J. Membrane Biol. 107, 57–62 (1989)

The Journal of
Membrane Biology

$Na^+(Li^+)$/Branched-Chain Amino Acid Cotransport in *Pseudomonas aeruginosa*

Yoshihiko Uratani†, Tomofusa Tsuchiya‡, Yukiko Akamatsu‡, and Toshimitsu Hoshino†

†Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194, Japan, and ‡Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-naka, Okayama 700, Japan

Summary. A transport system for branched-chain amino acids (designated as LIV-II system) in *Pseudomonas aeruginosa* requires Na^+ for its operation. Coupling cation for this system was identified by measuring cation movement during substrate entry using cation-selective electrodes. Uptakes of Na^+ and Li^- were induced by the imposition of an inwardly-directed concentration gradient of leucine, isoleucine, or valine. No uptake of H^+ was found, however, under the same conditions. In addition, effects of Na^+ and Li^+ on the kinetic property of the system were examined. At chloride salt concentration of 2.5 mM, values of apparent K_m and V_{max} for leucine uptake were larger in the presence of Na^+ than Li^+. These results indicate that the LIV-II transport system is a $Na^+(Li^+)$/substrate cotransport system, although effects of Na^+ and Li^+ on kinetics of the system are different.

Key Words *Pseudomonas aeruginosa* · cation cotransport · branched-chain amino acids

Introduction

Pseudomonas aeruginosa has two kinetically distinguishable transport systems for branched-chain amino acids, L-leucine, L-isoleucine, and L-valine (Hoshino, 1979*b*; Hoshino & Kageyama, 1982). One is a binding protein-dependent system (LIV-I) with high affinity to the substrates. The other is a sodium-dependent carrier system (LIV-II) with low affinity. The latter (LIV-II) transport system requires sodium ion for its operation in whole cells and membrane vesicles. An imposition of inwardly-directed concentration gradient of sodium ion drives leucine accumulation in nonenergized membrane vesicles (Hoshino, 1979*a*). Similarly, the addition of lithium ion also causes considerable accumulation of leucine. These findings strongly suggest that Na^+ and Li^+ are coupling cations for the LIV-II transport system. However, there is no direct evidence for cotransport between the three substrates and Na^+ or Li^+ via the transport system.

It has been reported that *Escherichia coli* and *Salmonella typhimurium* have Na^+/substrate cotransport systems (Hasan & Tsuchiya, 1977; Tokuda & Kaback, 1977; Tsuchiya et al., 1977*a*; Chen et al., 1985; Hama et al., 1987). Of them, melibiose and proline transport systems are coupled to Li^+ in addition to Na^+ (Tsuchiya, Oho & Shiota-Niiya, 1983; Tsuchiya et al., 1984).

In this communication, we describe uptake of leucine, isoleucine, and valine in the presence of Na^+ or Li^+, substrate-induced uptake of Na^+ and Li^+ in whole cells using Na^+ and Li^+-selective electrodes, and difference between effects of Na^+ and Li^+ on kinetic parameters of leucine uptake.

Materials and Methods

Bacterial Strains and Growth Conditions

P. aeruginosa PML14, wild-type strain, was mainly used throughout this study. Strains PML1459 and 1460, derivatives of PML14, are defective in LIV-I system and LIV-II system, respectively. Strain PML1455 is also a LIV-I-defective derivative of PML14, having an enhanced LIV-II transport activity with the same K_m and large V_{max} compared to those of the parental strain. All the strains were grown in modified G medium at 37°C (Hoshino, 1979*b*).

Uptake Measurement

Cells were harvested at early logarithmic phase of growth, washed three times with 100 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)/Tris-(hydroxymethyl)-aminomethane (Tris) buffer, pH 7.5, suspended in the same buffer, and kept at room temperature until use. For initial rate measurements, a 200-μl aliquot of cell suspension was preincubated for 1.5 min at 30°C with appropriate salt solution containing 50 mM glucose as a respiratory substrate and 10 mM alanine. Alanine was contained to repress uptakes of leucine, isoleucine, and valine via LIV-I transport system (Hoshino, 1979*a*). Uptake was initiated by the addition of 4 μl of [^{14}C]-amino acid to give a final concentration of 23 μM (0.9 μCi/ml). After 10 sec incubation, the

