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THE CHARACTERISTIC CHANGES OF AMINO ACID TRANSPORT DURING DEVELOPMENT IN BRUSH BORDER MEMBRANE VESICLES OF THE GUINEA PIG ILEUM

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The characteristic changes of transport systems for amino acids during development were studied with brush border membrane vesicles from the guinea pig ileum. There was a difference of K_m value for L-proline transport kinetics between the vesicles on the 10th day after birth and those on the 60th day after birth. Differences of fatty acid composition of phospholipids and unsaturated percentage of fatty acids were observed between these two vesicles. Fluorescence polarization of the vesicles on the 10th day after birth was larger than that of the vesicles on the 60th day after birth at each temperature examined. L-Proline transport at a substrate concentration below K_m was larger in the vesicles on the 60th day after birth than in the vesicles on the 10th day after birth at each corresponding temperature. Differences of L-leucine transport characteristics were not observed between these two vesicles. These results suggest that the characterisitic changes of L-proline and L-leucine transports during development are different and the characteristics of L-proline transport are regulated at least in part by membrane fluidity which is controlled by membrane lipid composition.

Introduction

In the small intestine nutrients are absorbed through the brush border membrane of the intestinal epithelial cells, and it has been demonstrated that sugars and amino acids are transported actively by electrochemical potential for Na^+ with brush border membrane vesicles [1–3]. The characteristic changes of transport systems for sugars and amino acids during development have been investigated previously with tissue slices and sacs and the changes of transport activity during development have been observed [4–7]. It is important to investigate these problems and factors which influence transport activity with brush border membrane vesicles. Studies with brush border membrane vesicles can elucidate the transport process without cellular metabolism.

With the intestinal brush border membrane vesicles, the difference of L-proline and L-leucine transport activities in guinea pigs between the 10th day and 60th day after birth was investigated and we observed that transport activity of L-proline in the vesicles on the 60th day after birth was larger than that in the vesicles on the 10th day after birth at substrate concentration below K_m and difference of transport kinetics was found in K_m value. There was no difference of L-leucine transport activity between these two vesicles.

Recently it has been observed that transport activity or membrane bound enzyme activity is influenced by membrane lipid composition in animal cells [8,9]. Therefore, in the present study we investigated the relationship between mem-

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

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brane lipid composition and transport activity and kinetics with the intestinal brush border membrane vesicles in guinea pigs on the 10th day and on the 60th day after birth.

Materials and Methods

Chemicals. All reagents used were of the highest purity commercially available. L-[U-^{14}C]Proline (270 mCi/mmol) and L-[U-^{14}C]leucine (351 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, U.K.

Isolation of brush border membrane vesicles. Membrane vesicles were prepared from the guinea pig ileum by the procedure of Fujita et al. [10] with slight modification as described previously [3] and 0.32 mM sucrose was used for sucrose-EDTA buffer. The final membrane pellet was usually suspended in a medium containing 100 mM D-mannitol, 0.1 mM $MgSO_4$, and 2 mM Tris-Hepes (pH 7.4).

Assay of transport activity. All assays of transport activity were carried out at 25°C according to the procedure described previously [3]. Assay conditions used are described in the legends of the tables and figures. The transport of substrate was terminated by diluting the samples (approx. 100 μg of membrane protein) with a 40-fold excess of an ice cold buffer containing 150 mM NaCl, 50 mM $MgCl_2$, 30 mM D-mannitol and 10 mM Tris-Hepes (pH 7.4). The diluted aliquot was immediately filtered through a Millipore filter (HAWP 0.45 μm) and washed once with 3 ml of the same ice-cold buffer. Radioactivity retained on the filter was counted in Bray's liquid scintillation fluid. All assays were performed in triplicate with membrane vesicles and each assay was repeated at least three times with different membrane preparations. Protein was determined according to the method of Lowry et al. [11].

