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Transport of β-Hydroxy-β-Methyl-Glutarate and β-Hydroxbutyrate by Renal Brushborder Membrane Vesicles

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Summary

The uptake of β-hydroxy-β-methyl-glutarate (HMG) and β-hydroxy-butyrate (β-HB) by renal brushborder membrane vesicles prepared from normal and starved rats was examined. HMG and β-HB uptake show a Na^+ gradient-induced overshoot, suggesting luminal cotransport of these organic acids. Kinetic analysis of HMG and β-HB uptake revealed a single component carrier system and a diffusional component for each compound. Vesicles from starved rats exhibit the same transport characteristics as those from normal rats. The transport interactions of other organic acids with HMG were examined and revealed that citrate is a competitive inhibitor, which implies that the compounds share a common organic acid carrier.

Speculation

The possibility exists that by administration of high oral doses of citrate, urinary citrate can be elevated sufficiently to competitively inhibit the tubular reabsorption of β-hydroxy-β-methyl-glutarate. This, in addition to the buffering properties of citrate, could be of significance in the treatment of the severe metabolic acidosis seen in patients with β-hydroxy-β-methyl-glutarate-CoA lyase deficiency.

The compound β-hydroxy-β-methyl-glutaric acid (HMG) is a key intermediate in the metabolic pathways of ketogenesis and cholesterol synthesis. Biosynthesis of HMG, mediated by HMG-CoA synthetase, occurs primarily from condensation of two acetoacetyl CoA molecules to form HMG-CoA. Subsequent hydrolysis of the acyl-CoA compound release the free organic acid HMG. Additional HMG-CoA is formed as an intermediate during leucine catabolism. A recent report documenting renal tubular transport of the organic acids citrate and α-ketoglutarate (11), which are also key metabolic intermediates, stimulated our interest in the renal handling of HMG.

The normal hepatic response to fasting is an increased rate of acetoacetyl CoA production, which is reacted with acetyl CoA to form HMG-CoA. The latter compound is then cleaved to release free acetoacetate. Because acetoacetate is normally in equilibrium with β-hydroxy-butyrate (β-HB) in blood, we also examined the transport of β-HB in renal brushborder membrane vesicles. Further, because starvation increases the synthesis rate of both HMG and β-HB, we observed the effects of starvation on the renal transport of these two compounds.

The clinical relevance of the present study is underscored by descriptions of children with an inherited deficiency of the enzyme HMG-CoA lyase, resulting in severe metabolic acidosis and mental retardation (5, 6, 20). In the face of this enzyme deficiency, large quantities of HMG are produced without further catabolism. Filtration and subsequent renal tubular reabsorption of this compound could contribute to a state of metabolic acidosis. It was, therefore, of interest to determine the characteristics of renal brushborder transport in normal animals in order to understand the mechanism underlying this disorder. The results of these studies form the basis for this report.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats weighing 150–200 g were obtained from Charles River Breeding Laboratories (Wilmington, MA). In some experiments, animals were divided into two groups: Group I received Purina rat chow and water *ad libitum* and Group II was given only water *ad libitum* for 72 h before sacrifice. Ketosis was documented in Group II animals by determination of plasma β-HB levels, which were found to be three times control values. Unless otherwise noted, only animals from Group I were used.

Membrane preparation. After decapitation, the rat kidneys were removed, decapsulated and placed in normal saline on ice. Cortical slices were made with a Stadie-Riggs microtome and the tissue weighed. Brushborder membrane vesicles were then prepared using the method of Booth and Kenny (2) modified as described by Weiss *et al.* (21). The final membrane pellet was suspended in THM buffer, pH 7.4 (2 mM Tris/HEPES + 100 mM mannitol) to a protein concentration of 0.3–0.4 mg/ml as determined by the method of Lowry *et al.* (13).

