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Glutamate Transport Driven by an Electrochemical Gradient of Sodium Ions in *Escherichia coli*

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The role of Na^+ in glutamate transport was studied in *Escherichia coli* B, strain 29-78, which possesses a very high activity of glutamate transport (L. Frank and I. Hopkins, J. Bacteriol., 1969). Energy-depleted cells were exposed to radioactive glutamate in the presence of a sodium gradient, a membrane potential, or both. One hundred- to 200-fold accumulation of the amino acid was attained in the presence of both electrical and chemical driving forces for the sodium ion. Somewhat lower accumulation values were obtained when either chemical or electrical driving forces were applied separately. A chemical driving force was produced by the addition of external Na^+ to Na^+-free cells. A membrane potential was established by a diffusion potential either of H^+ in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or of SCN^-. These results support the hypothesis of a Na^+-glutamate cotransport. Na^+-driven glutamate transport was also observed in wild-type *E. coli* B but not in a strain of K-12.

In 1969, Frank and Hopkins (7) isolated a mutant (strain 29-78) from *Escherichia coli* B and demonstrated that Na^+ stimulated growth of the mutant on glutamate as sole source of carbon and nitrogen. Furthermore, they showed that it was the transport process of glutamate that was stimulated by Na^+ in whole cells (7) and in membrane vesicles (15). Kinetic studies of transport in this strain revealed that Na^+ lowered the K_m value without affecting the maximum velocity (7). These studies were extended to *E. coli* K-12 by Halpern and co-workers (8, 11). Wild-type *E. coli* K-12 cannot grow on glutamate as sole source of carbon, although it has an Na^+-stimulated glutamate transport system.

Lanyi et al. (13) found a similar Na^+-stimulated glutamate transport system in *Halobacterium halobium*. An artificially produced Na^+ gradient caused glutamate and leucine accumulation by membrane vesicles treated with the proton conductor carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (CCFP) (13, 14). This provided direct evidence that a gradient for Na^+ and not H^+ was the immediate source of energy for accumulation of these amino acids in this microorganism. More recently they have demonstrated a similar Na^+-driven glutamate transport in vesicles of *E. coli* (J. K. Lanyi, personal communication).

In this paper we present evidence that the driving force for glutamate accumulation in strains of *E. coli* B is the electrochemical potential difference for the sodium ion.

MATERIALS AND METHODS

Strains. *E. coli* B (wild type) and its derivative strains 29-78 and 36-39 were used. The latter two strains were generously provided by L. Frank. Strain 36-39 was selected for its resistance to DL-α-methylglutamate (L. Frank and I. Hopkins-Williams, personal communication). The defect in this strain has not been characterized. Wild-type cells and strain 29-78 were grown on medium B7 (7) plus 10 mM NaCl and 20 mM glutamic acid in some experiments (see Fig. 3, 4, 5, and Table 2). In other experiments (see Fig. 1, 2, and Table 1) these two strains were grown in the same medium plus 0.2% glycerol. The transport-defective strain, 36-39, was grown on medium 63 (2) containing 0.2% glycerol and 0.2% Casamino Acids. In some experiments cells were energy depleted with 2,4-dinitrophenol as described by Berger (1). In this procedure cells were washed two times with medium 63 and incubated with shaking in medium 63 containing 5 mM 2,4-dinitrophenol at 37°C for 7 h. After this energy starvation method, cells were washed with appropriate buffer at least twice.

Transport assay. Cells used in the first two figures and Table 1 were washed three times with 0.1 M morpholinopropane sulfonic acid (MOPS) buffer, which was adjusted to pH 7.0 by tris(hydroxymethyl)aminomethane (Tris), and resuspended in the same buffer. The incubation medium for the transport assay consisted of MOPS-Tris buffer (pH

7.0) containing a solution of 10 mM As_2O_3, 20 mM NH_4Cl, and 50 μg of chloramphenicol per ml. The reaction was initiated by the addition of [^{3}H]-glutamate (final concentration, 1 μM), and samples were filtered on membrane filters (Millipore Corp.) at various time intervals.

Energy-starved cells were used in experiments where transport was energized by an artificial driving force. Energy-depleted cells were divided into four portions. One portion of cells (low Na^+, low pH) was washed with 100 mM KH_2PO_4, pH 6.0. A second portion of cells (low pH) was washed with 100 mM NaH_2PO_4, pH 6.0. A third portion of cells (low Na) was washed with 100 mM KH_2PO_4, pH 8.0. The last portion of cells (control) was washed with 100 mM NaH_2PO_4, pH 8.0. The pH of each buffer was adjusted with KOH. Each type of cell was resuspended at an optical density of 20,000 Klett units (no. 42 filter) in the same buffer as in the washing procedure. CCFP and KCN were added to the resuspending media to give final concentrations at 5 μM and 5 mM, respectively. The transport assay was initiated by the dilution of cells 100-fold into buffer containing 100 mM NaH_2PO_4 adjusted to pH 8.0 with KOH, 5 μM CCFP, 5 mM KCN, and 1 μM [^{3}H]glutamate.