Lipid determination Lipids were extracted by the method of Folch et al. [12]. Phospholipids were separated by thin-layer chromatography in Silica gel 60 plate with reference to phospholipid standards. The solvent system used was chloroform/methanol/acetic acid/water (25:15:4:2, v/v). Spots were visualized with I_2 vapor, scraped, and eluted with 5 ml of chloroform/methanol/acetic acid/water (5:5:1:1, v/v) followed by 2 ml of methanol. The extracts were combined, evaporated to dryness, and redissolved in chloroform/methanol/water (2:2:1.8, v/v). The chloroform phase was used for total phosphate analysis by the method of Bartlett [14]. Fatty acid composition was determined after separation of phospholipids from neutral lipids and glycolipids on a unisil column according to the method of Rouser et al. [15]. Fatty acids of phospholipid molecules were methylated with 0.1 M sodium methylate. Methyl esters were chromatographed on a 25% diethyleneglycol succinate on Celite 545A (60–80 mesh) column at 180°C. The flow rate of the carrier gas (N_2) was 50 ml/min. Fatty acids were identified by means of comparison with methyl ester standards and a semilogarithmic plot of carbon number versus retention time. The composition of fatty acids and phospholipids is the mean of triplicate determinations. Lipid composition is expressed as percentage of the total recovered. Cholesterol content was determined by the method of Courchaine et al. [13].

Membrane fluidity measurement. Membrane fluidity was measured by a fluorescence polarization technique. A suspension of 1 μM 1,6-diphenyl-1,3,5-hexatriene was prepared freshly by adding a known volume of 2 mM diphenylhexatriene dissolved in tetrahydrofuran to an appropriate volume of 100 mM NaCl, 100 mM D-mannitol, 2 mM Tris-Hepes (pH 7.4). One volume of the 1 μM diphenylhexatriene suspension was added to 1 volume of membrane suspension containing about 100 μg of membrane protein in 100 mM D-mannitol, 0.1 mM $MgSO_4$ and 2 mM Tris-Hepes (pH 7.4). The mixture was incubated at 25°C for 60 min. Fluorescence polarization was measured with Hitachi 650-10S fluorescence spectrophotometer using excitation and emission wavelengths of 366 and 430 nm, respectively. Measurements were made within illumination periods up to 10 s. The sample was left at each temperature until the reading of fluorescence polarization had stabilized.

Results

Time-course of L-proline transport

The uptake of L-proline by the membrane vesicles during different incubation times is shown

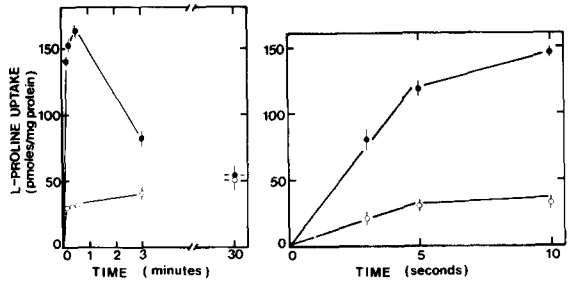

Fig. 1. The time-course of L-proline uptake by brush border membrane vesicles in the presence and absence of an Na^+ gradient. The prepared membrane vesicles were suspended in a medium containing 100 mM D-mannitol, 0.1 mM $MgSO_4$ and 2 mM Tris-Hepes (pH 7.4). L-Proline uptake was initiated by adding 50 μl of the membrane suspension to 50 μl of a reaction medium containing 40 μM L-proline, 100 mM D-mannitol, 0.1 mM $MgSO_4$, 2 mM Tris-Hepes (pH 7.4) and 200 mM NaCl. Both the membrane suspension and the reaction medium were preincubated independently at 25°C before mixing, followed by further incubation at 25°C. As control, NaCl in the reaction medium was replaced by an isosmotic concentration of D-mannitol (400 mM). ●——●, NaCl; ○——○, D-mannitol. Each point represents mean ± S.D.

