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AMINO ACID UPTAKE BY ISOLATED RENAL BRUSH BORDER MEMBRANE VESICLES IN VARIOUS BUFFERS

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The uptake of amino acids by isolated rat renal brush border membrane vesicles in a modified Krebs-Ringer bicarbonate buffer and a phosphate buffer was compared to the uptake in the standard membrane vesicle buffer, Tris-Hepes-mannitol. The uptake in the modified Krebs-Ringer bicarbonate buffer was similar to that in the Tris-Hepes-mannitol buffer. Removal of the ionic constituents other than NaCl and $NaHCO_3$ in the modified Krebs-Ringer bicarbonate buffer (KCl, $CaCl_2$, KH_2PO_4 and $MgSO_4$) did not affect the amino acid uptake by the isolated membrane vesicles. The timed uptake of proline under sodium gradient conditions in a phosphate buffer had a markedly dampened overshoot. Kinetic analysis of the initial rate of proline uptake in a phosphate buffer compared to a Tris-Hepes-mannitol buffer showed two entry systems for proline in each buffer with similar K_m values, but the maximal rate of transport (V) for each system in the phosphate buffer was much lower than that in the Tris-Hepes-mannitol buffer. From these data, phosphate buffer does not appear to be a suitable medium for the study of amino acid uptake by isolated brush border membrane vesicles.

Most transport studies using isolated kidney membranes have employed a non-physiologic buffer composed of tris(hydroxymethyl)aminomethane, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and mannitol (Tris-Hepes-mannitol buffer). However, the majority of transport studies with intact cells have used a physiologic buffer, such as Krebs-Ringer bicarbonate buffer or Krebs-Ringer phosphate buffer, which more closely approximates the extracellular buffering systems in vivo than the Tris-Hepes-mannitol buffer. The bicarbonate-CO_2 system is also important in intracellular buffering since intracellular pH is in part determined by the transport of bicarbonate ion and the diffusibility of CO_2. The use of phosphate or bicarbonate buffer, in contrast to a Tris-Hepes-mannitol buffer, may be physiologically important for other reasons in that the reabsorption of these anions is a necessary function of the proximal tubule cells which normally have their luminal surface membranes bathed by these ions. In fact, lower uptake velocities were observed in transport studies with renal cortex slices, isolated cortex tubules and brain synaptosomes employing a Tris buffer when compared with similar studies employing a bicarbonate buffer [1–3]. Because of these considerations, we compared the uptake of two amino acids, proline and lysine, by isolated brush border membrane vesicles in

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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a modified Krebs-Ringer bicarbonate buffer and a phosphate buffer to the uptake in the standard membrane vesicle Tris-Hepes-mannitol buffer.

Renal brush border membrane vesicles were prepared from adult male Sprague-Dawley rats according to the method described by Booth and Kenny [4] with minor modifications described by Weiss et al. [5]. The final membrane pellet was suspended in Tris-Hepes-mannitol buffer, pH 7.4, to a protein concentration of 3.0–4.0 mg/ml as determined by the method of Lowry et al. [6] using bovine serum albumin as the protein standard.

The uptake of 0.02 mM L-proline and 0.02 mM L-lysine was compared in various buffers. The initial bicarbonate buffer used was a modified Krebs-Ringer bicarbonate buffer containing 4.75 mM KCl, 2.53 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 75 mM NaCl and 100 mM mannitol in order to achieve the same osmolality and sodium concentration in all the buffers. This was further modified to a bicarbonate-mannitol buffer containing 25 mM $NaHCO_3$, 75 mM NaCl and 100 mM mannitol. Both bicarbonate buffers were gassed with O_2/CO_2 (95 : 5, v/v) at 22°C to give a final pH of 7.4. The phosphate buffer employed contained 2.4 mM NaH_2PO_4, 10.4 mM Na_2HPO_4, 75 mM NaCl and 100 mM mannitol with a final pH of 7.4. The uptake of proline in these buffers was compared to that obtained in the standard membrane buffer, Tris-Hepes-mannitol, which contains 1 mM Tris, 2 mM Hepes and 100 mM mannitol with a pH of 7.4. With the lysine uptake studies, a concentrated Tris-Hepes-mannitol buffer, containing 20 mM Tris-Hepes plus 60 mM mannitol with a pH of 7.4, was used in place of standard Tris-Hepes-mannitol buffer. This was necessary in order to neutralize the acidity of lysine HCl. In uptake studies with Tris-Hepes-mannitol or concentrated Tris-Hepes-mannitol buffer, NaCl was added to the incubation mixture such that the final NaCl concentration was 100 mM.

