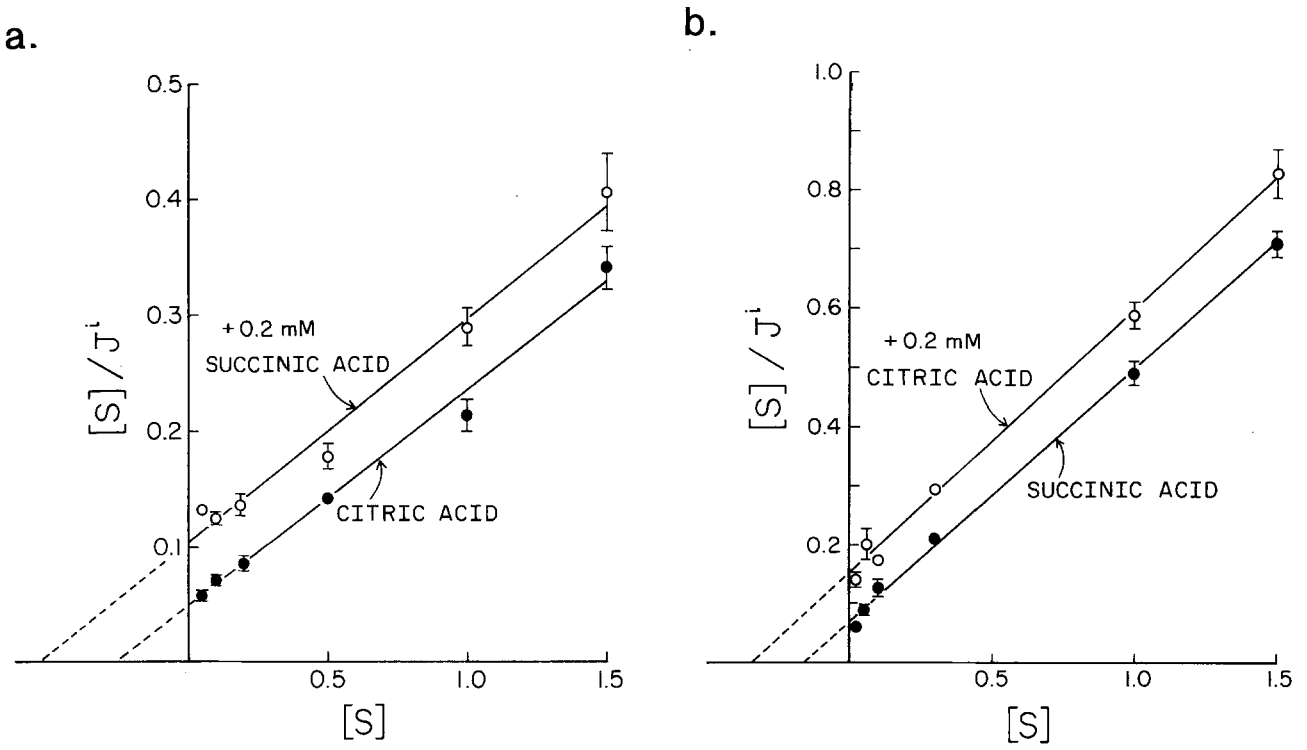


Fig. 3. Effect of succinate on the influx of citrate (*a*), and the effect of citrate on the influx of succinate (*b*), into brush border membrane vesicles. In these plots, the slope of the lines is proportional to $1/J^i_{max}$ or $1/J^i_{max}{}'$, the maximal rates of influx in the absence or presence of inhibitor, respectively. The *x*-intercepts are equal to $-K_t$ or $-K_a$, the Michaelis constants in the absence or presence of inhibitor, respectively. (*a*): J^i_{max} for citrate was 5.37 nmol/(mg-15 sec), and K_t was 0.265 mM; the $J^i_{max}{}'$ was 5.17 nmol/(mg-15 sec), the K_a was 0.538 mM; the inhibitory constant, K_i, for citrate was 0.194 mM. (*b*): The J^i_{max} for succinate was 2.34 nmol/(mg-15 sec), the K_t was 0.163 mM; the $J^i_{max}{}'$ was 2.21 nmol/(mg-15 sec), the K_a was 0.327 mM; the inhibitory constant, K_i, for citrate was 0.199 mM. Each point is the mean ±SEM from one experiment done in triplicate. In those cases where vertical bars are absent, the standard errors were smaller than the graphical representation of the mean. Lines were fit using a least-squares analysis