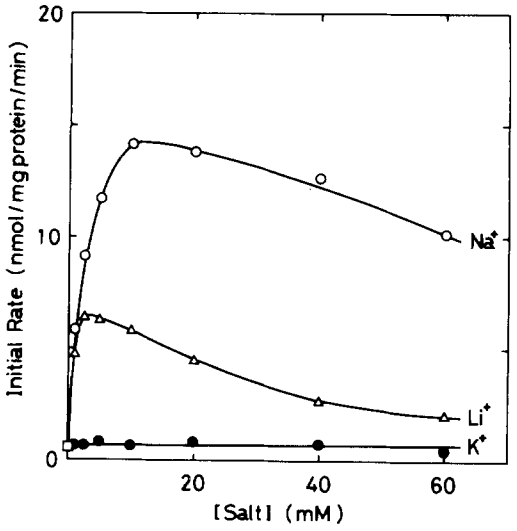

Fig. 1. Initial rate of leucine uptake as a function of salt concentration. Initial rates were measured in duplicate at 30°C as described in Materials and Methods. Salt solutions (1 M) were added to cell suspensions to give appropriate concentrations of NaCl (○), LiCl (△), KCl (●), or none (□). The final cell concentration was 0.25 mg protein/ml

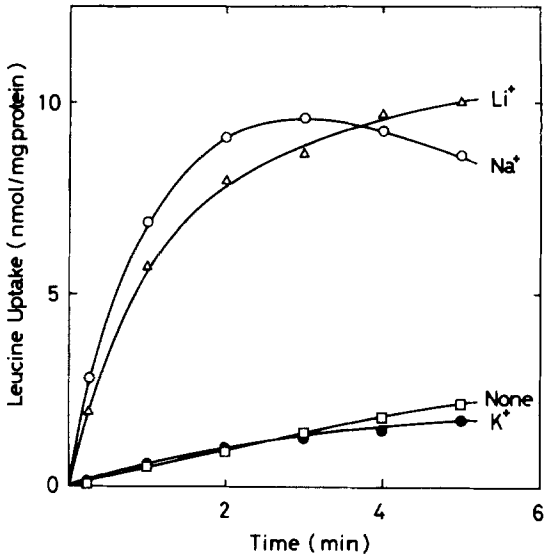

Fig. 2. Leucine uptake in the presence of various salts. Cell suspensions contained 2.5 mM NaCl (○), LiCl (△), KCl (●), or none (□). Uptake reaction was initiated by addition of [^{14}C]-leucine at a final concentration of 23 μM leucine. Cell concentration was 0.3 mg protein/ml

sample solution was diluted with 4 ml of dilution solution composed of 0.125 M KCl and 10 mM Tris-HCl, pH 7.5, to stop the uptake reaction, filtered on a 0.45 μm Millipore filter, and washed with the same volume of the dilution solution. Residual radioactivity on the filter was counted after drying it (Hoshino, 1979*b*). Time course of uptake was measured at 30°C by removing 100-μl aliquots of reaction mixture at various time intervals under the conditions of aeration with stirring.

Cation Transport Measurement

Uptakes of Na^-, Li^-, and H^- were measured at 28°C using cation-selective electrodes (Tsuchiya et al., 1983; Hama et al., 1987). For measurement of Na^+ and Li^+ uptakes, cells were harvested at late logarithmic phase, washed twice with 100 mM 3-(N-morpholino)-propanesulfonic acid (MOPS)/tetramethylammonium hydroxide (TMAH), pH 7.5, and suspended in a small volume of the MOPS/TMAH buffer. Protein concentration of cell suspensions was approximately 40 mg per ml. A portion of the cell suspension (0.3 ml) was diluted with 2.7 ml of 100 mM tricine/TMAH, pH 8.0, containing 100 μM NaCl or LiCl. Cells were incubated at 28°C in a plastic vessel with rapid stirring under water-saturated N_2 gas flow. An Na^+-electrode (Radiometer, Copenhagen, Denmark) or a hand-made Li^+-electrode with a reference electrode (Radiometer) was put into the vessel. An anaerobic solution (30 μl) of 0.1 M leucine, isoleucine, or valine was added to initiate the cation transport. The two electrodes were connected to a pH meter (PHM80, Radiometer), and Na^+ or Li^+ concentration was continuously recorded. Calibration was carried out by the addition of known amounts of NaCl or LiCl.