in Fig. 1. In the absence of an Na^+ gradient, a steady-state level of the uptake of L-proline was reached in about 30 min. The presence of an Na^+ gradient (out > in) stimulated the uptake of L-proline. The uptake of L-proline into the vesicles reached a maximal level in about 10 s in the presence of an Na^+ gradient and then decreased to the equilibrium level. This result suggests that the presence of an extravesicular to intravesicular Na^+ gradient affects the transient uptake of L-proline into membrane vesicles against its concentration gradient. L-Proline uptake increased linearly for about 5 s.

Kinetics of L-proline transport

The transport kinetics of L-proline were investigated with initial rate of L-proline transport. The uptake of L-proline was linear for 4 s at any substrate concentration used in this experiment, suggesting that the driving force due to an Na^+ gradient for L-proline transport was nearly constant during the first 4 s. The incubation time of 2 s was used for transport kinetics. Transport kinetics in the vesicles on the 10th day and 60th day after birth were examined with the substrate concentration from 0.25 mM to 2 mM in two different concentrations of NaCl (Fig. 2). In the vesicles on the 10th day after birth, K_m values of L-proline transport in 50 and 100 mM NaCl were 3.40 ± 0.09 and 2.96 ± 0.15 mM, respectively. V_{max} values in 50 and 100 mM NaCl were $8.16 \pm$

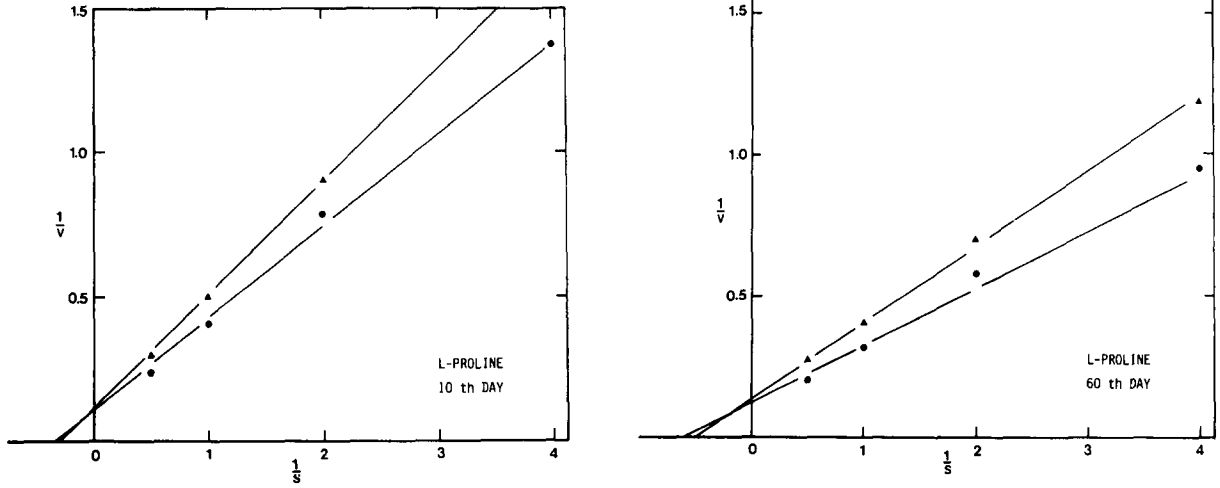

Fig. 2. The relationship between L-proline concentrations and the initial rates of L-proline transport. The uptake for 2 s was measured in a medium containing L-proline at the indicated concentrations, 100 mM D-mannitol, 0.1 mM $MgSO_4$ and 2 mM Tris-Hepes (pH 7.4) supplemented with 100 mM NaCl (●——●) or 50 mM NaCl (▲——▲) in final concentrations. Results were presented as double reciprocal plot of the transport in each NaCl concentration. Osmolarity of the medium was adjusted to be constant by addition of D-mannitol.

