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Na^+-GRADIENT-DEPENDENT TRANSPORT OF L-PROLINE AND ANALYSIS OF ITS CARRIER SYSTEM IN BRUSH-BORDER MEMBRANE VESICLES OF THE GUINEA-PIG ILEUM

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Summary

Transport of L-proline was studied with membrane vesicles prepared from the brush borders of the guinea-pig ileum.

The presence of an Na^+ gradient from outside to inside of the vesicles stimulated L-proline uptake. Accumulation of amino acid in the vesicles reached a maximum 30 s after incubation, then decreased due to efflux and finally equilibrated at a level nearly identical to that shown in the absence of an Na^+ gradient in 30 min. The peak level of the uptake was 3.5-times greater than the final equilibrium level.

The equilibrium level of L-proline uptake decreased with increasing medium osmolarity. Extrapolation to infinite medium osmolarity, that is, under the condition of zero intravesicular space, showed no uptake, indicating transport of L-proline into membrane vesicles.

The initial rate of uptake for 15 s was enhanced with increasing concentrations of Na^+ in the external medium. A small part of the L-proline transport occurred by simple diffusion in addition to Na^+-gradient-dependent transport.

When L-proline concentrations were varied and transport due to diffusion was subtracted, the initial rate of uptake dependent on Na^+ gradient (out > in) obeyed Michaelis-Menten kinetics with K_m and V values of 0.67 mM and 2.73 nmol/15 s per mg protein, respectively.

Evidence was obtained which indicates that L-cysteine is a substrate specific

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Abbreviation: ASC, alanine-, serine- and cysteine-preferring.

for transport through system ASC (alanine-, serine-, and cysteine-preferring) and that transport in the presence of an Li^+ gradient (out > in) also takes place by the ASC system.

The uptake of L-proline in the presence of an Na^+ gradient (out > in) was inhibited 90% by a large excess of α-(methylamino)-isobutyrate, the model substrate specific for the A system (alanine-preferring). This indicates that 90% of Na^+-gradient-dependent L-proline uptake is supported by the A system. The remaining 10% of L-proline uptake was found to be catalyzed by the ASC system, since L-proline uptake equivalent to this α-(methylamino)-isobutyrate-uninhibited part was demonstrated in the presence of an Li^+ gradient.

Introduction

Membrane vesicles from the intestinal brush border have been recently used to study Na^+-gradient-dependent uptake of amino acid [1] as well as D-glucose [2–4].

In Ehrlich ascites tumor cells, Christensen classified Na^+-dependent carrier systems of amino acid transport into two systems [5]: systems A and ASC. Amino acid transport mediated by the A system is inhibited by α-(methylamino)-isobutyrate, whilst the transport mediated by the ASC system is not inhibited by α-(methylamino)-isobutyrate.

Edmondson et al. [6] have recently described in liver cells that Li^+ might be used to elicit directly the contribution of the ASC system. Using this approach and the inhibition by α-(methylamino)-isobutyrate, we will present evidence in this paper that L-proline is transported into brush-border membrane vesicles from the guinea-pig ileum by two different Na^+-gradient-dependent carrier systems, which are comparable to the A system and the ASC system of Ehrlich ascites tumor cells.

Methods and Materials

Membrane isolation

Membranes were prepared from guinea-pig ileum by the procedure of Fujita et al. [7]. This will be briefly described. Mucosal scrapings from guinea-pig ileum were taken up in 0.25 M sucrose containing 5 mM Tris/Hepes buffer (pH 7.5) and 0.5 mM neutralized EDTA (sucrose-EDTA buffer). It was then homogenized in a Dounce-type homogenizer by 50 up-and-down strokes without revolving the pestle, followed by centrifugation for 5 min at 270 × *g*. The pellet was taken up in sucrose-EDTA buffer, homogenized by 30 strokes and centrifuged in the same way. The pellet was suspended in sucrose-EDTA buffer, homogenized by 20 strokes, then centrifuged for 5 min at 360 × *g* and finally suspended in 5 mM Tris/Hepes buffer (pH 7.5) containing 0.5 mM EDTA. The suspension was homogenized in a Potter-Elvehjem-type homogenizer and then centrifuged for 30 min at 200 000 × *g*. The pellet homogenized in 50% (w/v) sucrose solution was placed at the bottom of centrifuge tubes, and then was overlayered with 40% and 30% sucrose solutions in the order given. All sucrose solutions contained 5 mM Tris/Hepes buffer (pH 7.5) and 0.5 mM EDTA. The