Table 2. Apparent inhibitory constants ($K_i^\star$) for a series of inhibitors of succinate transport in brush border membrane vesicles.[a]

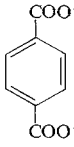
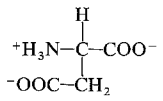
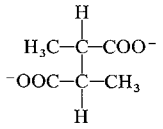
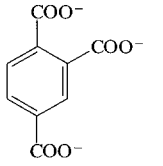
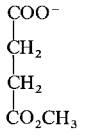
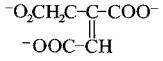
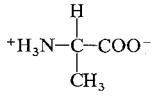
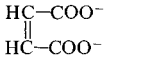
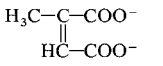
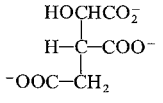
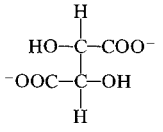
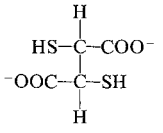
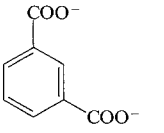
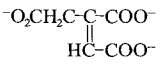
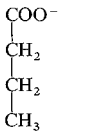
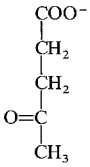
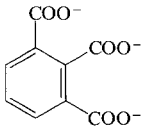
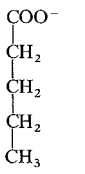
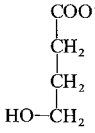
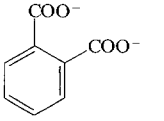
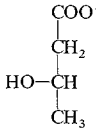
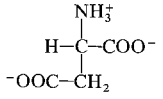
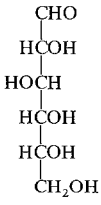
Compound	mol wt	Structure	pK_{a1}	pK_{a2}	$K_i^\star$ (mM)	*r*
Succinic acid	118.1	$H_2C(-COO^-)-COO^-$	4.21	5.64	0.05	0.93–0.99
D,L-Malic acid	134.1	$HO-CH(-COO^-)-CH_2-COO^-$	3.40	5.13	0.07	0.98
Oxaloacetic acid	132.1	$O=C(-COO^-)-CH_2-COO^-$	2.55	4.37	0.16	0.99
D,L-Methyl succinic acid	132.1	$H_3C-CH(-COO^-)-CH_2-COO^-$	4.21	5.63	0.16	0.99
Mesaconic acid	130.1	$H_3C-C(-COO^-)=CH-COO^-$	3.09	4.75	0.18	0.96
L-Malic acid	134.1	$HO-CH(-COO^-)-CH_2-COO^-$	3.40	5.13	0.20	0.95
Citric acid	192.1	$HO-C(-CH_2CO_2^-)(-COO^-)-CH_2-COO^-$	3.13	4.76	0.21	0.99
Fumaric acid	116.1	$HC(-COO^-)=CH-COO^-$	3.02	4.38	0.22	0.99
α-Ketoglutaric acid	146.1	$O=C(-COO^-)-CH_2-CH_2-COO^-$	—[b]	—	0.33	0.99
D,L-Mercapto succinic acid	150.2	$HS-CH(-COO^-)-CH_2-COO^-$	—[b]	—	0.34	0.96
Glutaric acid	132.1	$C(-COO^-)-CH_2-CH_2-COO^-$	4.34	5.41	0.55	0.99
Tricarballylic acid	176.1	$CH_2CO_2^--CH(-COO^-)-CH_2-COO^-$	—[b]	—	0.77	0.99
Terephthallic acid	166.1		3.54	4.46	0.63	0.99
Adipic acid	146.2	$C(-COO^-)-CH_2-CH_2-CH_2-COO^-$	4.43	5.41	1.87	0.98