0.56 and 8.89 ± 0.50 nmol/mg protein per 2 s, respectively. In the vesicles on the 60th day after birth, K_m values in 50 and 100 mM NaCl were 2.02 ± 0.04 and 1.63 ± 0.05 mM, respectively. V_{max} values in 50 and 100 mM NaCl were 7.27 ± 0.38 and 8.16 ± 0.23 nmol/mg protein per 2 s, respectively. Kinetically, there was a marked difference of K_m values between these two vesicles.

Purity of the brush border membrane vesicles

To exclude the possibility that the difference of kinetic parameters for L-proline transport shown in Fig. 2 was influenced by the purity of the brush border membrane vesicles, the degree of purification of the vesicles was compared between the vesicles on the 10th day after birth and those on the 60th day after birth (Table I). In the vesicles on the 10th day after birth, the specific activities of sucrase and alkaline phosphatase were about 15- and 12-fold greater than that of the first homogenate. In the vesicles on the 60th day after birth, the specific activities of both enzymes were about 15- and 11-fold greater than that of the first homogenate. There was thus no difference of the degree of purification of the brush border membrane vesicles between guinea pigs on the 10th day and those on the 60th day after birth.

These results suggest that the difference of kinetic parameters of L-proline transport between the vesicles on the 10th day and those on the 60th day after birth is not due to the difference of purity of the vesicles.

Lipid composition of brush border membrane vesicles

It has been suggested that K_m value of α-aminoisobutyric acid transport in Ehrlich ascites cells is influenced by changes of fatty acid composition [9]. In order to determine whether changes of K_m values for L-proline transport were influenced by membrane lipid composition, phospholipids and cholesterol composition of the brush border membrane vesicles were compared between guinea pigs on the 10th day after birth and those on the 60th day after birth (Table II). There was no significant difference of phospholipid composition but there was a significant difference of cholesterol content between the two vesicles. When fatty acid composition of phospholipids was analysed, a significant difference of each fatty acid composition except myristic acid and palmitic acid was observed between the two vesicles. Unsaturated ratios of fatty acids in the vesicles on the 10th day after birth and those on the 60th day

TABLE I

ENZYME ACTIVITIES IN THE FIRST HOMOGENATE AND BRUSH BORDER MEMBRANE VESICLES

Isolation procedure for brush border membrane vesicles is described in Materials and Methods, and the method of enzyme assay is described previously [3]. Specific activity is expressed as μmol product/mg protein per 30 min. Each value represents mean ± S.D.

	Specific activity	
	10th day	60th day
Sucrase		
First homogenate	3.84 ± 0.06	3.04 ± 0.06
Brush border membrane vesicles	57.33 ± 5.42	44.67 ± 1.78
Alkaline phosphatase		
First homogenate	6.72 ± 0.74	5.72 ± 0.74
Brush border membrane vesicles	82.72 ± 2.71	62.91 ± 12.60

TABLE II

LIPID COMPOSITIONS OF BRUSH BORDER MEMBRANE VESICLES

Individual phospholipids and cholesterol contents were determined as described in Materials and Methods. Results are expressed as percentage of the total recovered for phospholipids and μg per mg protein for cholesterol. Each value represents mean ± S.D. Sph, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; Chol, cholesterol. *P* refers to the *t*-test for differences between the vesicles on the 10th day after birth and those on the 60th day after birth. n.s., not significant.