H^+ movement was measured with a pH electrode in a manner essentially similar to the measurement of Na^+ or Li^+ uptake. Buffer solutions were replaced with 140 mM choline chloride. A portion (0.4 ml) of cell suspension was diluted with 2.1 ml of the same choline solution. H^- concentration was calibrated by the addition of known amounts of HCl.

Protein Determination

Protein content of cell suspensions was determined by the method of Schaffner and Weissmann (1973) for the samples of HEPES buffer or of Lowry et al. (1951) for the others with bovine serum albumin as standard.

Chemicals

L-[U-^{14}C]-leucine, isoleucine, and valine were purchased from Amersham (Japan). N,N'-diheptyl-N-N'-5,5-tetramethyl-3,7-dioxanonandiamide, ionophore for Li^+-selective electrode, was obtained from Fluka (Switzerland). All other chemicals were of reagent grade and commercially available.

Results

Effect of Monovalent Cations on Substrate Uptakes

Dependence of leucine uptake activity, a measure of LIV-II transport system, on salt concentrations of NaCl, LiCl, and KCl was examined by measuring the initial rate of leucine uptake (Fig. 1). The initial rate increased with the increase in NaCl concentration and reached maximal level around 10 mM NaCl. In the presence of LiCl, the initial rate was maximal at 2.5 to 5.0 mM and decreased above 5.0 mM. On the other hand, no stimulation of leucine uptake was observed with KCl up to 100 mM. Thus,

Table 1. Effect of cations on uptakes of leucine, isoleucine, and valine[a]

Strain	Salt added (2.5 mM)	Uptake (nmol/mg protein/min)		
		Leu	Ileu	Val
PML14	None	0.5	0.6	0.4
	LiCl	5.8	6.5	8.0
	NaCl	7.6	7.8	6.5
PML1460	None	0.03	0.14	0.0
	LiCl	0.07	0.03	0.04
	NaCl	0.03	0.04	0.03
PML1455	None	2.5	4.3	1.6
	LiCl	24.0	26.7	44.0
	NaCl	29.2	28.9	39.8

[a] Grown cells were suspended in 0.1 M HEPES/Tris, pH 7.5, after wash. Uptake reaction was initiated at 30°C by addition of [^{14}C]-leucine, isoleucine, and valine to give a final concentration of 23, 31, and 46 μM, respectively. Uptake values were taken at 1 min.

salt concentration was fixed at 2.5 mM for the following experiments of substrate uptake.

Figure 2 shows time course of leucine transport in the presence of Na^+, Li^+, or K^+. The presence of Na^+ or Li^+ simulated leucine accumulation, whereas that of K^+ did not. Leucine uptake in the absence of salt was as small as that in the presence of K^+. The effect of monovalent cation was examined for isoleucine and valine uptakes as well as leucine uptake (Table 1). Uptake activities of isoleucine and valine were high in the presence of Na^+ or Li^+, but low in the absence of these cations. Strain PML1460 lacking LIV-II transport activity did not exhibit any uptake activity even in the presence of Na^+ or Li^+. Strain PML1455 having high LIV-II activity exerted high uptake activities of leucine, isoleucine, and valine with Na^+ and Li^+. These results indicate that the LIV-II transport system is operated exclusively by Na^+ or Li^+ but not by K^+.

