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KETONE BODY TRANSPORT IN RENAL BRUSH BORDER MEMBRANE VESICLES

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Summary

Ketone body uptake by renal brush border vesicles has been investigated. Ketone bodies enter into the brush border vesicles by a carrier-mediated process. The uptake is dependent on an Na^+ gradient ($[Na^+]$outside > $[Na^+]$inside) and is electroneutral. The uptake is transport into an osmotically active space and not a binding artifact as indicated by the effect of increasing the medium osmolarity. A pH gradient (alkaline inside) also stimulates the ketone body uptake. Acetoacetate and 3-hydroxybutyrate share the same carrier as demonstrated by the accelerated exchange diffusion and mutual inhibitory effects.

Introduction

Ketone bodies (acetoacetate and D-(—)-β-hydroxybutyrate) are very important metabolic fuels in situations such as postnatal development and fasting when the ability to utilize glucose or the availability of this metabolic fuel is restricted [1,2]. In such situations a higher ketone body concentration is found in the blood [3,4]. Thus, to avoid the loss of these important metabolic fuels to urine, a reabsorption system in the luminal membrane of the proximal tubule of the kidney (brush border membranes) is to be expected.

Brush border membrane vesicle preparations have been demonstrated to be extremely useful for the study of the renal reabsorption process. In this system, it has been demonstrated that metabolite transport is dependent on an Na^+ gradient (ΔNa^+) or a membrane electrical potential ($\Delta\psi$), built up by the ($Na^+ + K^+$)-ATPase from the basolateral membrane [5].

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

In this paper, the general properties, the interaction of carrier and substrates, bioenergetics and inhibitors of ketone body transport in renal brush border membrane vesicles have been investigated. Acetoacetate and D-(—)-3-hydroxybutyrate share a transport system which is Na^+ gradient-dependent and electroneutral. The acetoacetate uptake can also be stimulated by a pH gradient (alkaline inside).

Methods

Isolation of brush border membranes. Adult male rats of the Wistar strain weighing 150—200 g were used. Luminal membranes derived from the proximal tubules were isolated by using the method of Thunenberg and Rostgaard [6] as modified by Aronson and Sacktor [7] and Mitchell et al. [8].

Membranes prepared in 0.5 M sucrose were suspended in a medium containing 120 mM KCl and 22 mM potassium phosphate buffer, pH 7.4, centrifuged at 27 000 × *g* for 5 min and the pellet washed three times with the same medium by resuspension and centrifugation at 12 000 × *g* for 5 min. The final pellet was resuspended in the KCl-phosphate medium described above.

Membrane protein was determined according to the method of Lowry et al. [9] using crystalline bovine serum albumin as standard.

Transport assays. Aliquots of 20 μl of the suspension of brush border membrane vesicles (about 0.2 mg of protein) having an internal medium of 120 mM KCl and 22 mM potassium phosphate buffer (pH 7.4) were preincubated for 1 min at 25°C. The uptake was started by adding 100 μl of a solution containing the radioactive substrate in 120 mM NaCl, 22 mM sodium phosphate (pH 7.4) (NaCl medium) or 120 mM KCl, 22 mM potassium phosphate (pH 7.4) (KCl medium), unless otherwise stated. The experiment was terminated by diluting with 5 ml of ice-cold external medium and immediately filtering through a moistened Millipore filter RAWP 02500 (1.2 μm pore size) attached to a vacuum assembly. The filters were rinsed twice with the ice-cold medium. The dilution, filtration and washing procedures were performed within 15 s. When the [^{3}H]tetraphenylphosphonium ion uptake was measured, Millipore EHWP 02500 filters (1.2 μm pore size) were used to avoid binding of tetraphenylphosphonium ion to the filter [10]. All the experiments were corrected for a control obtained by diluting the membrane suspension before adding the radioactive substrate solution. When ionophores were used membrane suspension was preincubated with them for 1 min before the uptake experiments.

All solutions used in the preparation of the membrane vesicles and in the uptake experiments were prepared with distilled-deionized water and were previously filtered through Millipore filters (0.22 μm) in order to avoid possible bacterial contamination. The osmolarity of all the solutions was kept constant during the uptake experiments. In order to replace equivalently Na^+ by choline, choline chloride and choline phosphate buffer (pH 7.4) replaced other chlorides and phosphates.