A membrane potential was induced with SCN^- rather than a proton diffusion potential in a series of experiments described in Fig. 4. Energy-depleted cells were treated as follows: one portion of cells (low Na^+, no SCN^-) was washed with 100 mM KH_2PO_4 adjusted to pH 8.0 with KOH. A second portion of cells (no SCN^-) was washed with 50 mM NaH_2PO_4-50 mM KH_2PO_4 adjusted to pH 8.0 with KOH. The third portion (low Na^+) was washed with 50 mM KSCN-50 mM KH_2PO_4 adjusted to pH 8.0. The last portion of cells (control) was washed with 50 mM NaSCN-50 mM potassium phosphate, pH 8.0. Each of the four types of cells was then resuspended in the same buffers used in the washing procedure. KCN was added to the resuspending medium to give a final concentration of 5 mM to lower the level of endogenous uptake. After 30 min of preincubation at room temperature, cells (optical density = 20,000 Klett units) were diluted 1:100 into a solution containing 50 mM NaSCN, 50 mM potassium phosphate, pH 8.0, 5 mM KCN, and 1 μM [^{3}H]glutamate. In one experiment CCFP (5 μM) was added.

Intracellular water space was determined as described (6).

Chemicals. L-[3-^{3}H]glutamic acid, 15 to 25 Ci/mmol, was purchased from New England Nuclear Corp. Stock solution of [^{3}H]glutamic acid was prepared by diluting with excess unlabeled L-glutamic acid to a final specific activity of 250 mCi/mmol. All other chemicals were reagent grade and obtained from commercial sources.

RESULTS

Effect of Na^+ on glutamate transport. The sodium ion markedly stimulated glutamate transport in strain 29-78 (Fig. 1), confirming the work of Frank and Hopkins (7). The stimulation of the initial rate by Na^+ was more than 30-fold. Similarly, Na^+ stimulation of glutamate transport was observed in wild-type strain B but to a lesser extent than that seen with strain 29-78. Very low uptake of glutamate and no stimulation by Na^+ were observed in strain 36-39, a cell which cannot grow on glutamate as sole source of carbon and nitrogen.

Figure 2 shows the effect of the external Na^+ concentration on the transport of glutamate in strain 29-78. Maximal stimulation was attained at 20 to 50 mM NaCl. In these experiments, the total concentration of NaCl and KCl was kept constant at 100 mM so that there would be no osmotic difference. No significant effect by K^+ on glutamate transport was detected.

Effect of metabolic inhibitors. Table 1 shows that though KCN and KN_3 show moderate inhibition of transport, the sum of the two

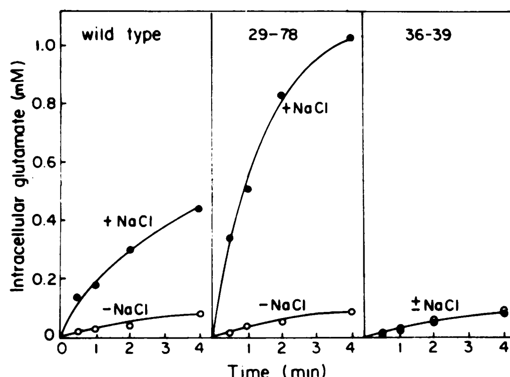

FIG. 1. *Stimulation of glutamate transport by Na^+. Glutamate transport was measured in the absence or presence of 25 mM NaCl.*

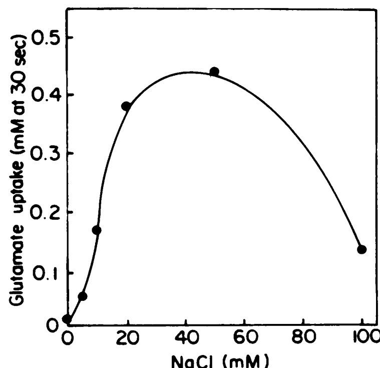

FIG. 2. *Glutamate transport in strain 29-78 as a function of Na^+ concentration. A mixture of NaCl and KCl was added so that the final concentration of Cl was 100 mM. Samples were taken at 30 s after the addition of [^{3}H]glutamate.*

TABLE 1. *Effect of metabolic inhibitors on glutamate uptake in strain 29-78*

Additions[a] (mM)	Glutamate uptake[b] (μM at 30 s)	%
Control	511	100
KCN (5)	113	22
KN_3 (2)	258	50
KCN (5) + KN_3 (2)	12	2
KN_3 (20)	2	1
CCFP (5 μM)	5	1

[a] Inhibitors were preincubated with cells for 10 min before addition of glutamate.

