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Stimulatory Effect of Lithium Ion on Proline Transport by Whole Cells of *Escherichia coli*

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The effect of monovalent cations on proline transport in whole cells of *Escherichia coli* K-12 has been examined. Lithium ion added to the uptake medium stimulated proline transport severalfold and K^+ and Na^+ were slightly effective, whereas Rb^+, Cs^+, and NH_4^+ were completely without effect. The stimulatory effect of Li^+ on proline transport was not due to an increase in osmolarity of the uptake medium, and 5 mM *p*-chloromercuribenzene sulfonic acid completely blocked this effect of Li^+ without having any effect on the basal rate of proline transport. The Arrhenius plots for Li^+-stimulated transport showed a clear transition point at 35°C in addition to 20°C which was also detectable in the basal transport. Lithium ion stimulated proline transport synergistically in the presence of glucose and succinate as a carbon source. The addition of 2.5 mM KCN or 0.5 mM arsenate did not inhibit this synergistic effect, although the presence of these inhibitors inhibited completely the stimulation of proline transport induced by the addition of carbon source. Carbonylcyanide *m*-chlorophenylhydrazone and 2,4-dinitrophenol blocked both the basal and Li^+-stimulated proline transport. When membrane potential of *E. coli* cells was measured by the dibenzyldimethylammonium uptake method, the incubation of Li^+ with the cells did not affect the preexisting membrane potential. These results suggest that Li^+ stimulates proline transport by intact cells of *E. coli* in a manner somehow affecting membrane component(s) different from the transport carrier of proline. It is uncertain whether the effect of Li^+ is directly involved in the mechanisms of energy coupling of proline transport.

Monovalent cations, especially Na^+ and K^+, have been recently reported to be required for the active transport of organic substances into microbial cells: Na^+ is involved in the transport of α-aminoisobutyrate in a marine pseudomonad (7, 8, 37, 38), of glutamate in *Escherichia coli* (12, 14), of melibiose in *Salmonella typhimurium* (36), and of glutamate in *Bacillus licheniformis* (29). Kinetic analysis of the Na^+-dependent transport in these cases revealed that Na^+ decreases the K_m for the solute, whereas the V_{max} remained constant (12, 14, 36–38). However, Na^+-stimulated proline transport by membrane vesicles of *Mycobacterium phlei* is different in kinetic behavior, in that the same K_m value for the substrate with the increased V_{max} value was demonstrated (20). In the case of Na^+-dependent K^+ ion transport in a marine pseudomonad, Na^+ increases the affinity and the capacity of the K^+-transport system (17).

Potassium ion was also found to be required for the transport of α-aminoisobutyrate in a marine pseudomonad (7, 8), of glutamate in *E. coli* K-12 (14), and of citrate in *Aerobacter aerogenes* (9). Potassium ion in the transport of α-aminoisobutyrate was shown to act at the intracellular level (38) and, in the case of glutamate, K^+ affected the capacity of the uptake but had no effect on the K_m (14). The transport of citrate was reported to be dependent on a gradient of K^+ concentration from inside to outside the cell (9). The other role of K^+ in the transport of glycine in *Saccharomyces carlsbergensis* was to accelerate the transport by counter transport of H^+ (10, 11). Furthermore, valinomycin-K^+-induced uptake of amino acids and sugars was recently demonstrated. This includes the uptake of proline in *E. coli* membrane vesicles (19), neutral amino acids in whole cells of *Streptococcus faecalis* (2), thiomethyl-β-*o*-galactoside in *Streptococcus lactis* (21), and glycine and lysine in *Staphylococcus aureus* (30). In these cases (2, 19, 21, 30) K^+ in the presence of valinomycin contributes by generating a membrane potential as a driving force for the transport. The role of monovalent cations other than Na^+ and K^+ in the transport of

solutes is not known, except for the stimulation by Li^+ of proline uptake by *M. phlei* membrane vesicles (20).