All the results are the mean of at least three typical experiments.

D-(—)-3-Hydroxy[3-^{14}C]butyrate and ethyl[3-^{14}C]acetoacetate were purchased from The Radiochemical Centre, Amersham. [3-^{14}C]Acetoacetate was obtained by hydrolysis of ethyl[3-^{14}C]acetoacetate as described by Edmon

[11]. [^{3}H]Tetraphenylphosphonium bromide was a gift from Dr. Sofia Ramos of Roche Institute, Nutley, NJ Lithium acetoacetate, sodium DL-3-hydroxybutyrate, sodium pyruvate, glucose, L-alanine, L-aspartate, ouabain and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were provided by Sigma. Nigericin and monensin were a gift from Lilly Laboratories, Indianapolis, IN. α-Cyano *p*-hydroxycinnamate was purchased from EGA-Chemie.

Results

The time course of ketone body uptake by membrane vesicles prepared in KCl medium is shown in Fig. 1. When an Na^+ gradient (outside > inside) was present, ketone body uptake was higher than that in the presence of either a K^+ (non-gradient conditions) or a choline$^+$ gradient.

To decide whether ketone body uptake represents transport across the membrane into an intravesicular osmotically active space or binding to the membrane, the equilibrium uptake was measured at different osmolarities of the incubation medium (Fig. 2). When the D-(−)-3-hydroxybutyrate uptake at equilibrium was plotted against the reciprocal of osmolarity of the incubation medium and extrapolated to infinite osmolarity (intravesicular volume zero), the value of the D-(−)-3-hydroxybutyrate uptake was zero both in the presence and absence of an Na^+ gradient, indicating that the D-(−)-3-hydroxybutyrate is

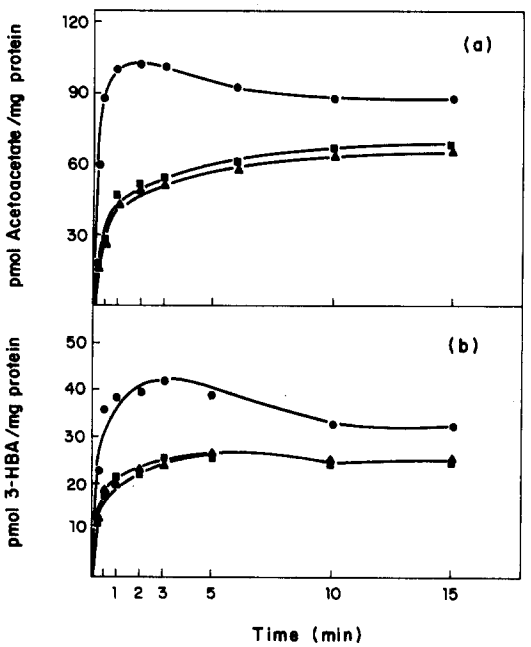

Fig. 1. Time course of ketone body uptake by renal brush border vesicles. The vesicles were incubated in the presence of 0.1 mM of the radioactive substrate: NaCl medium, ●; KCl medium ▲; choline chloride medium (120 mM choline chloride and 22 mM H_3PO_4 adjusted with choline base to pH 7.4), ■. (a) Acetoacetate uptake. (b) D-(−)-3-hydroxybutyrate (3-HBA) uptake.

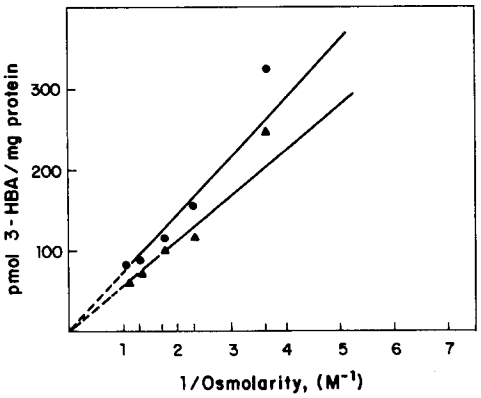

Fig. 2. Effect of the medium osmolarity on the D-(—)-3-hydroxybutyrate (3-HBA) uptake at equilibrium. The membranes were incubated for 15 min in the presence of 0.1 mM radioactive 3-HBA in the NaCl medium (●) or the KCl medium (▲) and sucrose to reach the indicated osmolarity.