tube was centrifuged for 90 min at 200 000 × *g*. The 50% sucrose layer was recovered, which was diluted with redistilled water and centrifuged for 30 min at 200 000 × *g*. The final pellet was usually suspended in 1 mM Tris/Hepes buffer (pH 7.5) containing 100 mM D-mannitol and 0.1 mM $MgSO_4$. This membrane vesicle preparation showed an alkaline phosphatase enrichment 14-times and a sucrase enrichment 15-times greater than the starting homogenates, whilst ouabain-sensitive ATPase was decreased one-fifth in specific activity, respectively. These activities were determined according to the method described by Fujita et al. [7].

Uptake method

All assays of the transport activity were carried out at 20 μM of substrate and at 25°C. The membrane vesicles were incubated in a medium containing 100 mM D-mannitol, 1 mM Tris/Hepes (pH 7.5), 0.1 mM $MgSO_4$ and labeled substrate. Other additions are described in the legends. The uptake of substrate was terminated by diluting the aliquot of the sample (approximately 100 μg of membrane protein) with a 40-fold excess of ice-cold buffer composed of 150 mM NaCl, 50 mM $MgCl_2$, 30 mM D-mannitol and 10 mM Tris/Hepes (pH 7.5). The diluted aliquot was filtered immediately through a Millipore filter (HA 025, 0.45 μm) and washed once with 3 ml of the same ice-cold buffer. Radioactivity retained on the filter was counted in a liquid scintillation fluid as described by Hopfer et al. [3]. Na^+-gradient-dependent uptake of a given amino acid is expressed by subtracting the control uptake from that in the presence of NaCl, when indicated.

Potassium-loaded vesicles

Membrane vesicles preloaded with K^+ were prepared by washing three times in 50 mM KCl, 0.1 mM $MgSO_4$ and 100 mM mannitol buffered with 1 mM Tris/Hepes (pH 7.5), followed by preincubation in the same medium for 20 min at 25°C.

All assays were performed in triplicate with freshly prepared membrane vesicles and each assay was repeated at least three times with different membrane preparations. Protein was determined according to Lowry et al. [8].

Chemicals

All reagents were of highest purity commercially available. L-[U-^{14}C]Proline, L-[U-^{14}C]cysteine and α-[1-^{14}C](methylamino)-isobutyric acid were purchased from New England Nuclear, Boston, MA, and α-(methylamino)-isobutyric acid and valinomycin from Sigma Chemical Co.

Results

Time course of Na^+ gradient-dependent uptake of L-proline

The uptake of L-proline by the membrane vesicles as a function of incubation time is shown in Fig. 1. In the absence of an Na^+ gradient, a steady-state level of the uptake was reached in about 30 min. The presence of the Na^+ gradient toward inside from outside the vesicles stimulated L-proline uptake: the uptake reached a maximum level at 30 s after incubation and then decreased with time