All measurements of uptake were performed using Millipore filtration on HAWP filters (0.45 μm) according to the technique described by McNamara *et al.* (14). The vesicle preparation was osmotically active and did not metabolize substrate. Results are expressed as nmoles of substrate uptake/mg protein *versus* time. Statistical significance was calculated using Student's *t* test. Preparation of isolated renal tubule fragments was by a method previously described (19).

[3-^{14}C]-β-OH-β-CH_3-glutarate, (40–60 mCi/mmole), and DL-β-[3-^{14}C]-hydroxybutyric acid (1–5 mCi/mmole) were obtained from New England Nuclear Corp. (Boston, MA). All unlabeled compounds were obtained from commercial sources and were of the highest purity available.

RESULTS

Organic acid uptake. The uptake of 0.092 mM β-hydroxy-β-methyl-glutarate (HMG) and 0.101 mM β-hydroxybutyrate (β-HB) by membrane vesicles incubated in 100 mM NaCl gradient was very rapid, reaching the peaks of their overshoots at 1 and 0.5 min., respectively. Brushborder vesicles, allowed to equilibrate in 100 mM NaCl before the start of incubation with substrate, showed no overshoot with either substrate, suggesting that uptake

of these organic acids was Na^+-dependent. These data are shown in Figure 1, panels A and C.

To determine whether the Na^+-gradient overshoot was due to cotransport of the negatively charged organic acid HMG or to HMG accumulation due to the assymetric charge distribution caused by Na^+-influx, vesicles were preequilibrated with 100 mM KCl in the presence of valinomycin (8 μg/mg protein) and the uptake of HMG under Na^+-gradient conditions was examined. If the overshoot was solely charge-dependent, it should have been absent with valinomycin as K^+ should equilibrate rapidly in the presence of valinomycin, to cancel any potential difference created by Na^+-influx. Figure 2 shows that an overshoot occurs in the presence of the ionophore suggesting that the overshoot is due largely to Na^+-HMG cotransport.

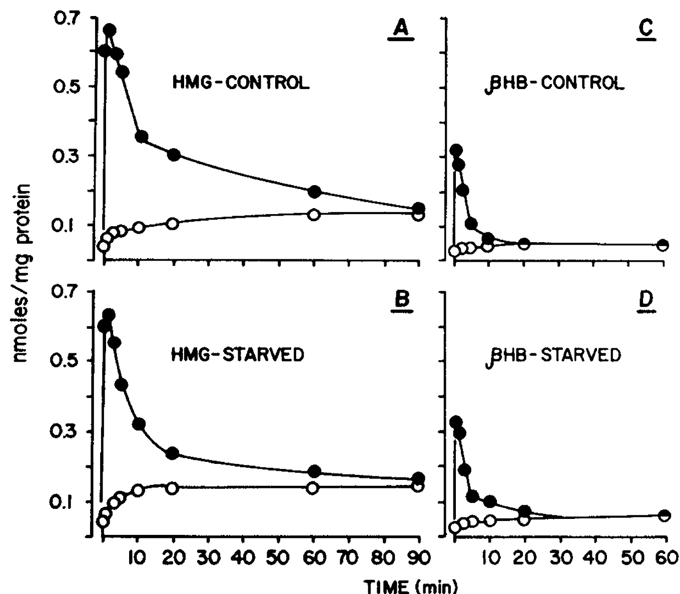

Fig. 1. Time-dependent uptake of 0.092 mM HMG (*panels A and B*) and 0.101 mM β-hydroxy butyrate (*panels C and D*) by renal brushborder membrane vesicles prepared from normal rats (*panels A and C*) and starved rats (*panels B and D*). The vesicles were suspended in THM buffer and substrate uptake was measured in the presence of a 100 mM NaCl gradient (●——●) or under conditions of equilibration of NaCl between extravesicular and intravesicular space (○——○), as described in the text for the times shown. Values shown are the means of at least 25 experiments done in triplicate.