Substrate-Induced Cation Uptake

In order to verify the mechanism of substrate/cation cotransport in the LIV-II transport system, Na^+ or Li^+ uptake during substrate entry was measured using an Na^+ or Li^+-selective electrode. In addition, H^+ uptake was examined. Figure 3*A* shows that the imposition of inwardly-directed concentration gradient of leucine, isoleucine, or valine induced Na^+ inflow into whole cells. The addition of valine caused slower Na^+ uptake than that of leucine and isoleucine. When an excess alanine (10 mM), a substrate for LIV-I system, was present to suppress branched-chain amino acid uptake via the

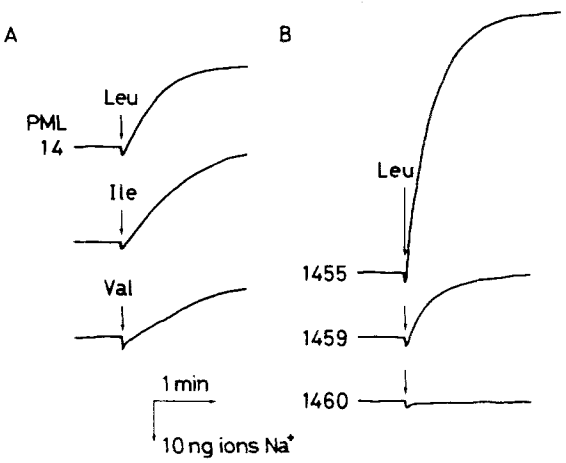

Fig. 3. Uptake of Na^+ induced by addition of leucine, isoleucine or valine. Na^+ uptake was measured at 28°C using an Na^+-selective electrode as described in Materials and Methods. The uptake was initiated by the addition of an anaerobic amino acid solution at the point indicated by arrows. Final concentrations of amino acids and cell suspension were 1 mM and 4 mg protein/ml, respectively. (*A*) Na^+ uptake induced by the addition of leucine, isoleucine, or valine in wild-type strain PML14. (*B*) Na^+ uptake after addition of leucine in mutant strains of PML1459 (LIV-I^-,II^+), 1460 (I^+,II^-), and 1455 (I^-,II^+)

LIV-I system, Na^+ uptake was induced by the addition of leucine, isoleucine, or valine (*data not shown*). The Na^+ uptake induced by leucine was observed in strain PML1459 (LIV-I^-,II^+), whereas it was not observed in strain PML1460 (LIV-I^+,II^-). Besides, an enhanced Na^+ uptake after the addition of leucine was observed in PML1455 strain having high LIV-II activity (Fig. 3*B*).

For the measurement of substrate-induced Li^+ uptake, NaCl was replaced with LiCl. The Li^+ uptake was detected after the addition of leucine, isoleucine or valine, although an initial rate of uptake was smaller than that of Na^+ uptake (Fig. 4). The Li^+ uptake induced by leucine was observed in PML1459 (LIV-I^-,II^+), but not in PML1460 (LIV-I^+,II^-). PML1455 mutant strain exerted an increased Li^+ uptake after the addition of leucine. Initial rates of Na^+ and Li^+ uptakes were estimated from the initial slopes of cation uptake records and summarized in Table 2.

Similarly, H^+ movement during substrate entry was examined (Fig. 5). In the absence of Na^+, no H^+ influx was detected after addition of leucine, isoleucine, or valine. However, in the presence of Na^+, H^+ efflux after leucine addition was observed in wild-type strain and PML1459 having LIV-II system, but not in PML1460 which lacks it.

The above results demonstrate that the LIV-II transport system is a cotransport system of Na^+ or Li^+ and branched-chain amino acids, but not an H^+-coupled transport system.

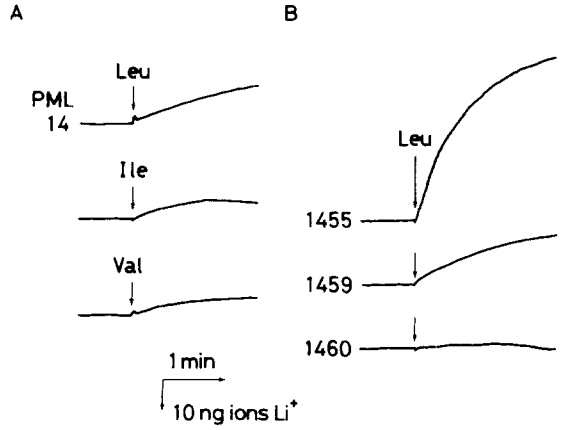

Fig. 4. Uptake of Li^+ induced by addition of leucine, isoleucine, or valine. Li^+ uptake was measured at 28°C using a Li^+-selective electrode. Conditions for measurements were the same as those described for Fig. 3 except for replacement of NaCl with LiCl. (*A*) Li^+ uptake induced by the addition of leucine, isoleucine, or valine in PML14. The final concentration was 1 mM leucine, 1 mM isoleucine, or 5 mM valine. (*B*) Li^+ uptake induced by leucine in mutant strains of PML1459, 1460, and 1455