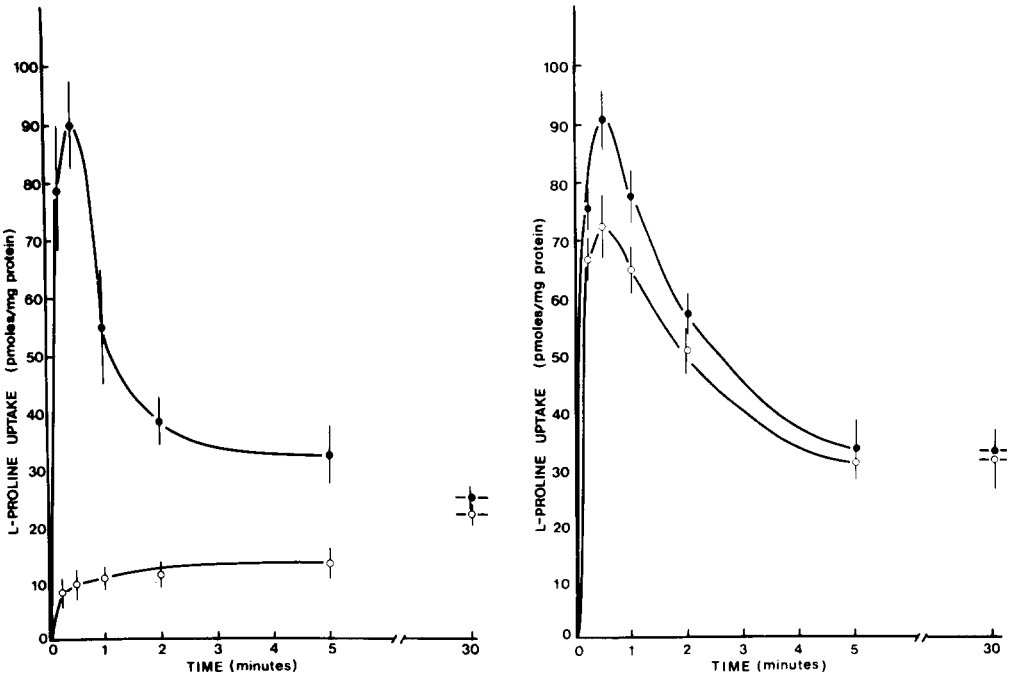

Fig. 1. Effect of Na^+ on the time course of L-proline uptake by brush-border membrane vesicles of the guinea-pig ileum. Membrane vesicles prepared were suspended in a medium containing 100 mM D-mannitol, 0.1 mM $MgSO_4$ and 1 mM Tris/Hepes (pH 7.5). L-Proline uptake was initiated by adding 50 μl of the membrane suspension to 50 μl of an incubation medium composed of 40 μM L-[U-^{14}C]proline, 100 mM D-mannitol, 0.1 mM $MgSO_4$, 1 mM Tris/Hepes (pH 7.5) and 200 mM NaCl. Both the membrane suspension and the incubation medium were preincubated independently at 25°C before mixing, followed by further incubation at 25°C. As control, NaCl in the incubation medium was replaced by an isosmotic concentration of D-mannitol (400 mM). NaCl (●———●); D-mannitol (○———○). Each point represents mean ± S.D.

Fig. 2. Effect of valinomycin on Na^+-gradient-dependent L-proline uptake. The vesicles were preloaded with 50 mM KCl as described in the text. L-Proline uptake was initiated by adding 20 μl of the membrane suspension to 180 μl of an incubation medium composed of 22 μM L-[U-^{14}C]proline, 100 mM D-mannitol, 0.1 mM $MgSO_4$, 1 mM Tris/Hepes (pH 7.5) and 110 mM NaCl. Valinomycin, when present, was 8 μg/mg membrane protein. Control suspensions received 95% ethanol alone in the same volume as valinomycin. With valinomycin (●———●); control (○———○). Each point represents mean ± S.D.

to the steady-state level.

The 'overshoot' of the uptake resulted from an electrochemical gradient formed by the Na^+ gradient and subsequent loss with time of the gradient due to Na^+-substrate cotransport. This was supported by an experiment using K^+-preloaded (in > out) membrane vesicles: when valinomycin was added outside the preloaded vesicles to enhance the electrochemical potential in the presence of the Na^+ gradient, the 'overshoot' of the uptake was further enhanced (Fig. 2).