Table 2 (continued)

Compound	mol wt	Structure	pK_{a1}	pK_{a2}	K_i^* (mM)	r
L-Aspartic acid	133.1		1.99	3.90	2.35	0.99
α,β-Dimethyl succinic acid	146.1		3.77	5.94	2.35	0.98
1,2,4-Benzene tricarboxylic acid	210.1		2.52	3.84	2.41	0.99
mono-Methyl succinic acid	132.1		—[b]	—	3.17	0.99
trans-Aconitic acid	174.1		2.80	4.46	4.60	0.99
L-Alanine	89.1		2.35	—	8.0	0.99
Maleic acid	116.1		1.97	6.24	11	0.97
Citraconic acid	130.1		3.77	6.17	11	0.92
n-Propionic acid	74.8		4.87	—	12	0.78
D,L-Isocitric acid	192.1		3.29	4.71	12	0.89
L-Tartaric acid	150.1		2.89	4.16	13	0.99
α,β-Dimercapto succinic acid	182.2		—[b]	—	13	0.88
Isophthallic acid	166.1		3.62	4.60	13	0.97
cis-Aconitic acid	174.1		—[b]	—	14	0.98
Malonic acid	104.1		2.85	5.70	15	0.94
Acetic acid	60.1		4.76	—	17	0.96
n-Butyric acid	88.1		4.82	—	20	0.53
γ-Ketovaleric acid	116.1		4.64	—	20	0.71
1,2,3-Benzene-tricarboxylic acid	210.1		2.80	4.20	21	0.78
Oxalic acid	90.1		1.27	4.29	24	0.96
n-Valeric acid	102.1		4.84	—	26	0.67
γ-Hydroxybutyric acid	104.1		—[b]	—	30	0.94
Phthallic acid	166.1		2.95	5.41	36	0.90
β-Hydroxybutyric acid	104.1		4.41	—	38	0.68
D-Aspartic acid	133.1		1.99	3.90	60	0.66
D-Glucose	180.2		—	—	60	0.81
Formic acid	46.0	$HCOO^-$	3.75	—	93	0.61

[a] K_i^*'s were determined using the method described in the text and Fig. 4. Values of K_i^* less than 1 mM were calculated from triplicate samples at inhibitor concentrations of 0.1, 0.5, and 1.0 mM; K_i^*'s greater than 1 mM utilized a fourth inhibitor concentration of 10 mM. Values for r represent correlation coefficients for least-squares regression lines fitted to the data. Values for pK_a's from Kortüm, Vogel and Andrussow (1961).

[b] Value unavailable.