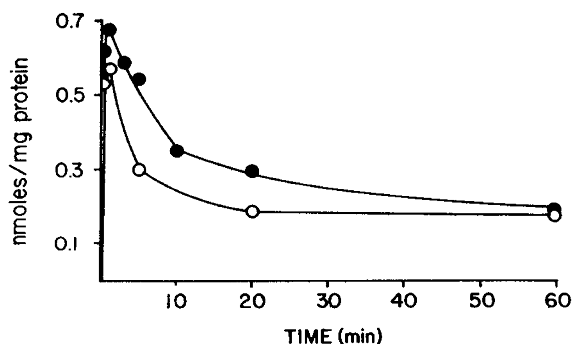

Fig. 2. Time-dependent uptake of 0.092 mM β-hydroxy-β-methyl-glutarate by renal brushborder membrane vesicles in the presence of a NaCl gradient with (○——○) and without (●——●) valinomycin (8 μg/mg protein). The vesicles were suspended in THM buffer and in experiments with valinomycin were allowed to preequilibrate with 100 mM KCl. Valinomycin was added in ethanol (0.9% final concentration); ethanol was added to the incubation fluid of nonvalinomycin containing vesicles as a control. Values shown are the means of at least three experiments done in triplicate.

Table 1. *Cation-dependence of HMG-transport, % of control uptake*[1]

Cation	Incubation time (min)		
	0.5	1	5
Control (100 mM Na^+)	100	100	100
RbCl (100 mM)	3.7	11.9	20.6
CsCl (100mM)	16.7	19.0	26.1
LiCl (100 mM)	16.5	18.3	20.7
KCl (100 mM)	16.3	14.0	23.1

[1] Vesicles were prepared in Na^+-free THM buffer (2 mM Tris-HEPES + 100 mM mannitol, pH 7.2). To initiate uptake, vesicles were added to tubes containing the substrate and one of the various cations. Results are expressed as a % of the uptake occurring in 100 mM NaCl and represent the means of at least six separate determinations.

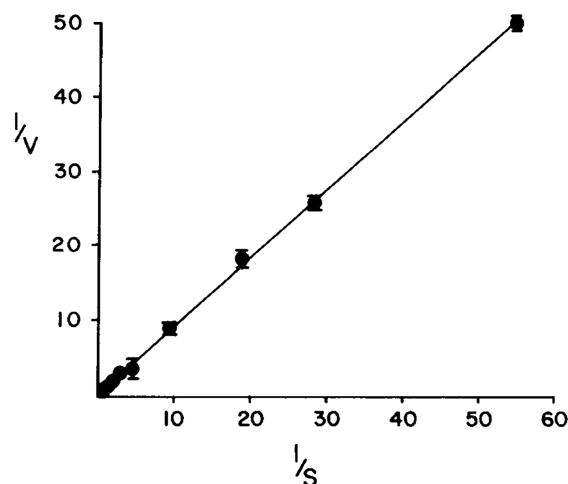

Fig. 3. Concentration-dependent uptake of β-hydroxy-β-methyl-glutarate (18 μM–3.9 mM) by renal brushborder membrane vesicles. β-Hydroxy-β-methyl-glutarate uptake was measured after a 0.5-min incubation in vesicles suspended in THM buffer, which were allowed to preequilibrate in 100 mM NaCl before the start of incubation. Values shown are the means of at least three experiments done in triplicate. 1/V, reciprocal of velocity of uptake (nmole substrate/mg protein/0.5 min); 1/S, reciprocal of substrate concentration (mmole/liter).

Sodium-dependence of uptake. The nature of cation-dependence of HMG uptake was examined. Vesicles were prepared in sodium-free THM buffer, and the initial phase of uptake of 0.09 mM HMG studied under gradient conditions using 100 mM concentrations of KCl, RbCl, CsCl, LiCl and choline chloride. No overshoot was apparent using cations other than sodium. The 1-min uptake of HMG under gradient conditions using these cations was less than 20% of the uptake with Na^+. The 1-min uptakes of HMG using these various cations were similar to those seen under NaCl-equilibrated conditions. These data are summarized in Table 1.