[b] Glutamate uptake was expressed as the intracellular concentration 30 s after addition of [^{3}H]glutamate.

gives complete inhibition. Presumably the two major sources of proton translocation, respiratory chain and membrane-bound adenosine triphosphatase, are blocked by the two inhibitors. The two ionophores CCFP and KN_3 (at high concentrations) completely inhibit transport. Whereas these data indicate the involvement of a protonmotive force, it is not clear whether the effect is direct or indirect. The subsequent studies further explore this question.

Na^+-coupled transport of glutamate. The aim of the next experimental series was to distinguish between two possible ionic mechanisms of transport: (i) Na^+ as a cofactor or activator in a proton-glutamate cotransport system; and (ii) Na^+-glutamate cotransport. The strategy of the experiment was to prevent an inwardly directed protonmotive force with the use of the proton conductor CCFP. In one series of experiments an adverse (outwardly directed) protonmotive force was established.

Energy-depleted cells were used to reduce the level of endogenous uptake and to minimize the effect of metabolism. A pH gradient across the membrane (interior acidic) was imposed in the presence of CCFP. This produced several results: (i) the direction of H^+ movement was outward; (ii) membrane potential (inside negative) was established; and (iii) CCFP reduced the level of endogenous uptake. A chemical gradient of Na^+ (ΔpNa) was also imposed in some experiments to establish a driving force to supplement the electrical driving force.

In the first experiment both a chemical gradient (ΔpNa) and an electrical gradient ($\Delta\Psi$) were imposed across the membrane. The Na^+ gradient was obtained by placing low Na cells into a medium containing 100 mM Na; the electrical potential difference (inside negative) was induced by a proton diffusion potential. The latter was accomplished by suddenly diluting a concentrated suspension of cells (pre-equilibrated at pH 6.0) into a medium at pH 8.0 in the presence of CCFP. In the presence of these two driving forces for Na^+, 120-fold accumulation of glutamate was observed (see $\Delta\Psi$ + ΔpNa, Fig. 3).

When only a chemical potential for the Na ion was present (ΔpNa, Fig. 3), a slightly lower level of accumulation was observed. Furthermore, glutamate uptake was of briefer duration. In this experiment CCFP was present, but no pH gradient was produced.

To produce a membrane potential alone the proton diffusion potential was again used, but the chemical gradient was minimal, since the sodium ion was present both inside and outside the cell. Under these conditions a 40-fold glutamate accumulation with a prolonged time course was observed ($\Delta\Psi$, Fig. 3).

Finally, in the control experiment, neither membrane potential nor chemical gradient was imposed. This was accomplished by exposing high-Na cells (preincubated at pH 8.0) to high-Na medium at pH 8.0. Under these conditions very little glutamate uptake was observed.

An alternative method of inducing a membrane potential is to add SCN^-. Being lipid soluble, SCN^- enters the cell and induces a membrane potential (inside negative). When NaSCN is added to an energy-depleted cell suspension, a chemical gradient of Na^+ is also

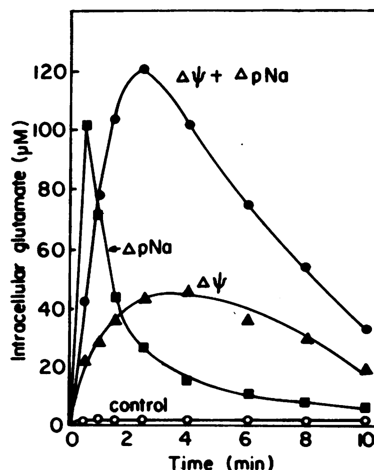

FIG. 3. *Glutamate transport energized by an electrochemical Na^+ gradient in strain 29-78. A membrane potential difference ($\Delta\Psi$), interior negative, was imposed as a diffusion potential of H^+ in the presence of CCFP. An inwardly directed chemical gradient of Na^+ (ΔpNa) was imposed by the addition of Na^+ to the external medium. pNa = $-log_{10}$ (sodium concentration).*

established in addition to membrane potential. Thus we can impose a large inwardly directed driving force for Na^+ by adding NaSCN. Figure 4 shows that electrochemical potential difference thus established resulted in a 200-fold accumulation of glutamate. The addition of CCFP reduced the uptake. The membrane potential established by influx of SCN^- would be reduced by flux of H^+ through CCFP. As in the previous experiment, a membrane potential alone or a chemical gradient alone could drive glutamate transport. When an Na^+ gradient was imposed in the presence of CCFP, very rapid glutamate uptake and subsequent rapid efflux was observed (Fig. 3). On the other hand slower uptake and slower efflux were observed when a Na^+ gradient was established in the presence of SCN^- (Fig. 4) due to slower movement of SCN^-.