Effect of medium osmolarity on the uptake of L-proline

As shown in Fig. 3, L-proline uptake was inversely proportional to medium osmolarity and no uptake could be estimated by extrapolation to infinite medi-

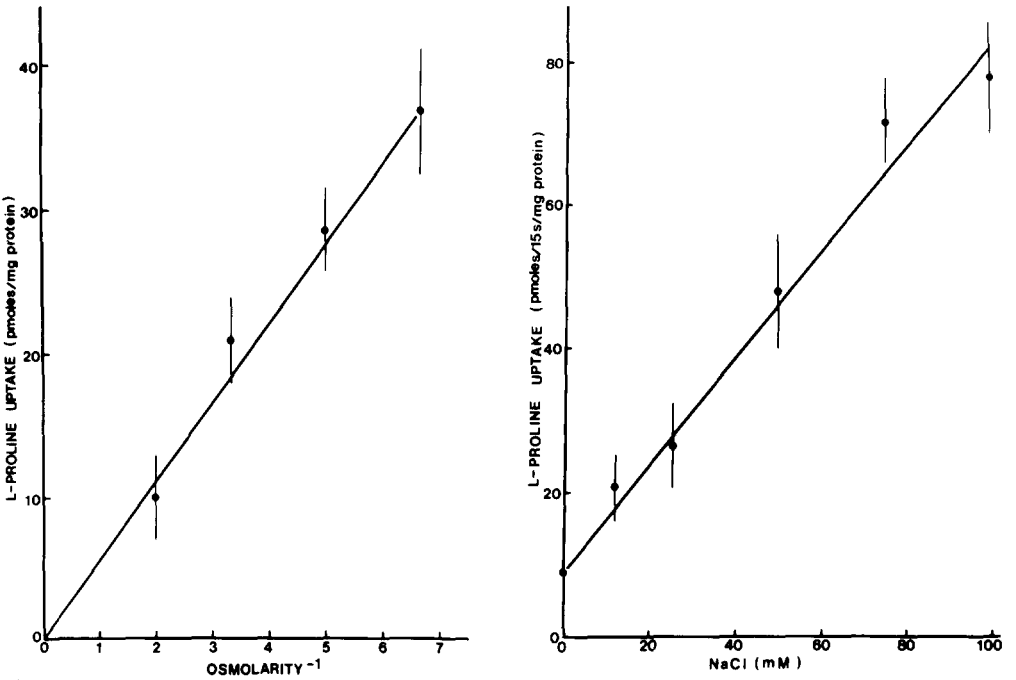

Fig. 3. Effect of medium osmolarity on L-proline uptake. L-Proline uptake was measured 30 min after incubation in a medium containing 20 μM L-[U-^{14}C]proline, 0.1 mM $MgSO_4$, 1 mM Tris/Hepes (pH 7.5) and 25 mM NaCl in final concentrations and D-mannitol was added to the medium to give the indicated osmolarities. Each point represents mean ± S.D.

Fig. 4. Effect of NaCl concentrations on the rate of L-proline uptake. The uptake for 15 s was determined under the same conditions as described in the legend of Fig. 1 except for changes in final NaCl concentration (0—100 mM). Osmolarity of the medium was maintained constant by adjusting the added concentration of D-mannitol. The uptake rates are given as mean ± S.D.

um osmolarity. This indicates that L-proline was transported into the intravesicular membrane space, but not simply adsorbed by the vesicles.

Effect of Na^+ concentrations on the rate of L-proline uptake

The effect of external Na^+ concentrations on the initial rate of L-proline uptake was determined (Fig. 4). Concentrations of D-mannitol were adjusted to maintain medium isosmolarity. There is a linear relationship between increase in the uptake rate and increase in the Na^+ gradient (out > in). At an extravesicular concentration of 100 mM NaCl, the rate of L-proline uptake was stimulated to a level 8-times greater than that obtained in the absence of Na^+, whilst the uptake rate shown in the absence of Na^+ seemed to be due to diffusion, and the same equilibrium level of the uptake in both the presence and absence of an Na^+ gradient (Fig. 1) supports this assumption.