Concentration-dependence of uptake. The 0.5-min uptake of HMG under Na^+-gradient conditions was studied, using substrate concentrations from 0.95 μM to 3.9 mM. Similar studies were carried out at 0.25 min using β-HB with concentrations from 6.3 μM to 9.8 mM. Correction of the data for a sodium-independent and/or diffusional component was made by carrying out identical concentration-dependent studies under Na^+-equilibrated conditions. The resulting data points, when plotted by the double-reciprocal method, resulted in a line that passed through the origin (Fig. 3), indicating that Na^+-equilibrated uptake is diffusional. The slope of the line was calculated and used as a value for K_D in all subsequent substrate velocity calculations describing uptake under Na^+-gradient conditions ($K_D = 0.93 \pm 0.11$).

Computer analysis by a method reported previously (19) of the

Table 2. *Effects of starvation on vesicle uptake and derived kinetic parameters*[1]

Substrate	Uptake[1]			
	Control		Starved	
HMG[2] (0.092 mM) 1 min uptake	0.675 ± 0.04 (n = 56)		0.636 ± 0.02 (n = 44)	
β-HB[2] (0.1 mM) 0.5 min uptake	0.315 ± 0.015 (n = 24)		0.330 ± 0.02 (n = 24)	
	Derived kinetic parameters			
	K_m	V_{max}	K_m	V_{max}
HMG	0.0106 ± 0.005	0.4019 ± 0.024	0.013 ± 0.0098	0.579 ± 0.05
β-HB	1.579 ± 0.135	3.649 ± 0.602	1.46 ± 0.21	3.74 ± 0.73

[1] Uptake is expressed as nmole substrate/mg protein. K_m; and V_{max} values were obtained as described in the text. K_m, mM; V_{max}, nmole/0.5 min/mg protein for HMG; and nmole/0.25 min/mg protein for β-HB.
[2] HMG, β-hydroxy-β-methyl-glutarate; β-HB, β-hydroxy-butyrate.

Table 3. *Transport interactions of HMG with organic acids*[1]

Control	Inhibitor (6 mM)			
	α-Ketoglutarate	Citrate	Acetoacetate	Maleic acid
0.675 ± 0.21	0.0382 ± 0.0063	0.0614 ± 0.0086	0.1296 ± 0.011	0.3178 ± 0.011
(n = 18)	(n = 18)	(n = 18)	(n = 18)	(n = 18)
% of Control	5.7	9.1	19.2	47.1

[1] Vesicles were incubated for 0.5 min with 0.092 mM β-hydroxy-β-methyl-glutarate and 6 mM inhibitor under 100 mM Na^+-gradient conditions. Results are expressed both as nmole β-hydroxy-β-methyl-glutarate/mg protein and as a % of control uptake in the presence of inhibitor.

data obtained for HMG uptake under Na^+-gradient conditions showed a single transport system with an apparent transport K_m of 0.0106 mM ± 0.0055 and a V_{max} of 0.4019 ± 0.0240 nmoles/0.5 min/mg protein. Similar analysis of data obtained from vesicles made from kidneys of animals starved for 72 h before sacrifice gave an apparent transport K_m of 0.0130 mM ± 0.0098 and a V_{max} of 0.579 ± 0.050 nmoles/0.5 min/mg protein. Although the difference between the V_{max} values of the two groups is highly statistically significant ($P < 0.001$), we question any physiologic significance. To test this, we performed HMG uptake studies in isolated tubule fragments made from control and starved animals. The 30 min distribution ratios achieved did not differ significantly (control = 2.70 ± 0.2 *versus* starved = 2.36 ± 0.4). Starvation also had no effect on the uptake of β-HB by vesicles. These data are summarized in Figure 1, panels B and D and in Table 2.