Previously we had demonstrated that proline transport by whole cells of *E. coli* K-12 was stimulated by Li^+ but not by Na^+ and K^+ in medium composed of 0.25 M sucrose–10 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$ (24). This stimulation of proline transport was due to an increase in the V_{max} value without affecting the K_m value. It was also found that ionophores, valinomycin, and gramicidin, with appropriate monovalent cations inhibited proline transport in this medium. The present paper describes the results obtained from studies performed to further characterize the properties of the Li^+ stimulation of proline transport by intact cells of *E. coli* and to find out any relation between the Li^+ effect and energy coupling of proline transport.

MATERIALS AND METHODS

Organism. *E. coli* K-12 was used through out the experiments and grown aerobically on a minimal medium (6) containing 0.2% glucose as the carbon source. Cells were harvested at an absorbancy at 560 nm of 0.30 measured by a Bausch and Lomb-Schimadzu spectrophotometer (Spectronic 20).

Preparation of cell suspension. Harvested cells were washed twice in 10 mM Tris-hydrochloride buffer, pH 7.5, containing 10 mM $MgCl_2$ and then resuspended in the same medium to give the absorbancy of 0.25 at 560 nm (0.18 mg of dry weight of cells per ml) for the assay of proline transport activity. To determine oxygen consumption by cell suspensions, washed cells were resuspended to give an approximate absorbancy of 0.35 at 560 nm (0.25 mg of dry weight of cells per ml).

Assay method of [^{14}C]proline transport. The cell suspensions with or without cations and other supplements were preincubated for 5 min at 37°C with constant shaking, and then [^{14}C]proline was added at 10 μM, followed by continuous shaking at 37°C. At time intervals shown in the text, 1 ml of the cell suspensions was collected on a membrane filter (type HA, 0.45 μm; Millipore Corp., Bedford, Mass.) and washed once with 10 ml of the medium, and then the radioactivity transported into the cells was counted as described previously (25).

Assay method of oxygen consumption. The rate of oxygen consumption by the cell suspensions was measured at 37°C in a vessel equipped with an oxygen electrode probe (model 54; Yellow Springs Instrument Co., Yellow Springs, Ohio) and a thermocirculator.

Measurement of Li^+ content. Washed cells prepared as described above were resuspended in the same medium as used for washing the cells to give a concentration of 2 to 6 mg of cell dry weight per ml. Cell suspensions were incubated with various concentrations of Li^+ in the presence or absence of 1 μM gramicidin, and determination of intracellular Li^+ was then carried out by flame emission spectroscopy (5). To calculate cellular concentration of Li^+, cellular water space of *E. coli* cells was taken as 2.55 ml per g of dry weight (33).

Estimation of membrane potential from DDA^+ uptake. The cell suspension was treated with Tris and ethylenediaminetetraacetic acid by the method of Leive (27) and stored at 0°C in 0.25 M sucrose. The accumulation of diphenyldimethylammonium chloride (DDA^+) in the cell suspension was measured by a modified method of Harold and Papineau (16) as described by Griniuvienė et al (13).

Chemicals. L-[U-^{14}C]proline (290 mCi/mmol) was obtained from the Radiochemical Centre, Amersham/Searle Corp., Des Plaines, Ill. *p*-Chloromercuribenzene sulfonic acid (PCMBS), carbonylcyanide *m*-chlorophenyl hydrazone (CCCP), and gramicidin were obtained from Sigma Chemical Co., St. Louis, Mo., and valinomycin was from Calbiochem, Los Angeles, Calif. DDA^+, sodium tetraphenylborate, 2,4-dinitrophenol, potassium cyanide, and arsenic acid were obtained from Nakarai Chemicals Co., Kyoto, Japan. Other chemicals were standard commercial products.