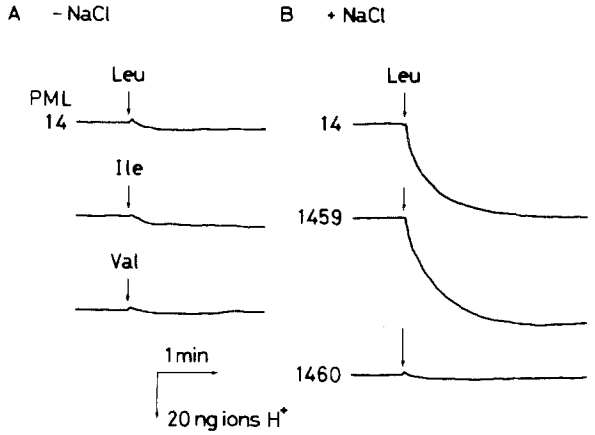

Fig. 5. Movement of H^+ after addition of leucine, isoleucine, or valine. H^+ flux was measured at 28°C by monitoring pH change of extracellular medium with an H^+ electrode as described in Materials and Methods. Final concentrations of amino acids and cell suspensions were 1 mM and 5 mg protein/ml, respectively. (*A*) pH of the medium after the addition of amino acids in the absence of NaCl to PML14 cell suspension. (*B*) H^+ efflux induced by leucine addition in the presence of 5 mM NaCl from PML14, 1459, and 1460

Table 2. Initial rate of Na^+ or Li^+ uptake induced by the addition of leucine, isoleucine, and valine[a]

Strain	Initial rate (ng ions/mg protein/min)					
	Na^+ uptake			Li^+ uptake		
	Leu	Ile	Val	Leu	Ile	Val
PML14	1.40	2.39	0.57	0.43	0.81	0.26
PML1459	2.30	1.97	1.05	0.25	0.15	0.06
PML1460	ND	ND	ND	ND	ND	ND
PML1455	8.79	5.20	2.65	1.28	1.37	1.31

[a] Uptakes of Na^+ and Li^+ after addition of leucine, valine, or valine were monitored at 28°C using an Na^+ or Li^+-selective electrode. Initial rate of Na^+ or Li^+ uptake was estimated from initial slope in records. Final concentrations of leucine, isoleucine, and valine were 1, 1, and 5 mM, respectively. ND, not detected.

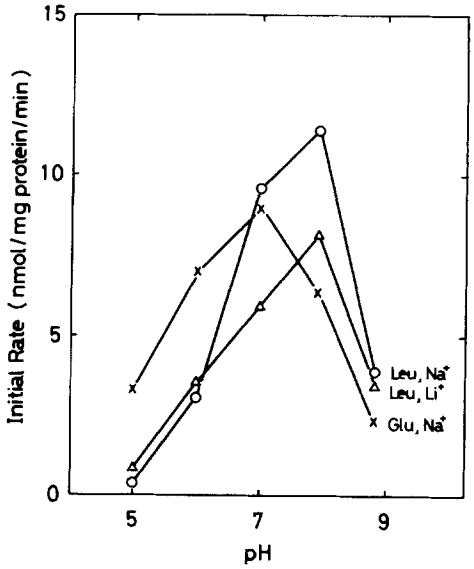

Fig. 6. Effect of pH on leucine uptake in the presence of Na^+ or Li^+. Washed cells were resuspended in buffer solutions of 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES)/Tris, pH 5 and 6, HEPES/Tris, pH 7 and 8, and 2-(cyclohexylamino)ethanesulfonic acid (CHES)/Tris, pH 9, and kept at room temperature before use. Initial rate was calculated from 15-sec values of leucine uptake at 30°C in the presence of 2.5 mM NaCl (○) or LiCl (△) and from those of glutamic acid uptake at 2.5 mM NaCl (×). The final concentration of [^{14}C]-leucine or glutamic acid was 23 μM