The measurement of amino acid uptake using Millipore filtration on HAWP filters (0.45 μm) was performed using the technique described by McNamara et al. [7]. Trapped and diffused space was measured using L-[^{3}H]glucose as previously described [7] and the results are expressed as uptake in excess of diffusion. The standard uptake experiment, which was under the conditions of an Na^+ gradient unless otherwise stated, was performed by adding 50 μl of freshly prepared membrane vesicles (150–200 μg of protein) in Tris-Hepes-mannitol buffer at 22°C to a test tube containing 0.1 μCi of ^{14}C-labeled amino acid, 0.2 μCi L-[^{3}H]glucose, and 0.5 ml of the various buffers. This mixture was stirred for 6 sec. on a Vortex genie. At various times ranging from 0.5 to 15 min, the incubation mixture was stopped by rapid filtration through a Millipore filter and washed once with 5 ml of a solution containing 154 mM NaCl plus 1 mM Tris-Hepes at pH 7.4. The filters were air dried and assayed for radioactivity as previously described [7]. In experiments where uptake in the absence of an Na^+ gradient was to be studied, the membrane vesicles in Tris-Hepes-mannitol were preincubated for 40 min with 100 mM NaCl. Krebs-Ringer bicarbonate and bicarbonate-mannitol buffers were continuously gassed with O_2/CO_2 (95 : 5, v/v) at 22°C until the buffers were added to the incubation tube which was just prior to the addition of the membrane vesicles. The tubes were then sealed until the filtration step in order to maintain a 5% CO_2 atmosphere.

To study the effect of a phosphate buffer on the kinetic parameters of proline uptake, the concentration dependence of proline uptake was assessed over the range of from 0.18 to 4 mM. The observed transport parameters were determined from an Eadie-Hofstee plot of these data with a Monroe model 1775 calculator to obtain the best fit by the least-squares method.

In Fig. 1(A), the uptake of 0.02 mM proline by brush border membrane vesicles in Tris-Hepes-mannitol buffer is shown. These determinations represent the uptake in excess of diffusion as measured by L-glucose under either sodium gradient or sodium equilibrated conditions as are the curves in the other figures. In the presence of a sodium gradient, an 'overshoot' phenomenon of stimulated uptake occurred with no overshoot for the sodium equilibrated state. In Fig. 1(B), a similar uptake curve with an overshoot occurred for 0.02 mM proline in Krebs-Ringer bicarbonate buffer under sodium gradient conditions. Systematic removal of $CaCl_2$, KH_2PO_4, KCl and $MgSO_4$, from the modified Krebs-Ringer bicarbonate buffer, which resulted in the bicarbonate-mannitol buffer, did not alter the uptake curves for 0.02 mM proline shown in Fig. 1(B). Similar studies with a phosphate buffer having the same pH, osmolal-

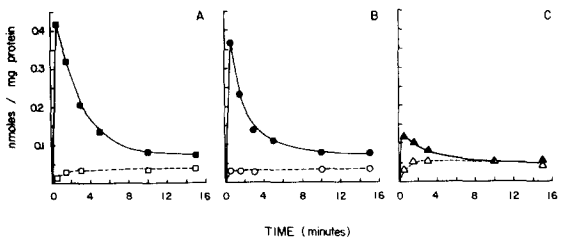

Fig. 1. Time course of 0.02 mM L-proline uptake by isolated rat renal brush border membrane vesicles in a Tris-Hepes-mannitol buffer (A), Krebs-Ringer bicarbonate buffer (B) and phosphate buffer (C). Closed symbols represent uptake under a 100 mM sodium gradient and open symbols represent uptake under sodium equilibrated conditions. The Tris-Hepes-mannitol buffer (pH 7.4) contained 2 mM Tris-Hepes plus 100 mM mannitol. The Krebs-Ringer bicarbonate buffer (pH 7.4) contained 75 mM NaCl, 2.53 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 100 mM mannitol under a 95% O_2–5% CO_2 atmosphere. The phosphate buffer (pH 7.4) contained 2.4 mM NaH_2PO_4, 10.4 mM Na_2HPO_4, 75 mM NaCl and 100 mM mannitol. Each point represents the mean of at least seven determinations with a standard error of 10% or less.