Effect of L-proline concentrations on the initial rate of uptake

Effect of different concentrations of L-proline on the initial rate of uptake in the presence and absence of 100 mM NaCl is illustrated in Fig. 5. In the absence of Na^+, the rate of L-proline uptake increased linearly with increasing

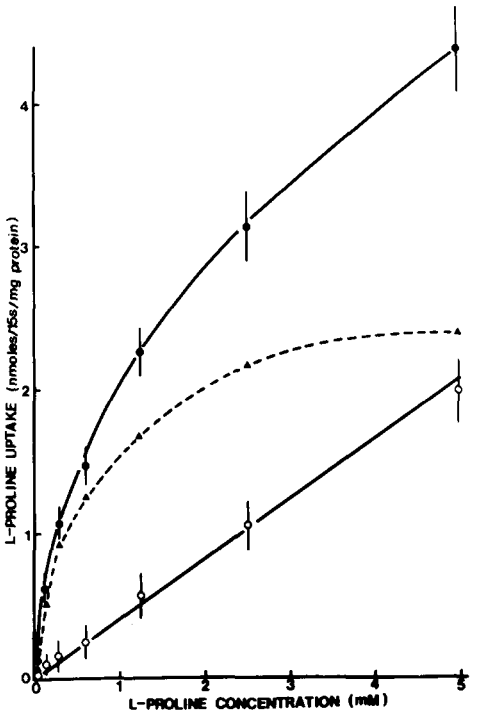

Fig. 5. Effect of L-proline concentrations on the initial rate of uptake. The uptake for 15 s was measured in the medium containing L-[U-^{14}C]proline, 100 mM D-mannitol, 0.1 mM $MgSO_4$ and 1 mM Tris/Hepes (pH 7.5) supplemented with either 100 mM NaCl (●———●) or 200 mM D-mannitol (○———○) in final concentrations. The uptake dependent on Na^+ gradient (▲- - - - -▲) was obtained by subtracting the uptake obtained in the absence of Na^+ from that in its presence. Values of the uptake are given as mean ± S.D.

L-proline concentrations throughout the range 20 μM to 5 mM. The presence of a Na^+ gradient (out > in) gave a non-linear relationship between L-proline concentrations and the uptake rates. The uptake rate solely dependent on Na^+ gradient, which was obtained by subtracting the rate in the absence of Na^+ from that in the presence of Na^+, showed a saturable hyperbolic curve that obeyed Michaelis-Menten kinetics. Lineweaver-Burk plots corresponding to the Na^+ gradient-dependent uptake rates showed a straight line. The calculated K_m and V values for the Na^+ gradient-dependent transport of L-proline were 0.67 mM and 2.73 nmol/15 s per mg of protein, respectively.

Effect of monovalent cations on L-proline transport

Table I shows the effect of monovalent cations on the initial rate of L-proline transport. The membrane vesicles were incubated with L-[U-^{14}C]proline in 300 mM mannitol as the control or in 100 mM NaCl, KCl, LiCl or choline chloride, which was isosmotically replaced for mannitol. The uptake rate was enhanced 9-fold by Na^+ and slightly but significantly by Li^+, whilst neither K^+ nor choline influenced the uptake rate (Table I).

Edmondson et al. [6] recently described that Li^+ might be used to elicit directly the contribution of the ASC system in liver cells. In order to investi-

TABLE I

EFFECT OF MONOVALENT CATIONS ON L-PROLINE TRANSPORT

The uptake for 15 s was carried out under the same conditions as described in the legend of Fig. 1, except for replacement of NaCl with an equal concentration of either choline chloride, KCl or LiCl. Amounts of the uptake are represented as mean ± S.D.

Addition	L-Proline uptake (pmol/15 s per mg protein)
Mannitol (300 mM)	9.01 ± 0.87
Mannitol (100 mM) + choline chloride (100 mM)	8.74 ± 0.65
Mannitol (100 mM) + KCl (100 mM)	9.28 ± 0.92
Mannitol (100 mM) + LiCl (100 mM)	15.17 ± 0.57
Mannitol (100 mM) + NaCl (100 mM)	80.13 ± 13.21

gate whether or not this might be the case in brush-border membranes of the ileum, the following experiments were carried out.