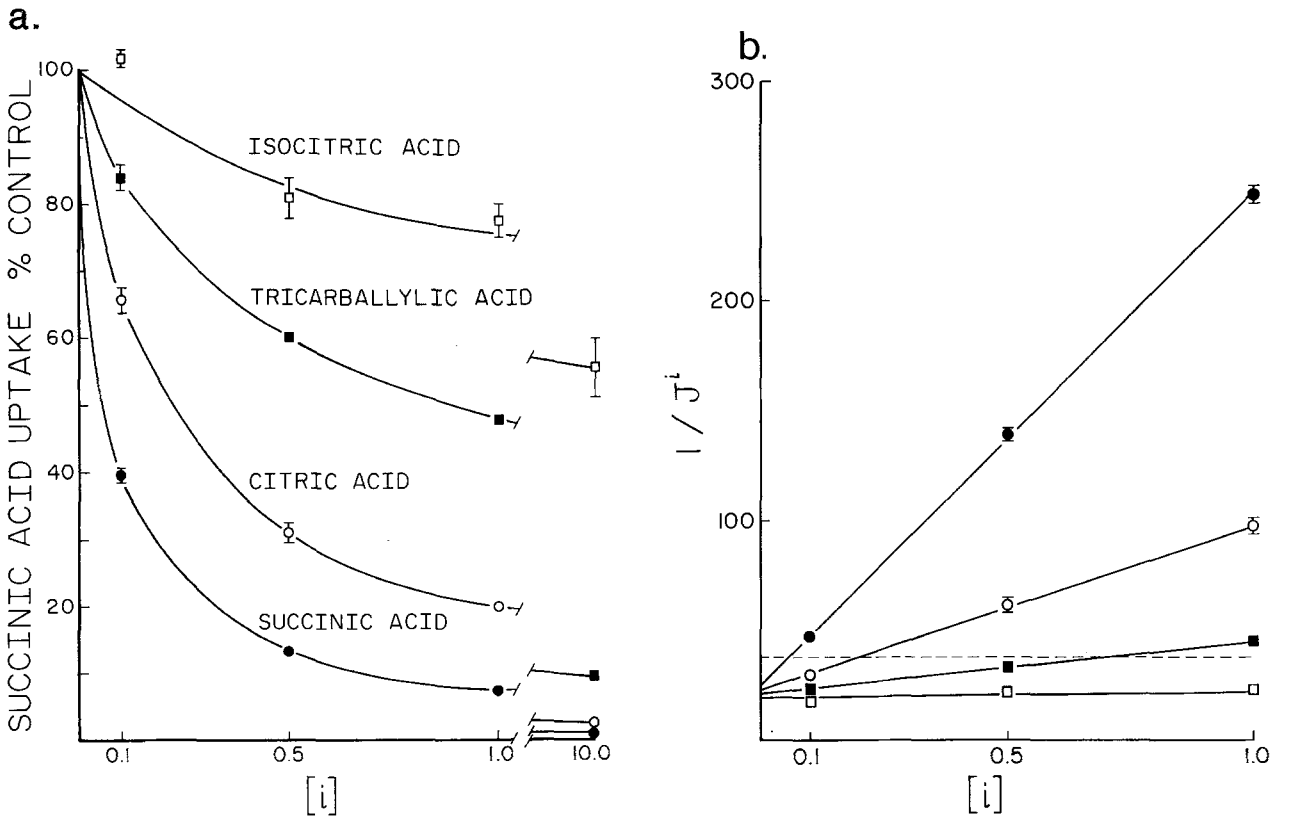


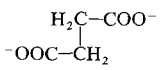
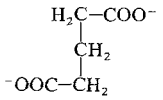
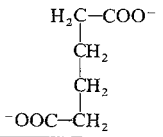
Fig. 4. Effect of increasing concentration of inhibitor on succinate transport in brush border vesicles. (*a*): Influx of succinate is expressed as the percentage of influx in the absence of inhibitor. Units of the abscissa are in millimoles of inhibitor per liter. Concentration of ^{14}C-succinate was kept constant at 0.001 mM. Each point is a mean ± SEM from one experiment done in triplicate. (*b*): Dixon plot of the data in 4*a*; symbols are as defined in 4*a*. Units of J^i are picomoles × 10^{-3}/(mg-15 sec). The horizontal dashed line indicates the value of $1/J^i$ that represents 50% inhibition of succinate influx; intercepts of the dashed line by the calculated regression lines for each inhibitor signify the apparent inhibitory constants or K_i^*, of each inhibitor: succinate, $K_i^* = 0.051$ mM, regression coefficient = 0.99; citrate, 0.21 mM, 0.99; tricarballylate, 0.77 mM, 0.99; isocitrate, 12 mM, 0.89

estimate of the relative affinity of substrates for a transport system. Thus this ordering of K_t's suggests that, of the three substrates, succinate most closely approximates the "ideal" structure for recognition by the transport receptor site.