RESULTS

Effect of monovalent cations. The rate of proline transport was determined in the medium composed of 10 mM Tris-hydrochloride, pH 7.5, 10 mM $MgCl_2$, and 10 mM monovalent cations including, K^+, Na^+, Li^+, Rb^+, Cs^+, and NH_4^+, respectively, and compared with that of the control without cations (Table 1). The addition of Li^+ stimulated proline transport 7.4 times, Na^+, 2.4 times, and K^+, 1.8 times as compared to that of the control. Other cations (Rb^+, Cs^+, and NH_4^+) tested have negligible effect on the transport of proline (Table 1). The presence of Mg^{2+} in the uptake medium did not affect proline transport in the presence of 10 mM Li^+; values of 12.9 and 11.6 μmol per g of dry weight per 5 min, respectively, were obtained with and without 10 mM $MgCl_2$.

Effect of Li^+ concentrations. The rate of proline transport was determined as a function of extracellular concentrations of Li^+ (Fig. 1). The transport rate was maximally enhanced at 10 mM Li^+ and then decreased with an increase in its concentration. Proline transport was rather inhibited in the presence of 0.1 M Li^+. Lithium ion transported into the cell of *E. coli* measured photometrically by flame was scarcely detectable when 5 to 10 mM Li^+ was added to the uptake medium. Intracellular concentration of Li^+ on the addition of 0.1 M Li^+ to the uptake medium was 35.3 mM and was almost identical to that of the addition of 1 μM gramicidin plus 50 mM Li^+. Under the latter condition, proline transport was inhibited as indicated in the previous paper (24).

TABLE 1. *Effect of monovalent cations on proline transport by whole cells of E. coli K-12 in the absence of added energy source*[a]

Cations (10 mM)	Proline uptake (μmol/g of dry wt per 5 min)
None	1.86
LiCl	13.3
NaCl	4.42
KCl	3.41
RbCl	2.32
CsCl	2.00
NH_4Cl	1.95

[a] Each monovalent cation was added as chloride salt to the cell suspension, which was then preincubated for 5 min at 37°C. [^{14}C]Proline at 10 μM (290 mCi/mmol) was then added, and the incubation with constant shaking was continued for 5 min at 37°C.

The stimulatory effect of Li^+ on proline transport is not due to a change in osmolarity of the assay medium. When the rates of proline uptake were determined in the assay medium containing sucrose in a range of concentrations from 5 mM to 0.4 M, the rates were nearly identical at sucrose concentrations up to 0.25 M. The osmolarity of the assay medium containing 0.1 M LiCl was found to be 200 mosM/kg when measured with an Advanced osmometer (model L. W.) and was lower than that of the assay medium containing 0.25 M sucrose (300 mosM/kg). Yet, proline transport under the latter condition was 1.58 μmol per g of dry weight per 5 min, which was identical to that in the assay medium without LiCl.

Effect of sulfhydryl reagents. Sulfhydryl reagents were known to inhibit many microbial transport systems (30). Therefore, PCMBS in different concentrations was selected to study its effect on the Li^+-stimulated transport. Addition of 0.5 mM PCMBS to the uptake medium completely blocked the stimulatory effect of Li^+ without affecting the basal rate of proline transport (Fig. 2). The basal and Li^+-stimulated proline transport were both greatly reduced at 1 mM PCMBS, probably due to its inhibition of proline transport carrier (Fig. 2). This result suggests that Li^+ may interact with a protein(s) sensitive to sulfhydryl reagents, which is different from the transport carrier, to stimulate proline transport.

Effect of temperature. The effect of temperature on the rate of proline transport was examined in the presence and absence of 10 mM Li^+. The transport rates in the absence of Li^+ increased up to 30°C and decreased slightly above 30°C. On the other hand, the rate in the presence of Li^+ increased gradually in the range from 0 to 35°C and then increased more markedly with the rise of temperature above 35 to 45°C. The difference in the effect of temperature on proline transport in the presence and absence of Li^+ is further emphasized by the Arrhenius plots (Fig. 3). It was clear from the Arrhenius plots that Li^+-stimulated proline transport showed a transition point of 35°C, which is completely different from that shown in the absence of Li^+. Another identical transition temperature, 20°C, is detectable in both transport conditions.