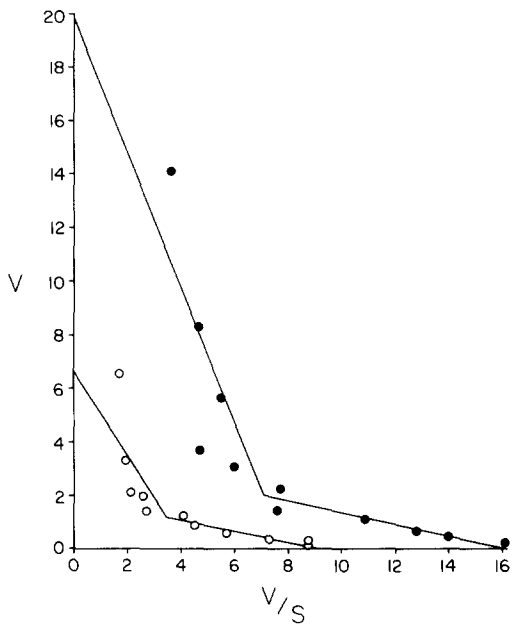

Fig. 2. Eadie-Hofstee plot of the concentration dependent uptake of L-proline by isolated rat renal brush border membrane vesicles in Tris-Hepes-mannitol buffer (•) or phosphate buffer (○). Values represent uptake after 15 s of incubation of L-proline over the concentration range of 0.02 to 4.5 mM. Each point represents eight determinations. V represents velocity of uptake (nmol/mg protein per 15 s) and S represents the proline concentration (mM).

ity and sodium concentration as the bicarbonate-mannitol or Tris-Hepes-mannitol buffer resulted in a significant dampening of the overshoot phenomenon under sodium gradient conditions (Fig. 1C). The height of the overshoot is only 1/4 of that seen with the Krebs-Ringer bicarbonate, bicarbonate-mannitol or Tris-Hepes-mannitol buffer. The height and shape of the proline uptake curve in the sodium equilibrated state with the four different buffers are the same.

Since the phosphate buffer inhibited the sodium gradient uptake of proline, the effect of this inhibition on the kinetic parameters of proline uptake was examined (Fig. 2). An Eadie-Hofstee plot of proline uptake in both a phosphate and a Tris-Hepes-mannitol buffer revealed a two-limbed curve consistent with multiple transport systems for proline entry. This is similar to previously published data on proline uptake into isolated brushborder membrane vesicles [7]. The observed kinetic parameters for proline uptake in the Tris-Hepes-mannitol buffer were $K_{m_1} = 0.21$ mM, $V_1 = 3.51$ nmol/mg protein per 15 s and $K_{m_2} = 2.50$ mM, $V_2 = 19.72$ nmol/mg protein per 15 s. These parameters in the phosphate buffer were $K_{m_1} = 0.19$ mM, $V_1 = 1.83$ nmol/mg protein per 15 s and $K_{m_2} = 1.56$, $V_2 = 6.68$. From these data, the phosphate buffer appears not to affect the affinity (K_m) of proline for each transport system, but does appear to lower the maximal rate (V) of proline uptake by both systems.

In addition to studying the uptake of the neutral amino acid, proline, the uptake of the dibasic amino acid, lysine, was compared in the bicarbonate-mannitol buffer and in the concentrated Tris-Hepes-mannitol buffer. As can be seen from Fig. 3(A) and (B), there was no difference in the uptake curves observed with the two buffers under either sodium gradient or sodium equilibrated conditions. However, there is a marked difference in the uptake of lysine under the sodium gradient condition compared to the sodium equilibrated condition in either buffer, although the 'overshoot' for lysine is not nearly as steep as that observed with proline.