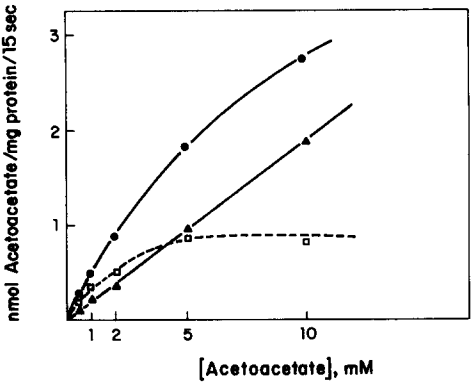

Fig. 3. Kinetics of ketone body uptake. The membranes were incubated for 15 s in the NaCl medium (●) or the KCl medium (▲) and radioactive acetoacetate at the indicated concentrations. The specific Na^+-dependent acetoacetate uptake (□) was obtained by subtracting the uptake in the NaCl medium from the uptake in the KCl medium at each concentration of acetoacetate.

not bound to the membrane but transported into an osmotically active space. Similar results were found with acetoacetate.

Accelerated exchange diffusion

Table I shows that vesicles preloaded with non-radioactive acetoacetate have, when an Na^+ gradient is established, a faster initial D-(—)-3-hydroxybutyrate uptake rate than vesicles not previously preloaded. The stimulation of the uptake is dependent on the existence of an Na^+ gradient, since in a K^+ medium no stimulation is found. Vesicles preloaded with D-(—)-3-hydroxybutyrate showed a similar enhancement of acetoacetate uptake. This fact demonstrates that the uptake of ketone bodies by the brush border membrane vesicles represents transport into an osmotically active space rather than membrane

TABLE I

Na^+-DEPENDENT KETONE BODY ACCELERATED EXCHANGE DIFFUSION

The vesicles were incubated for 15 min in the presence of 5 mM acetoacetate or 10 mM of DL-3-HBA in the KCl medium. After this time, 20-μl aliquots were added to 100 μl of the incubation medium containing 0.1 mM of the labelled substrate. The values represent the mean ± S.E. of at least three experiments. 3-HBA, D-(—)-3-hydroxybutyrate. Values of uptake are expressed as pmol/mg protein per 15 s.

	NaCl medium	KCl medium
Acetoacetate uptake		
control	60.0 ± 3.2	18.2 ± 0.8
preloaded with 5 mM 3-HBA	103.8 ± 5.3	17.5 ± 0.7
3-HBA uptake		
control	25.4 ± 1.5	16.9 ± 0.6
preloaded with 5 mM acetoacetate	42.4 ± 2.9	17.8 ± 0.7

binding, since preincubation with unlabelled substrates would saturate the binding sites and would have inhibited the ketone body binding.

Kinetics of ketone body uptake

In the presence of an Na^+ gradient the initial uptake rates approach saturation kinetics, whereas in the absence of an Na^+ gradient both acetoacetate and D-(−)-3-hydroxybutyrate uptake rates increase linearly when the concentrations of these compounds are augmented.

This saturability is even clearer when the uptake rates in the presence of an Na^+ gradient are corrected for the rates under non-gradient conditions (i.e., KCl medium) (Fig. 3). The ketone body uptake system specifically dependent on an Na^+ gradient (corrected for non-gradient conditions) follows Michaelis-Menten kinetics and from a Lineweaver-Burk plot an apparent K_m value of 4 mM for acetoacetate and 10 mM for D-(−)-3-hydroxybutyrate were obtained (Fig. 4).

Inhibitors

Among the compounds tested, pyruvate, acetoacetate and D-(−)-3-hydroxybutyrate strongly inhibit the initial rate of the specifically Na^+-dependent ketone body uptake, showing no effect on the uptake in the absence of an Na^+ gradient (Table II).

D-Glucose, L-aspartate and L-alanine have no effect on the ketone body uptake. Ouabain, an inhibitor of $(Na^+ + K^+)$-ATPase, does not inhibit ketone body uptake demonstrating the independence of this enzyme with regard to the place where the reabsorption processes are carried out. α-Cyano *p*-hydroxycinnamate, described as inhibitor of pyruvate and ketone body mitochondrial transport [12], does not show any effect in renal brush border membrane vesicles.