These results suggest that Li^+ stimulates proline transport by probably inducing some alteration in a membrane component(s) other than proline transport carrier.

Effect of carbon source on proline transport. The effect of Li^+ on active transport of proline driven by oxidation of glucose and succinate (succinic acid was adjusted to pH 7.5 with Tris) was examined (Fig. 4). Proline transport for 5 min was stimulated by 10 mM glucose and succinate by 4.0- and 1.7-fold, respectively, and these values were lower than those obtained in

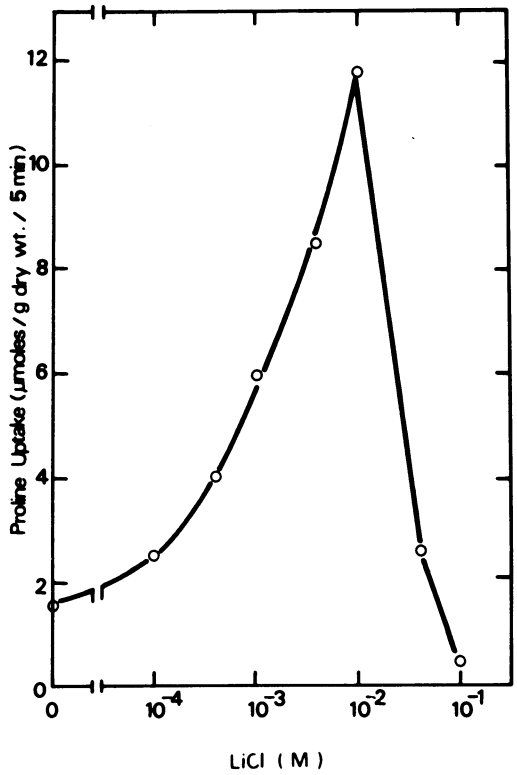

FIG. 1. *Effect of Li^+ concentrations on proline transport. LiCl was added to the cell suspensions 5 min prior to the addition of [^{14}C]proline. Proline taken up for 5 min at 37°C was determined.*

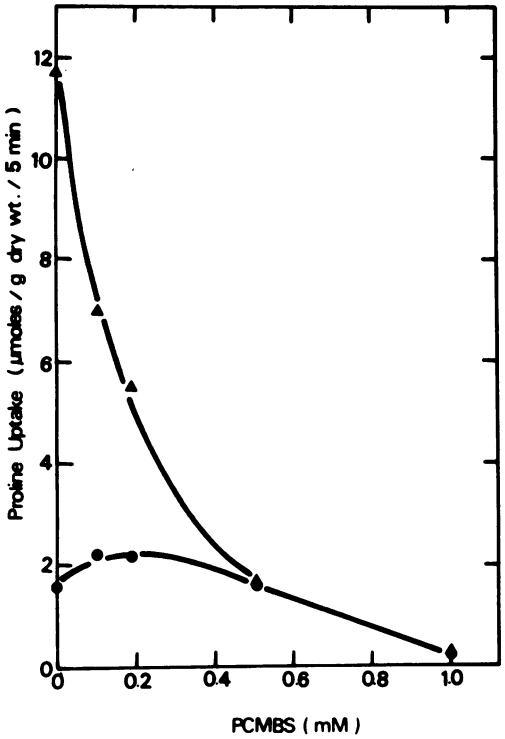

FIG. 2. *Effect of sulfhydryl reagent on proline transport in the presence and absence of Li^+. PCMBS varying in concentrations was added to the suspension 5 min before initiating the uptake, followed by incubation for further 5 min at 37°C to measure the amount of proline taken up. Symbols: ●, Without Li^+; ▲, with 10 mM Li^+.*

the presence of 10 mM Li^+. However, the transport was synergistically stimulated by glucose and succinate in the presence of Li^+.