Effect of pH on Leucine Uptake and Its Kinetics

First, effect of pH on leucine uptake activity, a measure of the LIV-II system, in the presence of Na^+ or Li^+ was examined in a pH range between 5 and 9. The uptake activity with Na^+ or Li^+ was maximal around pH 8 and low at pH 5 and 9 (Fig. 6). A big difference in the activity was observed between pH 6 and 7. Similarly, pH effect on glutamic acid uptake activity in the presence of Na^+ was examined to compare with the effect on the leucine transport activity. Glutamic acid transport by *P. aeruginosa* cells is suggested to be independent of Na^+ (Hoshino, 1979*a*). The activity of glutamic acid transport was maximal around pH 7 and higher at pH 5 than the leucine uptake activity with Na^+. Its activity at pH 9 was as low as the activity of leucine

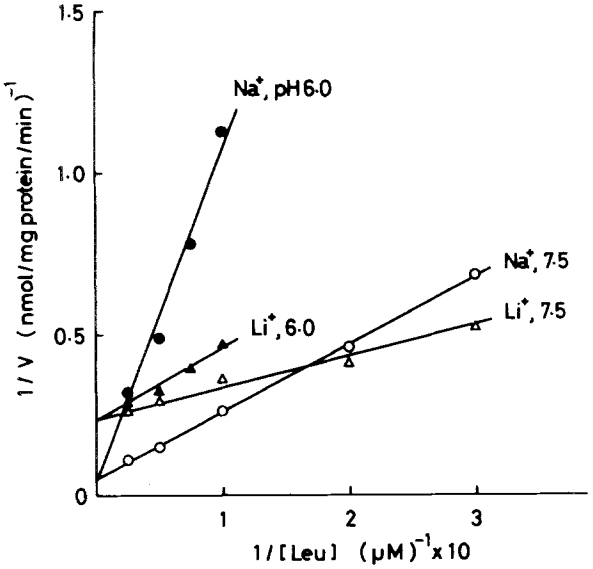

Fig. 7. Effect of Na^+ and Li^+ on kinetic parameters of leucine uptake at different pH. Initial rate was estimated from leucine uptake values at 10 sec and 30°C in the presence of 2.5 mM NaCl at pH 6.0 (●) and 7.5 (○) or LiCl at pH 6.0 (▲) and 7.5 (△). The results were presented as double reciprocal plots of the initial rate and leucine concentration

uptake in the presence of Na^+ or Li^+. Effect of pH on the Na^+ and Li^+-coupled leucine transport appears similar, but leucine and glutamate transport systems exhibit different pH dependence.

Next, effect of pH on kinetics of Na^+ or Li^+-coupled leucine transport was examined by determining kinetic parameters at pH 6.0 and 7.5. Initial rates of leucine uptake were measured in the concentration range of 0.3 to 40 μM leucine in the presence of Na^+ or Li^+ at pH 6 and 7.5. Results are presented in a double reciprocal fashion (Fig. 7). The kinetic parameters were estimated and are given in Table 3. At pH 7.5, the values of apparent K_m and V_{max} with Na^+ were 8 and 4 times larger than those with Li^+, respectively. At pH 6.0, the K_m values with Na^+ and Li^+ were 2.5–5 times larger than those at pH 7.5, whereas the V_{max} values with Na^+ and Li^+ were the same with those at pH 7.5. These results indicate that kinetic property of Na^+ and Li^+-coupled leucine transports is different from each other and strongly suggest that pH change influences K_m values rather than V_{max} values in the two cation/leucine cotransports.