Effects of other organic acids on HMG uptake. The interaction of the HMG transport system with other organic acids was examined by studying 0.09 mM HMG uptake in the presence of 6 mM maleate, 6 mM acetoacetate, 6 mM citrate, and 6 mM α-ketoglutarate. Each of these compounds produced significant inhibition of HMG uptake although greater degrees of impairment were seen for the endogenous organic acids than for maleate (Table 3). Concentration-dependent uptake of HMG over a concentration range of 0.95–18 μM was examined with and without 1 mM citrate as an inhibitor. These results were analyzed by Lineweaver-Burk plots and the nature of the resulting lines suggests that citrate competitively inhibits HMG uptake by renal brushborder vesicles (Fig. 4).

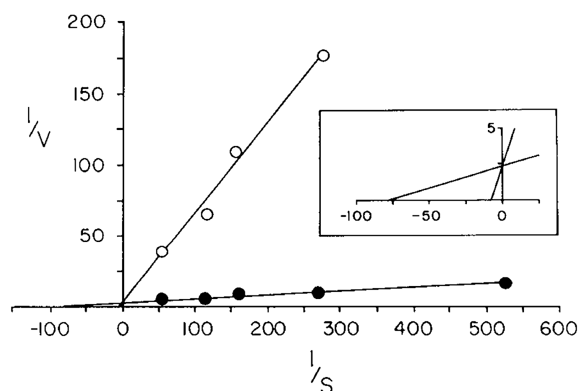

Fig. 4. Concentration-dependent uptake of β-hydroxy-β-methyl-glutarate (0.95 μM to 18 μM) by renal brushborder membrane vesicles. β-Hydroxy-β-methyl-glutarate uptake was measured after a 0.5-min incubation in vesicles suspended in THM buffer in the presence of a 100 mM NaCl gradient, with (○——○) and (●——●) without 1 mM citrate present. Values shown are the means of at least three experiments done in triplicate. The entire range of substrate concentration studied was used for data analysis; all data are not shown. The inset shows an enlargement of the intercepts used for determining kinetic parameters. 1/V, reciprocal of velocity of uptake (nmole substrate/mg protein/0.5 min); 1/S, reciprocal of substrate concentration (mmole/liter).

DISCUSSION

The availability of a technique for the isolation of renal brushborder membrane vesicles makes it possible to analyze the characteristics of luminal membrane transport without the need to consider the contribution of the basolateral surface. We have utilized this preparation to examine luminal HMG transport in rat renal brushborder vesicles.

The rapid initial rate and the overshoot achieved with 0.09 mM HMG by rat renal membrane vesicles is consistent with a carrier-mediated, cotransport mechanism (9). Others have shown the same to be true of citrate and α-ketoglutarate transport by the isolated brushborder vesicle (11) made from rabbit kidney. Our experiments with valinomycin suggest that the overshoot is due to cotransport, and not to a potential difference created by Na^+-influx. Studies utilizing 100 mM gradients of various cations gave clear evidence of the specific Na^+-dependence of the observed uptake phenomenon. Experiments carried out under sodium-equilibrated conditions show that the absence of a Na^+-gradient produced little, if any, substrate entry beyond that occurring by diffusion.

Since brushborder membrane vesicles contain a Na^+-H^+ ex-

change system (15), the intravesicular alkalinization that occurs in response to an inwardly directed Na^+-gradient could have stimulated HMG accumulation by nonionic diffusion of the undissociated acid. Our demonstration of a lack of stimulation by Li^+ (Table 1) together with the demonstration by Kinsella and Aronson (10) that imposition of a Li^+ gradient induced alkalinization similar to that induced by a Na^+-gradient eliminated pH gradient generation as a factor in accounting for the observed stimulation of HMG uptake by Na^+.

Several studies have shown an effect of substrate deprivation on cellular substrate uptake. The removal of amino acids (8, 18) and glucose (3, 4) from the incubation medium of various cell types results in an increased rate of substrate uptake with subsequent addition of the deprived compound. Starvation results in, among other things, an increased production of ketone bodies and a state of ketosis; thus, we examined the possibility that starvation leading to a state of ketosis in the rat might influence renal brushborder uptake of HMG and the ketone body β-HB. In neither case, at a time when the animal's blood level of β-HB was three times normal, was there a detectable difference in substrate uptake by isolated tubule fragments made from control and starved rats. These observations were consistent with the minimal differences between the apparent transport K_m and V_{max} values determined for both groups of animals for β-HB and HMG using brushborder membrane vesicles.