	10th day	60th day	*P*
Sph	17.48 ± 0.41	19.44 ± 2.29	n.s.
PC	31.22 ± 1.39	28.02 ± 0.17	n.s.
PI + PS	20.76 ± 0.62	21.55 ± 2.41	n.s.
PE	30.54 ± 2.42	30.87 ± 0.44	n.s.
Chol	38 ± 1.4	43 ± 2.1	< 0.05

TABLE III

RATIOS OF THE DIFFERENT FATTY ACID OBTAINED BY METHANOLYSIS OF PHOSPHOLIPIDS FROM DIFFERENT PREPARATIONS OF BRUSH BORDER MEMBRANE VESICLES

Fatty acid compositions are expressed as percentage of the total recovered. Each value represents mean ± S.D. *P* refers to the *t*-test for differences between the vesicles on the 10th day after birth and those on the 60th day after birth. n.s., not significant.

	10th day	60th day	*P*
Fatty acid			
14:0	0.82±0.27	0.48±0.12	n.s.
16:0	18.62±0.55	15.97±1.84	n.s.
16:1	0.36±0.03	0.30±0.01	<0.05
18:0	27.19±0.90	22.72±0.68	<0.01
18:1	10.55±0.23	13.51±1.12	<0.02
18:2	23.49±2.62	36.80±1.82	<0.01
18:3	3.54±0.13	2.22±0.24	<0.01
20:4	15.40±0.45	8.00±0.04	<0.001
% unsaturated	53.36±1.50	60.83±2.64	<0.02

after birth were 53.36 ± 1.50% and 60.83 ± 2.64%, respectively, indicating a significant difference between these two vesicles (Table III).

Measurement of membrane fluidity

Fluidity of the brush border membrane vesicles was inferred from measurement of fluorescence polarization of a fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene (Fig. 3). When measurement temperature was increased, fluorescence polarization decreased. The values obtained for the vesicles on the 10th day after birth were larger than those on the 60th day after birth at each temperature examined except 283 K. These results suggest that the brush border membrane vesicles on the 60th day after birth are more fluid than those on the 10th day after birth.

The comparison of L-proline transport activity between the vesicles on the 10th day after birth and those on the 60th day after birth at each temperature

L-Proline transport at different temperatures is shown in Fig. 4. L-Proline transport activity in the brush border membrane vesicles from guinea pigs on the 60th day after birth is larger than that from guinea pigs on the 10th day after birth at each

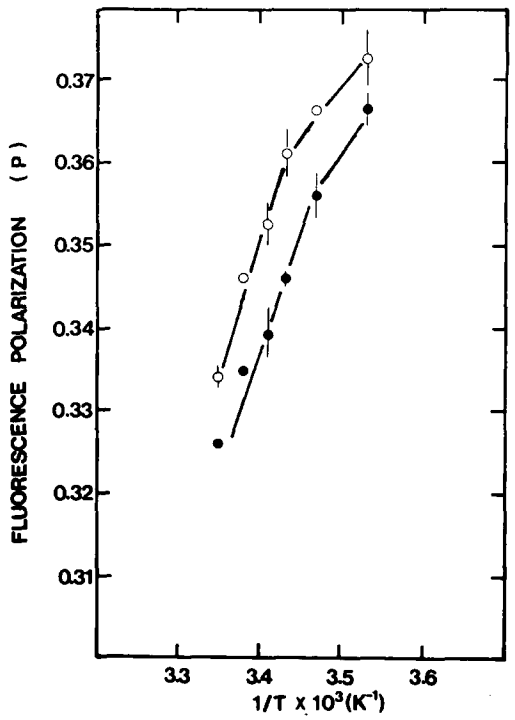

Fig. 3. Temperature dependence of the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in the vesicles on the 10th day after birth (○——○) and in the vesicles on the 60th day after birth (●——●). For experimental details see Materials and Methods. The ordinate is a fluorescence polarization term ($P=(I_{\parallel}-I_{\perp})/(I_{\parallel}+I_{\perp})$) and the abscissa is the reciprocal of the absolute temperature. Each value represents mean ± S.D.

temperature examined. The results shown in Figs. 3 and 4 indicate that the increase in L-proline transport activity was accompanied by the increase in membrane fluidity. A similar result was reported showing that D-glucose transport activity in adipocytes was enhanced by increase of plasma membrane fluidity [20].