Structural Specificity of the Transport System

Further examination of the structural specificity of this transport system was accomplished in the following manner. Succinate was chosen as a model substrate against which the inhibitory effects of a wide variety of suspected inhibitors were compared. The experimental protocol utilized a fixed concentration of ^{14}C-succinic acid (1 μM) in the presence of increasing concentrations of inhibitor. Shown in Fig. 4*a* are the results of a typical experiment, comparing the inhibitory effects of three tricarboxylic acids and succinate on succinic acid transport. We assumed that any observed inhibition was strictly competitive in nature and utilized a reciprocal transformation of the Michaelis-Menten equation to estimate the concentration of inhibitor producing 50% inhibition of succinic acid transport (i_{50}) (Fig. 4*b*). Because the concentration of ^{14}C-succinic acid used was much lower than the K_t for succinate transport, the i_{50} is a reasonable estimate of the K_i for inhibitor (Neame & Richards, 1972). It should be noted that it is only for citric acid that the adequacy of the assumption of a strictly competitive interaction between inhibitor and succinate has been experimentally established. Thus it is appropriate to label these values "apparent K_i's" or K_i^*, and emphasize that calculation of K_i^* serves only as a means for comparing the relative inhibitory effects of the compounds examined. For the three tricarboxylates presented in Fig. 4, the K_i^*'s were: citrate, 0.21 mM; tricarballylate, 0.77 mM; isocitrate, 12 mM. The value for succinate was 0.053 mM. Table 2 contains a list of the 40 compounds examined for inhibition of succinate transport, presented in order of increasing K_i^*. Each of the K_i^*'s presented in Table 2 represents the results of single experiments, each of which examined the inhibitory effects of 5 to 7

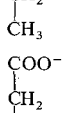
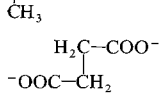
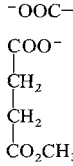
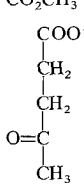
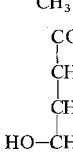
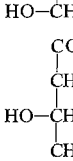
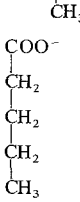
Table 3. Inhibitory constants for an homologous series of terminal dicarboxylic acids

Compound	Structure	K_i^* (mM)
Oxalic acid		24
Malonic acid		15
Succinic acid		0.05
Glutaric acid		0.55
Adipic acid		1.87

compounds on succinate transport. Each experiment tested succinate as an inhibitor, effectively serving as an internal control. The resulting K_i^*'s for succinate were very repeatable: in eight experiments the mean K_i^* was 0.052 ± 0.010 mM, with a range of 0.029 to 0.103 mM. These values for K_i^* correspond quite well with the measured K_t for succinate transport, determined in separate experiments (0.12 ± 0.03 mM). The K_i^* for citrate (0.21 mM) was also quite close to the value of K_t for citrate transport (0.28 ± 0.05). Finally, in the course of the study several compounds were tested two or more times (succinate, citrate, L-malate, fumurate and L-aspartate). The resulting K_i^*'s corresponded within an acceptable margin of experimental error (e.g., L-aspartate, 2.23 *vs.* 2.46 mM). Considering the range of K_i^*'s for the different compounds studied, 0.05 to 100 mM, the observed variation did not affect our interpretation of the results.

The compounds examined as potential inhibitors of succinate transport fell into several structural categories, each of which provided information concerning the requirements for recognition by the succinate-citrate transport system. Table 3 presents inhibitory constants of a homologous series of terminal dicarboxylic acids. From the first to the second number of the series (oxalate to malonate, respectively), the K_i^* decreased from 24 to 15 mM. The addition of another methylene group (i.e., succinate) drastically reduced the K_i^* down to 0.05 mM. For the higher members of the series the K_i^* increased with chain length. This latter effect must be due to steric factors rather than lipophilic interactions. A similar trend was observed for a homologous series of monocarboxylic acids (Table 4). However, a striking difference is noted between the K_i^*'s of the 4-carbon monocarboxylate, butyrate, and the 4-carbon dicarboxylate succinate: butyrate has a K_i^* 400 times higher than succinate. Thus it appears that the key constitutive elements for the binding of succinate to the transport site are its two terminal carboxyl groups. The requirement for the charged carboxyl group at the β-carbon, as in the case of succinate at pH 7.5, is demonstrated by the comparatively poor inhibitory effectiveness of

Table 4. Inhibitory constants for an homologous series of monocarboxylic acids[a]

Compound	Structure	K_i^* (mM)
Formic acid		93
Acetic acid		17
n-Propionic acid		12
n-Butyric acid		20
Succinic acid		0.05
mono-Methyl succinic acid		3.17
γ-Ketovaleric acid		20
γ-Hydroxybutyric acid		30
β-Hydroxybutyric acid		38
n-Valeric acid		26

[a] The K_i^* for succinate is presented for purposes of comparison.