Effect of cyanide. Based on the synergistic stimulation of proline transport by Li^+ plus succinate, it was examined whether Li^+ stimulates directly electron transport. The rate of oxygen consumption by cells of *E. coli* was not altered in the presence and absence of Li^+, regardless of the presence of glucose or succinate as a carbon source (data not shown).

Effect of KCN on Li^+-stimulated proline transport was then examined in the presence and absence of succinate (Fig. 5). Basal proline transport was not affected by KCN at up to 10 mM concentration. The stimulation of proline transport by the addition of succinate alone was completely inhibited at 2.5 mM KCN. Whereas the stimulated level of the transport caused by the presence of Li^+ plus succinate was reduced by increasing the concentration of KCN from 2.5 to 20 mM, the synergistic effect of Li^+ plus succinate on proline transport was still detectable in the range of 2.5 to 10 mM KCN.

The effect of KCN concentrations on oxygen consumption by *E. coli* K-12 cells was also checked under the same conditions used for the transport experiments. Oxygen consumption, 43.6 and 9.2 nmol of O_2 consumed per mg of dry weight per min, respectively, with and without 10 mM succinate, was completely inhibited by 0.5 mM KCN in the presence of Li^+. These results suggest that Li^+ does not affect directly the electron transfer.

Effect of arsenate. The proline transport by *E. coli* cells was also synergistically enhanced by Li^+ when glucose was present (Fig. 4), suggesting that Li^+ may affect the process to produce a high-energy membrane state through the hydrolysis of adenosine 5'-triphosphate (ATP), which is formed by glycolysis, by membrane bound Mg^{2+}-adenosine triphosphatase [EC 3.6.1.3]. Incubation of *E. coli* cells with sodium arsenate caused a drastic reduction in intracellular ATP level (26). If Li^+ affects this

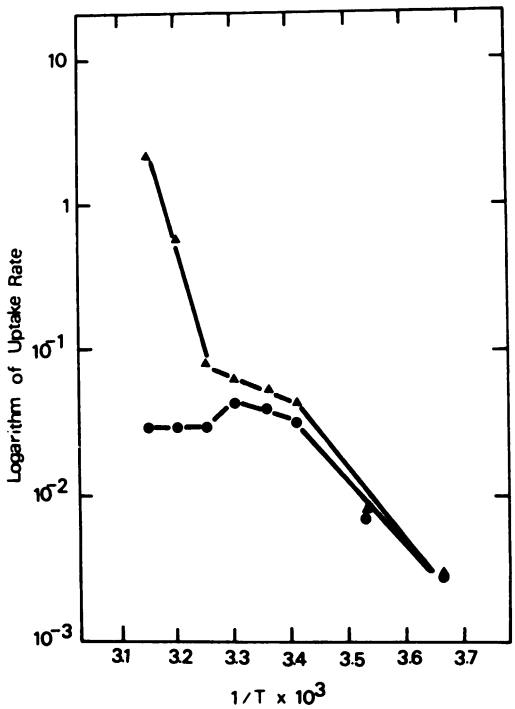

FIG. 3. *Arrhenius plots of the effect of temperature on proline transport in the presence and absence of Li^+. Preincubation of the suspensions at temperatures ranging from 0 to 45°C was carried out for 5 min in the presence and absence of 10 mM LiCl, and then [^{14}C]proline transport for 5 min at the same temperature as used for the preincubation was determined. Symbols: ●, Without Li^+; ▲, with Li^+.*