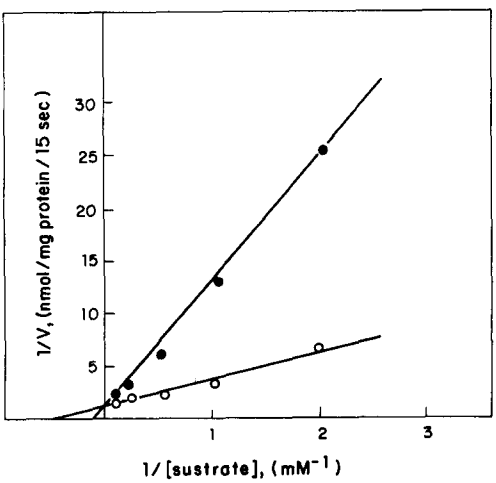

Fig. 4. Double-reciprocal plot of initial (15 s) specific Na^+-dependent uptake of acetoacetate (○) and 3-HBA (●). Experimental conditions as in Fig. 3.

TABLE II

INHIBITION OF THE KETONE BODY UPTAKE BY DIFFERENT COMPOUNDS

Addition (1 mM)	[3-^{14}C]Acetoacetate uptake (nmol/mg protein per 15 s)			D-(—)-3-Hydroxy[3-^{14}C]butyrate uptake (nmol/mg protein per 15 s)		
	NaCl medium	KCl medium	NaCl-KCl (% control)	NaCl medium	KCl medium	NaCl-KCl (% control)
None	60.0 ± 3.2	18.2 ± 0.8	100	25.4 ± 1.5	17.0 ± 0.6	100
Acetoacetate	19.9 ± 0.8	16.3 ± 0.6	7	15.6 ± 1.1	14.7 ± 0.5	10
3-HBA	21.6 ± 0.9	17.3 ± 0.4	10	13.48 ± 0.9	15.4 ± 0.9	<0
Pyruvate	18.3 ± 0.8	16.8 ± 0.6	4	18.3 ± 1.3	15.4 ± 0.9	34
D-Glucose	54.6 ± 3.0	16.7 ± 0.7	90	26.5 ± 1.5	13.3 ± 0.7	120
L-Alanine	52.6 ± 3.0	18.4 ± 0.8	82	24.5 ± 1.5	15.7 ± 1.0	103
L-Aspartate	55.6 ± 2.7	17.5 ± 0.5	91	25.0 ± 1.2	15.9 ± 0.9	110
Ouabain	58.7 ± 3.0	17.8 ± 0.8	98	25.6 ± 1.8	14.8 ± 0.8	126
α-Cyano *p*-hydroxycinnamate	58.7 ± 3.2	18.2 ± 0.8	97	25.0 ± 1.6	15.1 ± 1.0	116

The vesicles were incubated in the presence of the different compounds at a concentration of 1 mM. Experimental conditions as in Table I. 3-HBA,D-(—)-3-hydroxybutyrate.

Effect of specific ionophores

Both nigericin and monensin, which collapse the Na^+ gradient across the membrane through an Na^+, K^+ or an Na^+, H^+ antiport mechanism, decrease the Na^+-dependent acetoacetate uptake (Fig. 5). The difference between the uptake in the presence of NaCl plus monensin or nigericin and the uptake in a KCl medium could be explained by the effect of the Na^+ itself on the carrier even under non-Na^+-gradient conditions.

Valinomycin produces a transient increase in the membrane electrical potential ($\Delta\psi$) (negative inside) when a K^+ gradient (inside > outside) is