Reciprocal inhibitions of α-(methylamino)-isobutyrate and L-cysteine uptakes and Li^+ effect on these transports

To explore the specificity of amino acid transport systems in brush-border membranes of the guinea-pig ileum, reciprocal inhibitions between α-(methylamino)-isobutyrate and L-cysteine transports were examined. α-(Methylamino)-isobutyrate at 10 mM could not inhibit Na^+-gradient-(out > in)-dependent uptake rate of 20 μM L-cysteine. On the other hand, 10 mM L-cysteine inhibited Na^+-gradient-(out > in)-dependent uptake rate of 20 μM α-(methylamino)-isobutyrate approximately 50% (Table II). These results are similar to those described in the rat hepatocytes [9].

When Na^+ in the medium was replaced by Li^+, the rate of L-cysteine uptake was comparable to that in the presence of an Na^+ gradient. Furthermore, the rate

TABLE II

RECIPROCAL INHIBITIONS OF L-CYSTEINE AND α-(METHYLAMINO)-ISOBUTYRATE UPTAKES AND THE EFFECT OF Li^+

The uptake for 15 s was carried out under the same conditions as described in the legend of Fig. 1, except for use of 20 μM L-[U-^{14}C]cysteine or 20 μM α-[1-^{14}C](methylamino)isobutyrate as transport substrate. When the uptake in the presence of Li^+ was determined, 100 mM NaCl was replaced by 100 mM LiCl. Unlabeled amino acids added were 10 mM and osmolarity of the medium was adjusted to be constant by adding D-mannitol. The Na^+ or Li^+ gradient-dependent uptake of either L-cysteine or α-(methylamino)-isobutyrate (MeAIB) was obtained by subtracting the uptake in the absence of Na^+ or Li^+ from that in the presence of Na^+ or Li^+. All values obtained for the uptake are given as mean ± S.D.

Test amino acid (20 μM)	Cation (100 mM)	Inhibitor amino acid (10 mM)	Na^+ or Li^+ gradient-dependent test amino acid uptake (pmol/15 s per mg protein)
L-Cysteine	Na^+	—	39.5 ± 16.6
L-Cysteine	Na^+	MeAIB	38.1 ± 1.2
L-Cysteine	Li^+	—	49.9 ± 15.1
L-Cysteine	Li^+	MeAIB	55.5 ± 31.4
α-(Methylamino)-isobutyrate	Na^+	—	21.9 ± 6.2 (100%)
α-(Methylamino)-isobutyrate	Na^+	L-cysteine	10.7 ± 0.4 (49%)
α-(Methylamino)-isobutyrate	Li^+	—	1.0 ± 0.4 (4.6%)

TABLE III

RELATIVE CONTRIBUTION OF SYSTEMS A AND ASC TO THE TOTAL Na^+-GRADIENT-DEPENDENT TRANSPORT OF L-PROLINE

The uptake for 15 s was carried out under the same conditions as described in the legend of Fig. 1. When the uptake in the presence of Li^+ was determined, 100 mM NaCl was replaced by 100 mM LiCl. Unlabeled amino acids added were 10 mM and osmolarity of the medium was adjusted to be constant by adding D-mannitol. The Na^+ or Li^+ gradient-dependent uptake of L-proline was obtained by subtracting the uptake in the absence of Na^+ or Li^+ from that in the presence of Na^+ or Li^+. All values obtained for the uptake are given as mean ± S.D. MeAIB, α-(methylamino)-isobutyrate.

Test amino acid (20 μM)	Cation (100 mM)	Inhibitor amino acid (10 mM)	Na^+ or Li^+ gradient-dependent L-proline uptake (pmol/15 s per mg protein)
L-Proline	Na^+	—	71.1 ± 12.4
L-Proline	Na^+	MeAIB	7.5 ± 1.9
L-Proline	Li^+	—	6.2 ± 0.3
L-Proline	Li^+	MeAIB	5.2 ± 0.2

of Li^+-gradient-(out > in)-supported L-cysteine uptake was not inhibited by 10 mM α-(methylamino)-isobutyrate (Table II). On the other hand, when Na^+ in the medium was replaced by Li^+, the rate of α-(methylamino)-isobutyrate uptake was markedly reduced (Table II).