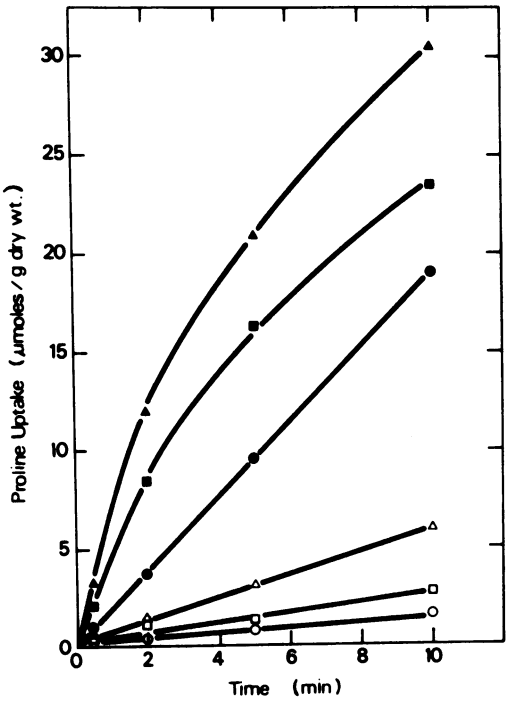

FIG. 4. *Effect of carbon source on proline transport in the presence and absence of Li^+. Glucose or succinate at 10 mM was added to the cell suspensions with or without 10 mM Li^+ and the suspensions were then preincubated for 5 min at 37°C before the addition of [^{14}C]proline. Uptake of [^{14}C]proline by these cell suspensions was measured at 37°C as described in Methods. Symbols: ○, without supplements; △, with glucose; □, with succinate; ●, with Li^+; ▲, with Li^+ plus glucose; ■, with Li^+ plus succinate.*

process, arsenate is expected to inhibit the synergistic stimulation of the transport by Li^+ plus glucose.

Arsenate (arsenic acid was adjusted to pH 7.5 with 2 M Tris) at 0.2 mM concentration blocked almost completely the increase in proline transport by glucose alone, whereas arsenate at the concentration inhibited the uptake in the presence of 10 mM Li^+ only by 8%. The addition of glucose plus Li^+ to the medium enhanced the uptake synergistically, and arsenate that was further added inhibited again the transport only by 9%. This indicates that Li^+ does not affect the process to produce an energized membrane state through the hydrolysis of ATP by membrane Mg^{2+}-adenosine triphosphatase.

Effect of uncoupler. Uncouplers of oxidative phosphorylation dissipate the energized membrane state of microbial cells by promoting proton permeability of the membrane (see review in reference 16) and thus inhibit such transport systems that utilize this state directly as a source of energy for active transport. Although both basal and Li^+-stimulated transport of proline were inhibited by CCCP, the Li^+ effect remained even at 2 to 3 μM CCCP (Fig. 6). Dinitrophenol at concentrations from 0.1 to 0.5 mM produced a result identical to that with CCCP (data not shown).

Effect of Li^+ on preexisting membrane potential. To determine whether Li^+ added to the transport medium contributes to an increase in membrane potential of *E. coli* cells, membrane potential of the cells incubated with or without Li^+ was determined by the method of lipid-soluble cation DDA^+ uptake (13, 14). The membrane potential estimated with the cells without the addition of Li^+ was −73.8 mV, interior negative, and this value was not altered after the addition of Li^+ to the medium (Table 2). When cells were incubated with valinomycin