From these studies, it appears that a bicarbonate buffer with 25 mM $NaHCO_3$, 75 mM NaCl, and 100

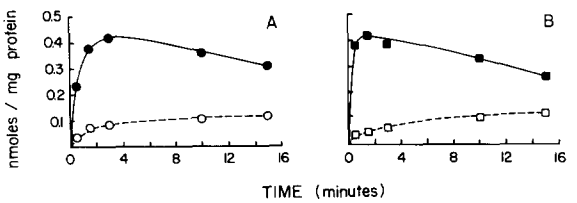

Fig. 3. Time course of 0.02 mM L-lysine uptake by isolated rat renal brush border membrane vesicles in a bicarbonate-mannitol buffer (A) and concentrated Tris-Hepes-mannitol buffer (B). Closed symbols represent uptake under a 100 mM sodium gradient and open symbols represent uptake under sodium equilibrated conditions. The bicarbonate-mannitol buffer contained 75 mM NaCl, 25 mM $NaHCO_3$, and 100 mM mannitol under a 95% O_2–5% CO_2 atmosphere. The concentrated Tris-Hepes-mannitol buffer contained 20 mM Tris-Hepes plus 60 mM mannitol. Each point represents the mean of eight determinations with a standard error of 10% or less.

mM mannitol gassed with O_2/CO_2 (95 : 5, v/v) can serve as a suitable medium for studying transport into membrane vesicles. The other constituents of Krebs-Ringer bicarbonate ($CaCl_2$, KCl, KH_2PO_4 and $MgSO_4$) in the concentrations normally employed do not seem to affect the vesicular transport of amino acids. This is in contrast to transport studies using renal cortex slices where these ions were important [8], reflecting obvious differences between intact cells and isolated membrane vesicles.

A similar contrast for the uptake of amino acids in a bicarbonate buffer compared to the uptake in a Tris buffer exists between kidney cortex slices and membrane vesicles. The uptake of proline by brush border membrane vesicles was the same in bicarbonate buffer or Tris buffer. The uptake of glycine whose transport is shared with proline in renal cortex slices is significantly lower when Tris buffer is substituted for bicarbonate buffer [1]. Tris is also known to inhibit the uptake of lysine by brain synaptosomes [3] but we have not observed such an effect with isolated brush-border membrane vesicles. Previous reports have demonstrated that Tris can enter cells [9] and can interact with various substrates and enzymes altering cellular metabolism [10,11]. An alteration by Tris on an aspect of cellular metabolism necessary for amino acid uptake, rather than an effect on the membrane per se appears to be the best explanation for this discrepancy between whole cell amino acid uptake and isolated membrane vesicle amino acid uptake.

The use of a phosphate buffer, however, resulted in a markedly reduced initial rate of uptake for proline under sodium gradient conditions when compared to uptake in either the Krebs-Ringer bicarbonate or Tris-Hepes-mannitol buffer. At later time points for proline uptake under sodium gradient conditions or throughout the uptake curve under sodium equilibrated conditions, the uptake of proline in the phosphate buffer was similar to that observed with the bicarbonate or Tris-Hepes-mannitol buffer. This dampening of the 'overshoot' was in spite of similar pH, final sodium concentration and osmolality when compared to the bicarbonate or Tris-Hepes-mannitol buffer.

Proline has been shown previously by our laboratory to enter isolated renal brush border membrane vesicles via two saturable systems [12]. Uptake via the high affinity, low K_m system is stimulated by a sodium gradient. A decrease in the medium sodium concentration will increase the K_m of this system for proline. Because the use of a phosphate buffer did not affect the K_m of the high affinity system for proline uptake, the effect of the phosphate buffer on proline uptake did not appear to be related to an alteration of the sodium gradient. On the other hand, the low affinity, high K_m system for proline entry shows no sodium dependence. The use of a phosphate buffer lowered the maximal velocity of uptake via this system, again suggesting that the effect of the phosphate buffer is not related to an effect on the sodium gradient. Rather, it appears that the phosphate buffer may have a general effect on either the brush border membrane or the carriers themselves, resulting in a decreased velocity of proline transport.

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