Table 5. Effect of *cis-trans* configuration on inhibition of succinate transport

Compound	Structure	K_i^* (mM)
Succinic acid	$H_2C(-COO^-)-{}^-OOC-CH_2$	0.05
Fumaric acid	$HC(-COO^-)=CH-{}^-OOC$	0.22
Maleic acid	$HC(-COO^-)=HC-COO^-$	11
Mesaconic acid	$H_3C-C(-COO^-)=CH-{}^-OOC$	0.18
Citraconic acid	$H_3C-C(-COO^-)=HC-COO^-$	11
trans-Aconitic acid	$^-O_2CH_2C-C(-COO^-)=CH-{}^-OOC$	4.6
cis-Aconitic acid	$^-O_2CH_2C-C(-COO^-)=HC-COO^-$	14

4-carbon monocarboxylates having polar but non-charged substitutions at the γ-carbon (Table 4). *Mono*-methyl-succinate, γ-ketovalerate, and γ-hydroxybutrate had K_i^*'s of 3.2, 20, and 30 mM, respectively.

Recognition of potential substrates is given preferentially to 4 carbon dicarboxylic acids in the *trans*-configuration. As indicated in Table 5, the K_i^* for fumaric acid (0.22 mM) is 50 times lower than that for maleic acid (11 mM). The disparity between the inhibitory effectiveness of the *trans*- and *cis*-isomers, mesaconic acid and citraconic acid is equally clear (0.18 *vs.* 11 mM, respectively). In the *trans*-configuration, succinate presents two identical faces to the receptor site. Substitution on the α-carbon, but not both α and β, still leaves one "succinate-like" face, and as such might be expected to result in compounds retaining a good inhibitory potential. As presented in Table 6, α-substitution of $-OH$, $-CH_3$, or $-SH$ moieties results in effective inhibitors, while substitution on both the α and β-carbons precludes effective inhibition of succinate transport. For example, the K_i^* for methyl succinate is 0.16 mM, while the di-substituted α, β-dimethyl succinate has a K_i^* of 2.4 mM (Table 6). The α-residues that are compatible with binding are all neutral. When a positively charged residue such as the amino group of D- or L-aspartate is substituted at the α-carbon, inhibitory effectiveness is reduced 10 to 100 times. This suggests that the receptor site contains protonated amino residues near the binding site for the α-carbon of succinate. Thus aspartate would not be expected to bind effectively due to electrostatic repulsion between the charged amino groups. The differences in K_i^* between D- and L-aspartate (60 *vs.* 2.35 mM, Table 6) probably reflect

Table 6. Effect of α or α, β substitution on inhibition of succinate transport

Compound	Structure	K_i^* (mM)
Succinic acid	$H_2C(-COO^-)-{}^-OOC-CH_2$	0.05
Methyl succinic acid	$H(-CH_3)(-COO^-)-{}^-OOC-CH_2$	0.16
α,β-Dimethyl succinic acid	$H(-CH_3)(-COO^-)-{}^-OOC-C(H)(-CH_3)$	2.35
Mercapto succinic acid	$H(-SH)(-COO^-)-{}^-OOC-CH_2$	0.34
α,β-Dimercapto succinic acid	$H(-SH)(-COO^-)-{}^-OOC-C(H)(-SH)$	13
D,L-Malic acid	$H(-OH)(-COO^-)-{}^-OOC-CH_2$	0.07
L-Tartaric acid	$H(-OH)(-COO^-)-{}^-OOC-C(H)(-OH)$	13
L-Aspartic acid	$H(-{}^+NH_3)(-COO^-)-{}^-OOC-CH_2$	2.35
D-Aspartic acid	$NH_3^+(-H)(-COO^-)-{}^-OOC-CH_2$	60

differences in the position of charged residues at the receptor site.