L-Leucine transport in the brush border membrane vesicles

Transport kinetics of L-leucine were examined and compared between the vesicles on the 10th day after birth and those on the 60th day after birth. In the vesicles on the 10th day after birth, K_m values of L-leucine transport in 50 and 100 mM NaCl were 3.81 ± 0.18 and 2.67 ± 0.17 mM, respectively, and V_{max} values in 50 and 100 mM NaCl were 10.88 ± 0.88 and 11.25 ± 1.76 nmol/mg

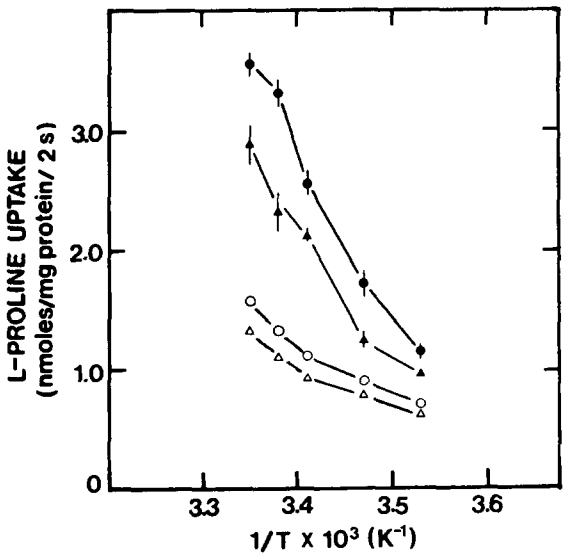

Fig. 4. The comparison of L-proline transport activity. L-Proline uptake was measured as described in the legend of Fig. 1 at each temperature except for the use of 1 mM L-proline. Symbols: L-proline uptake in the vesicles on the 60th day after birth in the presence (●——●) and absence (○——○) of an Na^+ gradient; L-proline uptake in the vesicles on the 10th day after birth in the presence (▲——▲) and absence (△——△) of an Na^+ gradient.

protein per 4 s, respectively. In the vesicles on the 60th day after birth, K_m values of L-leucine transport in 50 and 100 mM NaCl were 3.65 ± 0.35 and 2.55 ± 0.05 mM, respectively, and V_{max} values were 9.75 ± 0.25 and 9.80 ± 0.71 nmol/mg protein per 4 s, respectively (Fig. 5).

These results suggest that there is no difference of L-leucine transport activity between these two vesicles.

Discussion

In the present study with the brush border membrane vesicles, there was a difference of L-proline transport kinetics between the vesicles on the 10th day after birth and those on the 60th day after birth and the difference was shown in K_m values but not in V_{max} values. Amino acid transport model proposed by Curran et al. [16] has shown that V_{max} value changed with the number of carrier proteins, but the K_m value was not influenced by it. It is, therefore, suggested that there is no difference in the number of carrier proteins between the vesicles on the 10th day after birth and those on the 60th day after birth. Thus we