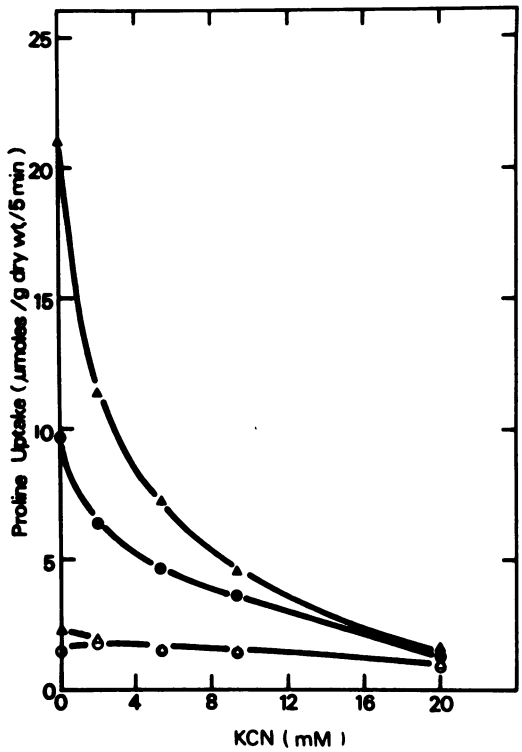

FIG. 5. *Effect of various concentrations of KCN on the basal and Li^+-stimulated proline transport in the presence and absence of succinate. Potassium cyanide was adjusted to pH 7.5 with 5 N HCl and added to the cell suspensions at final concentrations indicated with or without 10 mM Li^+ and 10 mM succinate. The reaction mixtures were incubated for 5 min at 37°C, and then 10 μM [^{14}C]proline was added. [^{14}C]Proline uptake for 5 min was measured as described in Methods. Symbols: ○, without supplements; △, with succinate; ●, with Li^+; △, with Li^+ plus succinate.*

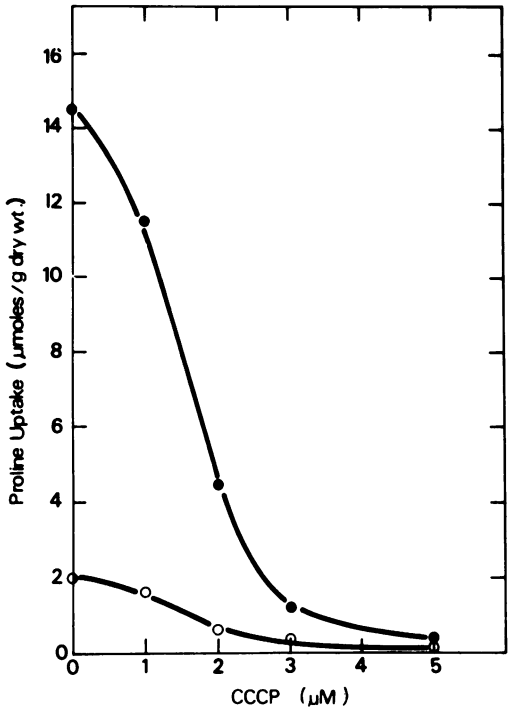

FIG. 6. *Effect of various concentrations of CCCP on the basal and Li^+-stimulated proline transport. CCCP was dissolved in ethanol, added to the cell suspensions at final concentrations indicated in the figure, and incubated for 5 min at 37°C with or without 10 mM Li^+. The reaction was started by the addition of 10 μM [^{14}C]proline, and the uptake for 5 min was measured as described in Methods. Symbols: ○, Without Li^+; ●, with Li^+.*

plus 50 mM K^+ and a membrane potential was measured by the DDA^+ uptake method, the potential was reduced (Table 2). This indicates that Li^+ added to the uptake medium did not affect the generation of the membrane potential of *E. coli* cells.

Effect of DDA^+. A permeant cation, DDA^+, is taken up by *E. coli* cells according to the membrane potential, followed by disrupting the potential (1, 13, 18, 28). The rate of proline uptake was determined under the conditions in which DDA^+ was added to the uptake medium at concentrations ranging from 0.2 to 0.5 mM with 1 μM sodium tetraphenylborate, a permeant anion, in the presence and absence of Li^+. Although proline uptake in the absence of Li^+ was inhibited by DDA^+, the stimulatory profiles and magnitude of proline uptake by Li^+ found in the presence of DDA^+ were quite similar to that in its absence. The result suggests that the stimulatory effect of Li^+ on proline transport by *E. coli* cells is not explained by any change in the membrane potential.