Discussion

P. aeruginosa cells accumulated leucine, isoleucine, or valine in the presence of Na^+ or Li^+ through the LIV-II transport system. Furthermore, the addition of leucine, isoleucine, or valine to the cell suspension caused uptake of Na^+ and Li^+. Neither uptake of substrates in the absence of Na^+ or Li^+ nor uptake of Na^+ or Li^+ induced by the substrates was observed. These findings indicate that the LIV-II transport system for the branched-chain amino acids in *P. aeruginosa* is a Na^+ or Li^+/substrate cotransport system, but not an H^+-coupled system. Hoshino and his collaborator (1979*a*,*b*) have already described that the LIV-II transport system is a Na^+-coupled transport system and that Li^+ ion works as a coupling cation for leucine transport, suggesting that not only Na^+ but also Li^+ is a coupling cation for LIV-II system. Our present study has confirmed their suggestion. Several transport systems that couple to Li^+ as well as Na^+ have been found in *E. coli*. They are melibiose and proline systems (Tsuchiya et al., 1983; Tsuchiya et al., 1984). Our finding provides evidence that $Na^+(Li^+)$-cotransport system is present in bacterial species such as *P. aeruginosa* other than *E. coli*.

The addition of leucine did not cause H^+ movement in the absence of Na^+, while it caused H^+ efflux from whole cells in the presence of Na^+. This observation is interpreted by a view that a membrane potential generated by an electrogenic Na^+/leucine cotransport elicited secondary electrophoretic H^+ extrusion (Tsuchiya et al., 1977*b*; Hama et al., 1987).

The leucine transport by the LIV-II system decreased more greatly by lowering pH from 7 to 5 than the glutamate transport did. Both transport activities were low at pH 9. It is considered that low activities at pH 9 may be attributed to either inactivation of the transport systems or reduction of a driving force generated by intact cells, but that a difference in pH dependence of the two transport activities between pH 5 and 7 should be due to the property of the respective systems. The big difference in the leucine transport activity between pH 5 and 7 could be interpreted as titration of histidine with a pK_a around 6.0 involved in the cotransport pathway (Bindslev & Wright, 1984). The possibility of involvement of histidine remains to be investigated.

Table 3. Effect of Na^+ and Li^+ on kinetic parameters of leucine uptake[a]

Salt added (2.5 mM)	pH	K_m (μM)		V_{max} (nmol/mg protein/min)	
		6	7.5	6	7.5
NaCl		200	40	20	20
LiCl		13	5	4.3	4.3

[a] Kinetic parameters were calculated from the data given in Fig. 7.

Effect of Na^+ and Li^+ on kinetic property of the LIV-II system was different. The apparent K_m values for leucine uptake were 40 and 5 μM in the presence of 2.5 mM NaCl and LiCl, respectively. NaCl concentration of 2.5 mM is not, however, optimal for Na^+/leucine cotransport. The K_m value has been found to be 11 μM when examined with 20 mM NaCl (Hoshino & Kageyama, 1982). On the other hand, it has been shown that V_{max} is constant with varying NaCl concentration (Hoshino, 1979*a*). In the present study, V_{max} value at 2.5 mM NaCl was four times larger than that at the same concentration of LiCl. These findings lead to a conclusion that under each optimal concentration of Na^+ and Li^+, K_m value and V_{max} with Na^+ are two and four times larger than those with Li^+, respectively. A difference in optimal salt concentration between NaCl and LiCl seems to be due to a difference in K_m for the cations, although the K_m value for Li^+ has not been estimated. This suggests that the affinity of the LIV-II carrier for Li^+ is greater than that for Na^+. In *E. coli,* the K_m value for thiomethyl-β-galactoside uptake via melibiose transport system with Na^+ is two times larger than that with Li^+ without changing V_{max} (Lopilato, Tsuchiya & Wilson, 1978). It is likely that effect of Na^+ and Li^+ on transport kinetics is characteristic of individual $Na^+(Li^+)$-coupled cotransport systems. There are two plausible explanations on the difference in effect of the two cations on kinetics of the LIV-II system. One is that both cations work in the same coupling pathway but behave differently on the ground of their physicochemical property. The other is that the two cations work in different coupling pathways. Further investigation is necessary to settle the above possibilities.

A decrease in pH caused an increase of K_m value with V_{max} unchanged. This means that high H^+ concentration reduces an affinity of the carrier for substrates but does not affect its turnover rate. This effect of H^+ is similar to that of Na^+ concentration on kinetics (Hoshino, 1979*a*), suggesting that H^+ may occupy binding site(s) for Na^+ and Li^+, although it is not able to work as a coupling cation of the LIV-II cotransport system.

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Received 13 June 1988; revised 3 October 1988