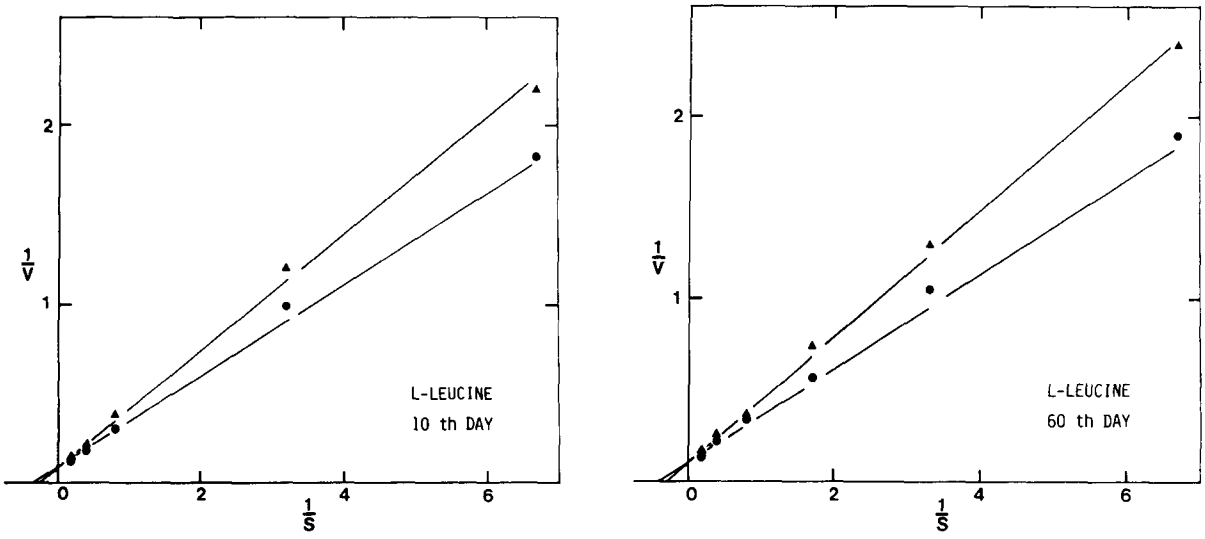

Fig. 5. The relationship between L-leucine concentrations and the initial rates of L-leucine transport. The uptake for 4 s was measured in a medium containing L-leucine at the indicated concentrations, 100 mM D-mannitol, 0.1 mM $MgSO_4$ and 2 mM Tris-Hepes (pH 7.4) supplemented with 100 mM NaCl (●——●) or 50 mM NaCl (▲——▲) in final concentrations. Results were presented as double reciprocal plot of the transport in each NaCl concentration. Osmolarity of the medium was adjusted to be constant by addition of D-mannitol.

investigated the lipid composition of brush border membrane vesicles and observed a difference of fatty acid composition and cholesterol content between these vesicles. These differences of membrane lipid composition seemed to be associated with the difference of membrane fluidity, which was inferred from measurements of fluorescence polarization of the fluorescence probe. The membrane fluidity in the vesicles on the 60th day after birth was larger than that in the vesicles on the 10th day after birth at each temperature examined. Because the initial rate of L-proline transport in the vesicles on the 60th day after birth was larger than that in the vesicles on the 10th day after birth at each corresponding temperature at substrate concentration below K_m, it is suggested that there is a positive correlation between membrane fluidity and L-proline transport activity. Probably membrane fluidity may influence K_m values by changing such component as dissociation constant of substrate and carrier binding and regulate L-proline transport activity.

It is reported that enzymes and transport system which are contained in the membrane are regulated at least in part by the surrounding lipids [8,17]. For example, adenylate cyclase activity in Chinese hamster ovary cells is controlled by membrane cholesterol content and the effect of cholesterol on enzyme activity is mediated through increase in acyl chain ordering [18]. In this case, adenylate cyclase activity is increased by decrease of membrane fluidity. On the contrary, (Na^+-K^+)-stimulated ATPase activity decreases with increase of membrane acyl chain ordering [19] and D-glucose transport activity in plasma membranes of adipocytes is enhanced by increase of membrane fluidity [20]. Furthermore, α-aminoisobutyric acid transport in Ehrlich ascites cells is influenced by change in the fatty acid composition of cell membrane and K_m for Na^+ dependent component of α-aminoisobutyric acid uptake is regulated [9]. This result resembles our data for L-proline transport. L-Leucine transport kinetics were not influenced by development and changes of membrane lipid composition (Fig. 5). This result suggests that the carrier system for L-proline is different from that for L-leucine and the regulation of transport activity by membrane lipid composition is not homogenous in brush border membrane vesicles of the guinea pig ileum.