These observations suggest that L-cysteine is a substrate specific for transport system ASC and that α-(methylamino)-isobutyrate is that for transport system A in brush-border membranes of the guinea-pig ileum. Therefore, the inhibition by α-(methylamino)-isobutyrate of a given amino acid uptake may be used to explain the direct contribution of the A system and that the Li^+-gradient-supported uptake occurs solely through the ASC system. L-Cysteine may not be used to define the transport system ASC, because its inhibitory action is not sharply limited, as shown by the fact that α-(methylamino)-isobutyrate uptake was inhibited 50% by L-cysteine (Table II).

Relative contribution of systems A and ASC to the total Na^+-gradient-dependent transport of L-proline

The role of the ASC system in L-proline transport was determined by inhibition due to the presence of α-(methylamino)-isobutyrate and by measuring Li^+-gradient-dependent uptake (Table III). α-(Methylamino)-isobutyrate at 10 mM decreased the rate of Na^+-gradient-dependent uptake of L-proline from 71.1 ± 12.4 to 7.5 ± 1.9 pmol/15 s per mg protein. The rate of Li^+-gradient-dependent uptake of L-proline was 6.2 ± 0.3 pmol/15 s per mg of protein (Table III), which is nearly identical to the residual rate of Na^+-gradient-dependent uptake of L-proline inhibited by α-(methylamino)-isobutyrate.

These results indicate that the relative contribution of system A and ASC involved in the total L-proline uptake dependent on Na^+ gradient is 90% and 10%, respectively, in brush-border membrane vesicles of the guinea-pig ileum.

Discussion

This paper describes the properties of L-proline transport system in brush-border membrane vesicles of the guinea-pig ileum, which has not been reported previously.

In the presence of an Na^+ gradient (out > in) L-proline uptake showed a typical 'overshoot' phenomenon (Fig. 1), indicating an active Na^+ gradient-dependent transport of L-proline. Similar evidence has been presented in the transport of other amino acids and glucose with brush-border membrane vesicles prepared from the intestine and kidney [1,10—13].

Na^+-dependent transport of amino acid in Ehrlich ascites tumor cells [5] was ascribed to the contribution of A and ASC systems. However, characterization of the ASC system is not yet complete, even in the case of Ehrlich ascites tumor cells.

Edmondson et al. [6] recently described that lithium might be used to elicit directly the contribution of the ASC system in rat liver cells. Kilberg et al. [9] reported that the hepatocyte ASC system tolerated Li^+-for-Na^+ substitution better than did system A and added evidence which indicates that L-cysteine is a specific substrate for transport system ASC in rat hepatocytes. The role of the ASC system involved in Na^+ gradient-dependent transport of amino acid in the intestinal brush-border membranes has not yet been analyzed. With brush-border membrane vesicles of the guinea-pig ileum, the rate of L-cysteine and α-(methylamino)-isobutyrate transports in the presence of an Na^+ or Li^+ gradient (out > in) was therefore measured together with reciprocal inhibitions by these substrates (Tables I—III).

The results obtained suggest that L-cysteine is a substrate specific for transport system ASC and α-(methylamino)-isobutyrate is that for transport system A, which are consistent with that of rat hepatocytes [9]. The results further support the assumption that the Li^+-gradient-supported part of L-cysteine and L-proline transports are mediated by the ASC system. Although L-cysteine is suggested to be a specific substrate for the ASC system, it may not be used to define the ASC system in the transport of amino acids, since L-cysteine inhibited transport of α-(methylamino)-isobutyrate, which is the specific substrate for the A system. The reason why L-cysteine inhibited the α-(methylamino)-isobutyrate transport is not clear.

The relative contribution of the ASC system to the total Na^+-gradient-dependent transport of L-proline was calculated by Li^+-gradient-dependent uptake and α-(methylamino)-isobutyrate inhibition (Table III) and the contributions of systems ASC and A were 10% and 90%, respectively.

In addition to analysis of contribution of systems A and ASC in amino acid transport in rat hepatocytes [6,9], it appears important to define the transport systems for amino acids in order to elucidate the regulatory mechanism of amino acid transport [14,15], the transport mechanism of melphalan, the phenylalanine derivative of nitrogen mustard [16], as well as genetic defects in the transport [17].

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