The differences in K_i^*'s between the tricarboxylates tested (Table 7) suggest that polar residues at the α-carbon can actually stabilize binding of substrate to the transport site. Citrate, tricarballylate, *trans*-aconitate and isocitrate all have comparatively large residues substituted at the α-carbon: the former three have acetate residues at the α-carbon, the latter a glycolate residue. The high K_i^*'s of tricarballylate, *trans*-aconitate, and isocitrate (0.77, 4.6, and 12 mM, respectively) suggest that such large substituents produce a sufficient degree of steric hindrance with the receptor that binding is severely limited. Citrate, however, has a rather low K_i^* (0.21 mM). This is probably due to the α-OH of citrate (Table 7) which could interact electrostatically with positively charged

Table 7. K_i^*'s of tricarboxylic acids on succinate transport

Compound	Structure	K_i^* (mM)
Succinic acid	H_2C-COO^- $^-OOC-CH_2$	0.05
Citric acid	$CH_2CO_2^-$ $HO-C-COO^-$ $^-OOC-CH_2$	0.21
Tricarballylic acid	$CH_2CO_2^-$ $H-C-COO^-$ $^-OOC-CH_2$	0.77
trans-Aconitic acid	$CH_2CO_2^-$ $C-COO^-$ $^-OOC-CH$	4.6
D,L-Isocitric acid	$HOCHCO_2^-$ $H-C-COO^-$ $^-OOC-CH_2$	12

amino residues in the receptor, the presence of which was discussed above, thereby stabilizing its binding. If the binding of citrate is sensitive to the presence of charged amino residues in the receptor, then binding should be sensitive to pH. At pH 7.5 amino residues are positively charged. At higher pH's dissociation of protons will make those amino groups neutral, reducing the effectiveness of electrostatic interactions with an α-OH. In fact, it has been observed that the initial rate of transport of 0.1 mM citrate into renal brush borders is 2.5 times higher at pH 7.5 than at pH 9.5 (Kippen et al.[3]). Other neutral residues at the α-carbon should also be effective at stabilizing substrate binding. In particular, it would be of interest to examine the effect of a negatively charged residue at the α-carbon, such as a sulfonyl group. We expect that a compound like α-sulfonyl succinic acid would be a particularly good inhibitor of succinate transport.

It is of interest to note that glucose and alanine were capable of a limited degree of inhibition of succinate transport (K_i^*'s of 60 and 8 mM, respectively, Table 2). These effects recall similar observations of interactions between the transport systems for sugars and amino acids which have been explained by competition of the various substrates for energy supplied by the sodium gradient (Murer, Sigrist-Nelson & Hopfer, 1975). However, further work is necessary to clarify the cause of the interactions between these disparate substrates.

[3] Kippen, I., Wright, S.H., Hirayama, B., Klinenberg, J.R., Wright, E.M. The sodium gradient-dependent transport of citrate in membrane vesicles from renal brush border. (*Submitted*)

Comparison with Mitochondrial Transport Systems

We are aware of only one other system that handles TCA cycle intermediates: the inner membrane of mitochondria. There are, however, three striking dissimilarities between the mitochondrial and brush border membrane transport systems. (i) The brush border system is Na^+-dependent and capable of uphill, concentrative transport; the inner mitochondrial membrane has a multiplicity of systems for dicarboxylates and tricarboxylates that are capable of only an electroneutral exchange of substrates (LaNoue & Schoolwerth, 1979). (ii) The tricarboxylate system of mitochondria transports isocitrate and *cis*-aconitate, as well as citrate, and is markedly inhibited by 1,2,3-benzentricarboxylic acid (LaNoue & Schoolwerth, 1979); the brush border system transports citrate but not these three other substrates (Table 2). (iii) The dicarboxylate systems of mitochondria have a greater affinity for malonate than for succinate (LaNoue & Schoolwerth, 1979); in the brush border, the system has its greatest apparent affinity for succinate, and the high K_i^* for malonate (15 mM; Table 3) suggests that it would be transported marginally at best. These differences in the transport systems of the brush border and mitochondria also serve as further evidence that the preparation of purified brush border used in the present study is free of mitochondrial contamination (Kippen et al., 1979*b*).