Development of D-glucose transport in brush border membrane vesicles of chick jejunum has been studied and it was shown that K_m values were the same, but V_{max} values were larger in the vesicles on the 2nd day than in the vesicles on the 21th day. In this case, it was suggested that D-glucose transport was regulated by the carrier number or maximal turnover rate per carrier [21]. These results suggest that there are many factors to regulate transport activity.

Development of amino acid transport by intestine of the guinea pig has been studied with tissue accumulation method and it was shown that the increase of L-proline accumulation began before birth and continued until just after birth and then the accumulation began to decline during the next several weeks to the adult tissue level [4]. In our results obtained with the brush border membrane vesicles, the initial rate of L-proline transport in the vesicles on the 60th day after birth was larger than in the vesicles on the 10th day after birth at each temperature examined in 1 mM L-proline concentration. This result does not seem to be consistent with the result obtained with tissue accumulation. There are many reasons to explain this discrepancy. The most reasonable reason is that there are other mechanisms except Na^+-dependent transport to accumulate amino acid into cells or tissue such as exchange mechnism in physiological cells or tissue.

Because brush border membrane vesicles prepared showed the same increase of marker enzyme specific activity in the vesicles on the 10th day after birth and in the vesicles on the 60th day after birth (Table I) and the Na^+ gradient imposed probably did not change during 2 s incubation period (Fig. 1), we consider that the difference of K_m values for L-proline transport between both vesicles is reliable and the transport kinetics of L-proline, especially K_m values, are regulated at least in part by membrane fluidity which is controlled by membrane lipid composition.

To establish this hypothesis, an experiment with purified carrier protein or a thermodynamic analysis must be conducted and these problems are now being studied.

References

1 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32

2 Sigrist-Nelson, K., Murer, H. and Hopfer, U. (1975) J. Biol. Chem. 250, 5674–5680

3 Hayashi, K., Yamamoto, S., Ohe, K., Miyoshi, A. and Kawasaki, T. (1980) Biochim. Biophys. Acta 601, 654–663

4 Butt, J.H. and Wilson, T.H. (1968) Am. J. Physiol. 215, 1468–1477

5 Holdsworth, C.D. and Wilson, T.H. (1967) Am. J. Physiol. 212, 233–240

6 Bamford, D.R. and Ingham, P.A. (1975) J. Physiol. London 248, 335–348

7 Thomson, A.B.R. (1979) Am. J. Physiol. 236, E685–E691

8 Sandermann, H. (1978) Biochim. Biophys. Acta 515, 209–237

9 Kaduce, T.L., Awad, A.B., Fontenelle, L.J. and Spector, A.A. (1977) J. Biol. Chem. 252, 6624–6630

10 Fujita, M., Ohta, H., Kawai, K., Matsui, H. and Nakao, M. (1972) Biochim. Biophys. Acta 274, 336–347

11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275

12 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509

13 Courchaine, A.J., Miller, W.H. and Stein, D.B., Jr. (1959) Clin. Chem. 5, 609–614

14 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468

15 Rouser, G., Kritchevsky, G., Simon, G. and Nelson, G.J. (1967) Lipids 2, 37–40

16 Curran, P.F., Schultz, S.G., Chez. R.A. and Fuisz, R.E. (1967) J. Gen. Phys. 50, 1261–1268

17 Amatruda, J.M. and Finch, E.D. (1979) J. Biol. Chem. 254, 2619–2625

18 Sinensky, M., Minneman, K.P. and Molinoff, P.B. (1979) J. Biol. Chem. 254, 9135–9141

19 Kimelberg, H.K. and Papahadjoupolos, D. (1974) J. Biol. Chem. 249, 1071–1080

20 Pilch, P.F., Thompson, P.A. and Czech, M.P. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 915–918

21 Shehata, A.T., Lerner, J. and Miller, D.S. (1981) Am. J. Physiol. 240, G102–G108