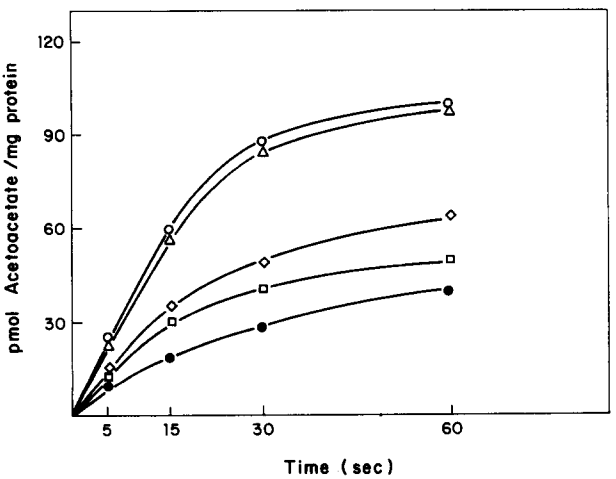

Fig. 5. Effect of ionophores on the acetoacetate uptake. The membrane vesicles were incubated in the presence of 0.1 mM radioactive acetoacetate in the NaCl medium (△, □, ◇) or the KCl medium (●) with the following additions. ○, none; △, valinomycin (8 μg/mg membrane protein); □, nigericin (8 μg/mg membrane protein); ◇, monensin (8 μg/mg membrane protein).

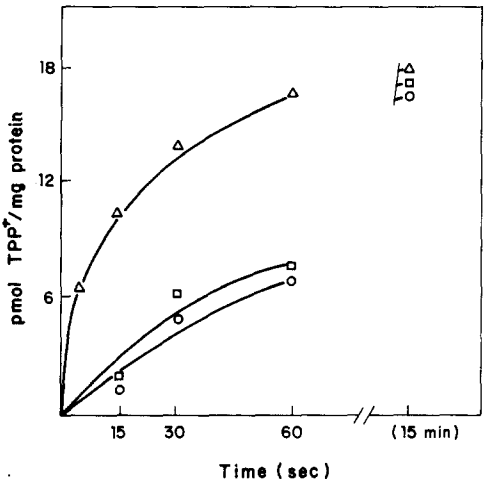

Fig. 6. Effect of ionophores on the [^{3}H]triphenylphosphonium ion (TPP^+) uptake. The membrane vesicles were incubated in the presence of 7 μM [^{3}H]TPP^+ in the NaCl medium, with the following additions. ○, none; △, valinomycin (8 μg/mg membrane protein); □, nigericin (8 μg/mg membrane protein).

present, as demonstrated in Fig. 6 by the accumulation of tetraphenylphosphonium ion, a lipophilic cation which is taken up in a $\Delta\psi$-dependent fashion [13]. However, acetoacetate uptake is not affected by the addition of valinomycin (Fig. 5), thus indicating that acetoacetate is transported electroneutrally, probably via an Na^+-acetoacetate co-transport. Another way to modify $\Delta\psi$ is by using anions with different permeabilities. The accumulation of tetraphenylphosphonium ion under these conditions is that expected according to anion permeability, $SCN^- > Cl^- > SO_4^{2-}$ (results not shown). However, when Cl^- is replaced by SCN^- or SO_4^{2-} the initial uptake rate of acetoacetate observed is

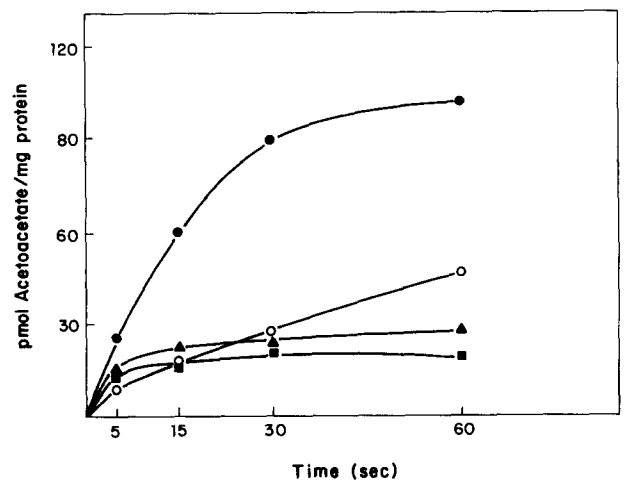

Fig. 7. Effect of anion replacement on the acetoacetate uptake. The membrane vesicles were incubated in the presence of 0.1 mM radioactive acetoacetate in the following media: ●, NaCl medium; ■, 120 mM NaSCN and 22 mM sodium phosphate buffer (pH 7.4); ▲, 60 mM Na_2SO_4, 30 mM KCl and 22 mM sodium phosphate buffer (pH 7.4); ○, KCl medium.