Conclusions

The results presented demonstrate that the rabbit kidney brush border membrane transports succinic acid, citric acid, and α-ketoglutaric acid by a Na^+-dependent process. The kinetic experiments suggest that these compounds are transported by a common system. A detailed examination of the structural specificity of this transport system demonstrated that it is highly specific for a well-defined class of structural analogs of succinic acid.

The schematic molecular structure presented in Fig. 5 summarizes the information on structural specificity of binding to the succinate receptor. Substrates must contain a 4 carbon, nonbranched dicarboxylate backbone in the *trans*-configuration. The boxed portion of the molecule signifies the requirement for an intact acetate moiety for successful binding. Substitution of certain groups at the α-carbon is compatible with binding: R_1 may be $-H$ (as in the case of succinate), $-CH_3$, $-OH$, $-SH$, but not $-NH_3^+$; R_2 can be $-H$, or CH_2OOH, but not CHOHCOOH. It should be emphasized that we have insufficient data to make a statement defining R_1 as the levorota-

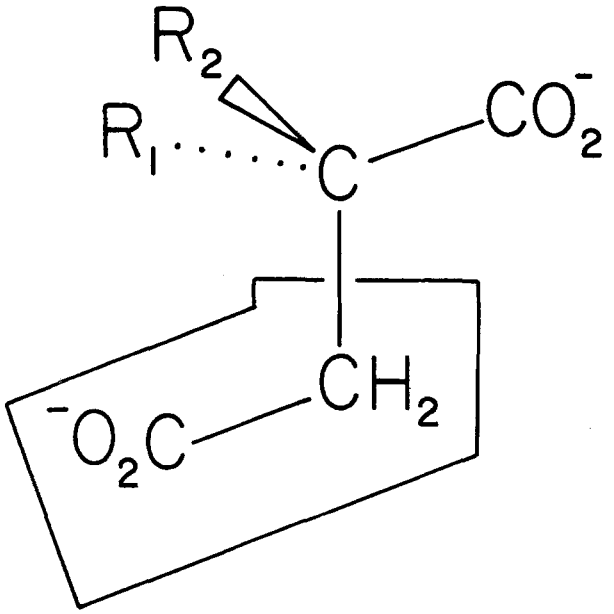


Fig. 5. Model of the basic structural elements required for binding of substrate to the receptor of the brush border succinate/citrate transport system. Bonds represented as solid lines lie in the plane of the page; the dotted line at the α-carbon (R_1) lies behind the page; the wedge (R_2) lies above the page. *See* text for discussion

tory position of the α-carbon. The difference in K_i^* between D- and L-aspartate indicates that the receptor structure is sensitive to the stereoconfiguration of substrates. However, the fact that L-malate and DL-malate had similar K_i^*'s suggests that for certain R groups, R_1 and R_2 are interchangeable. Thus the drawing of D and L positions on the α-carbon in Fig. 5 serves only as a reminder that stereospecificity must be considered a potential factor in successful binding of substrate to the transport receptor.

The strict structural requirements of this dicarboxylate transport system are effective in defining a transport process specific for TCA cycle intermediates. The succinate backbone with α-substitution allows the interaction of the transport receptor with succinate, fumarate, malate, oxaloacetate, citrate and, to a lesser extent, α-ketoglutarate. While the evidence presented here did not demonstrate that the effective inhibitors of succinate transport are themselves transported, the data are consistent with such a hypothesis. The role of such a transport system in the kidney brush border membrane in sparing the loss of important biochemical intermediates is obvious. Emphasis in the past has been placed on the study of brush border transport processes for a limited number of organic compounds such as sugars and amino acids. The present study indicates that the brush border has separate systems for the transport of a wide variety of biochemically important compounds.

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