It is interesting to note that although substrate deprivation induces increased transport capability, increased substrate levels do not result in the converse. This is not as unusual as initial consideration might suggest. Carriers presumably function in a regulatory capacity to expedite cellular solute entry. In the face of substrate abundance, as is the case for β-HB during starvation, an increase in the capacity or affinity for β-HB transport would not be expected, provided cellular nutritional requirements are met. From this, we must conclude that 72 h of starvation did not significantly impair renal cellular metabolism, except by changing the substrate available to the cell. Perhaps if the animals were deprived of free fatty acids, an alternate fuel used during starvation, the transport parameters describing organic acid and ketone body transport would change.

Concentration-dependent uptake studies showed a single transport system for both HMG and β-HB. The apparent K_m values for these systems were within the reported plasma concentration ranges of organic acids in rat (17); thus, we must conclude that in the rat, and possibly in the human, there is normally sufficient brushborder uptake of HMG to remove substantial amounts of this organic acid from the glomerular filtrate. As in the case of a child with HMG-CoA lyase deficiency, when large quantities of HMG are being produced without further catabolism, glomerular filtration and partial reabsorption of this organic acid could contribute significantly to a metabolic acidosis.

Our observations that citrate competitively inhibits HMG uptake by brushborder membrane vesicles and that other organic acids reduce vesicular HMG uptake are of particular interest in this regard. These findings imply the presence of a common organic acid carrier in these membranes. The possibility of a common organic acid carrier in renal membranes has previously been suggested: in dog, the sites of citrate reabsorption and PAH secretion appear to be coincident (7) and *p*-aminohippuric acid (PAH) secretion is inhibited by α-ketoglutarate (1); in rabbit, interactions between PAH and urate transport (12) and competition for luminal uptake between tricarboxylic acid intermediates (11) have been observed.

Recently, Wright *et al.* (22) published an extensive study of the interactions of a variety of organic compounds in brushborder membranes prepared from rabbit kidney. These authors demonstrated competitive inhibition between succinate and citrate transport; the calculated inhibitory constant (K_i) for citrate was similar to the K_m of succinate. They concluded from these data that succinate and citrate compete for the same transport site, which may also accommodate α-ketoglutarate. Our demonstration of the competitive interaction between HMG and citrate, and the inhibition of HMG uptake by α-ketoglutarate as well, supports the possibility that all of these compounds are transported at least in part by a common carrier mechanism. Of the compounds tested, maleic acid exerted the weakest effect on HMG uptake, which is consistent with the findings of Wright *et al.* who reported a relatively high K_i value for this compound.

On the other hand, the observation of Wright *et al.* (22) of a 400-fold difference between the inhibitory constant for β-HB, a monocarboxylate and succinate, is not consistent with our demonstration of significant inhibition of HMG uptake by acetoacetate, a compound structurally related to β-HB. These differences suggest the possibility of an additional transport system for monocarboxylic anions with structural requirements shared by the dicarboxylate carrier.

At the present time, there is no definitive treatment for patients with HMG-CoA lyase deficiency. Speculation based on these data suggest that citrate may be of use in the treatment of this disease. Citrate has been in use for many years in the treatment of distal renal tubular acidosis and has been used safely in fairly substantial doses (16). The possibility exists, therefore, that by administration of high oral doses of citrate, urinary citrate levels can be elevated sufficiently to competitively inhibit the tubular reabsorption of HMG. In addition, citrate functions as a buffer for hydrogen ions, providing an alkalinizing effect in blood. Thus, the dual actions of citrate could be of great significance in the chronic treatment of a patient with HMG-CoA lyase deficiency. Such an approach to therapy has been given a rational experimental basis by the present observations and deserves a clinical trial in conjunction with the current treatment utilizing dietary protein restriction (5).

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