DISCUSSION

The results presented in this paper indicate clearly stimulatory effect of lithium ion on proline transport by intact cells of *E. coli* K-12 under the condition in which exogenous energy source was absent. Such an effect of Li^+ on transport of a solute in microorganisms was not known until the previous paper (24) was presented, except for proline transport system of *M. phlei* membrane vesicles (20) in which Na^+ is, however, more effective to stimulate the transport than Li^+. Lithium ion stimulated proline transport not only in a wild strain of *E. coli* K-12 used in the experiment but in a thiamine auxotroph of *E. coli* W strain (data not shown).

Although the result of kinetic analysis showing a V_{max} effect with no inlfuence on K_m (24) may exclude a direct interaction of Li^+ with the transport carrier, evidence that is described in this paper indicates a plausible interaction of the cation with a membrane protein component(s) other than the carrier: (i) an optimal concentration of Li^+ to stimulate proline transport was observed (Fig. 1); (ii) a sulfhydryl reagent, PCMBS, at 0.5 mM blocks the stimulatory effect of Li^+ completely without affecting the basal transport level which is almost completely reduced by adding 1 mM PCMBS (Fig. 2), due to the inhibition of proline carrier itself.

Furthermore, the probable interaction of Li^+ with protein component(s) in the cell membrane may involve another relation to phospholipids around such protein. A transition temperature of 35°C was clearly demonstrated for

TABLE 2. *Estimation of the electrical potential in the presence and absence of Li^+*[a]

Additions	Final concn in medium and cells (μmol/ml)			Membrane potential (mV)
	DDA_o^+	DDA_i^+	DDA_i^+/DDA_o^+	
DDA^+, TPB^-	0.74	11.8	15.9	−73.8
DDA^+, TPB^- plus 5 mM Li^+	0.76	10.9	14.3	−71.0
DDA^+, TPB^-, and valinomycin plus 50 mM K^+	0.86	3.77	4.4	−37.9

[a] Washed cells of *E. coli* K-12 were suspended at a cell density of 8.6 mg of dry wt per ml in 0.25 M sucrose–3 mM glycylglycine (pH 7.2). DDA^+ and sodium tetraphenylborate (TPB^-) were added to 1 μmol/ml and 0.01 μmol/ml, respectively, in the presence and absence of 5 mM Li^+. Valinomycin and KCl were added at 1 μg/ml and 50 mM, respectively. The cell suspensions including these supplements were incubated for 5 min at 37°C and then pelleted by centrifugation. Concentrations of DDA^+ in and out of the cell were calculated from the data of three parallel measurements as described in Materials and Methods. Membrane potentials were estimated from the Nernst equation.

the effect of Li^+ on proline transport (Fig. 3), suggesting alteration(s) in membrane fluidity (35) and subsequently in the transport.

Active transport of proline in whole cells of *E. coli* occurs by coupling to an energized membrane state provided through substrate oxidation or hydrolysis of ATP (3, 4, 34). Mechanisms of the Li^+ effect to enhance proline transport specifically by *E. coli* cells might be, therefore, considered from the point of energy coupling to the transport. However, the stimulatory effect of Li^+ was not affected by KCN and arsenate (Fig. 5; see text). This suggests that lithium ion does not directly affect substrate oxidation via either electron transport or by a process generating an energized membrane state via hydrolysis of glycolytic ATP.

It has been demonstrated in recent studies (1, 18, 22, 23, 31) that a membrane potential is a direct driving force for active transport of some solutes in microorganisms. It was, therefore, determined whether Li^+ affects a preexisting membrane potential and found that the Li^+ effect cannot be related to alteration in the membrane potential (Table 2). Based on the fact that Li^+ stimulated proline transport even in the presence of CCCP (Fig. 6), it seemed to be reasonable to determine whether the effect of Li^+ is general phenomenon in many other transport systems in *E. coli*. However, Li^+ was found to be ineffective on the transport of other amino acids, such as phenylalanine, serine, glycine, cysteine, glutamine, arginine, histidine, and thiomethyl-β-galactoside under the same conditions used for proline transport. To explain the mechanisms of the specific stimulation of proline transport by Li^+, further investigations are in progress.

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