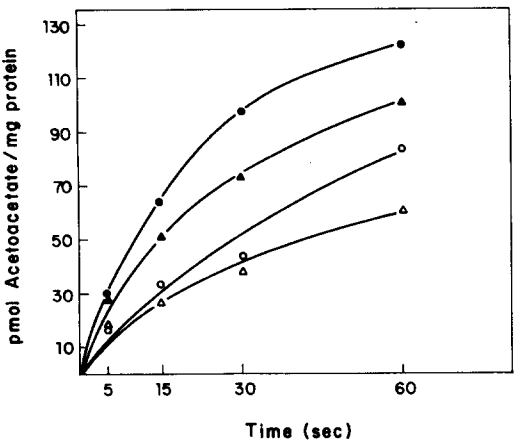

Fig. 8. Effect of a pH gradient on the acetoacetate uptake. The membrane vesicles were incubated in the presence of 0.1 mM radioactive acetoacetate in the following media: ● and ○, 120 mM NaCl and 22 mM sodium phosphate buffer (pH 6); ▲ and △, 120 mM KCl and 22 mM potassium phosphate buffer (pH 6). In ○ and △, CCCP (8 μg/mg membrane protein) was present.

even smaller than that found in the absence of an Na^+ gradient (Fig. 7). Since the effects of SO_4^{2-} and SCN^- are contradictory if they have to be interpreted as an effect on the membrane $\Delta\psi$ and considering they do not agree with the valinomycin effect, these results could be due to a direct interaction of SCN^- and SO_4^{2-} with the ketone body carrier.

Effect of a pH gradient (ΔpH)

A ΔpH (alkaline inside) can drive the transport of acetoacetate into the vesicles both in the presence and absence of Na^+ (Fig. 8). The uptake stimulation by a ΔpH is not additive with that produced by Na^+, however, when both gradients are present the uptake rate is higher than that found with only one present. The ΔpH effect can be abolished by CCCP, a proton ionophore, indicating that the stimulation of the uptake is not due to the protonation of specific membrane groups induced by the pH change. Moreover, CCCP has no effect on the acetoacetate uptake in the absence of a ΔpH (results not shown).

Discussion

The results reported herein clearly establish that ketone bodies are accumulated in brush-border membrane vesicles by an Na^+-gradient-dependent system analogous to those previously described for sugars and aminoacids (see Ref. 5). The uptake is not a binding artifact, rather the accumulation in an osmotically reactive space as demonstrated by the effect of increasing osmolarity (Fig. 2) and the accelerated exchange diffusion (Table I). This fact together with the reciprocal inhibition of acetoacetate and D-(—)-3-hydroxybutyrate uptake (Table II), are clear indications that the two compounds share the same carrier. This carrier has some of the properties of a mobile carrier such as accelerated exchange diffusion with unlabelled substrate, saturation and competitive inhibition as predicted by theoretical models [14]. It should be noted that the

ketone body uptake in the absence of Na^+ does not show any of the properties of a carrier-mediated system. This implies that the Na^+-independent component of the uptake is not a facilitated diffusion process.

Even though anion-replacement experiments provide confusing results, the effect of valinomycin clearly indicates that acetoacetate uptake is electroneutral. Similar results have been reported by Kippen et al. [15] who have shown an inhibition of citrate and 2-ketoglutarate uptake when Cl^- was replaced by SCN^- with no effect of valinomycin on the transport of this substrate by the renal brush border. Thus, under Na^+ gradient conditions, acetoacetate probably has to be taken up in cotransport with Na^+. In this way, the observation that monensin and nigericin, which collapse the Na^+ gradient, do not fully inhibit the effect of Na^+, agrees with a cotransport (acetoacetate$^-$, Na^+) electroneutral system which results in no net movement of charge across the membrane because the charges of the two transported species cancel each other. Na^+-dependent electroneutral transport systems have been reported for other metabolites such as phosphate at pH 7.4 [16] and ascorbate [17] in small intestine brush borders, and citrate and 2-ketoglutarate [15] in renal brush borders. However, up to now, the stimulation of the metabolite transport by ΔpH has not been reported in renal brush border vesicles. Considering the existence of an in vivo physiological ΔpH across the brush border membrane [5] the effect of a ΔpH on the acetoacetate uptake by the renal brush border vesicles may have a physiological significance in the in vivo mechanism for ketone body renal absorption.

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