Effect of glutamate analogs. Halpern and Even-Shoshan (9) reported that glutamate transport in a strain of K-12 was inhibited by several glutamate analogs. It was of interest to test the effect of such analogs on the artificially driven glutamate transport. In the experiments shown in Table 2, glutamate analogs were added at a 1 mM concentration, and the concentration of radioactive L-glutamate was 1 μM. D-Glutamate, L-glutamate γ-methyl ester, and L-glutamine inhibited Na^+-coupled transport of L-glutamate by 50 to 80%. DL-α-Methyl glutamate and L-aspartate were also inhibitory to some extent. Alanine, which is not a glutamate analog, did not inhibit at all. Thus, the glutamate

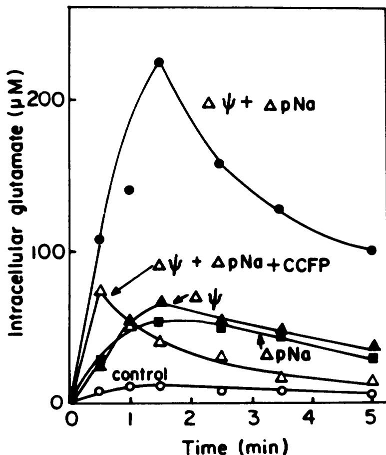

FIG. 4. *Glutamate transport driven by a gradient of NaSCN in strain 29-78. A membrane potential difference ($\Delta\Psi$), interior negative, was imposed by the addition of SCN^-, a permeable anion. An inwardly directed chemical gradient of Na (ΔpNa) was imposed by addition of Na^+ to the external medium. In one experiment CCFP (5 μM) was added.*

TABLE 2. *Effect of glutamate analogs on transport of L-glutamate induced by an artificial Na^+ gradient*[a]

Additions[b]	Glutamate uptake[c] (μM at 30 s)	%
Control	51	100
D-Glutamate	10	20
L-Glutamate γ-methyl ester	17	33
DL-α-Methyl glutamate	36	71
L-Glutamine	24	47
L-Aspartate	42	82
L-Alanine	50	98

[a] Low-Na^+, low-pH cells were diluted into medium containing 100 mM Na^+ at pH 8.0 in the presence of CCFP (see text).

[b] Concentration of each analog was 1 mM.

[c] Glutamate uptake was expressed as the intracellular concentration 30 s after addition of [^{3}H]glutamate.

transport system in strain 29-78 is similar to that of strain K-12 with respect to substrate specificity.

Na^+-coupled glutamate transport in wild-type *E. coli* B. A number of experiments were carried out with *E. coli* B in order to study glutamate transport in this strain. As shown in Fig. 1, the parental strain *E. coli* B possesses a glutamate transport system that is stimulated by Na^+, although the stimulation by Na^+ is less than that of strain 29-78. It was of interest to test whether wild-type *E. coli* B possessed an Na^+-glutamate cotransport system. For this purpose *E. coli* B was grown on glutamate, energy depleted, and incubated under conditions in which an electrochemical Na^+ gradient was imposed. Wild-type *E. coli* B has a high activity of Na^+-coupled glutamate transport (Fig. 5). Under the same conditions, strain 36-39 did not show glutamate uptake. Strain 36-39 is believed to be a mutant with a defect in Na^+-coupled glutamate transport system.

DISCUSSION

Cation-substrate cotransport systems in plasma membranes have been found in a wide variety of cells. In animal cells it is the Na^+ ion that is coupled to a wide variety of membrane transport events (3, 18). The first studies in microorganisms suggested that proton-coupled cotransport might be most important. Subsequently, reports of a variety of other cations activating transport in bacteria have appeared. The potassium ion has been implicated in the transport of citrate in *Aerobacter aerogenes* (5) and Mg^{2+} in citrate uptake by *Bacillus subtilis* (25). A number of studies have shown a marked stimulation of transport by Na^+, although the

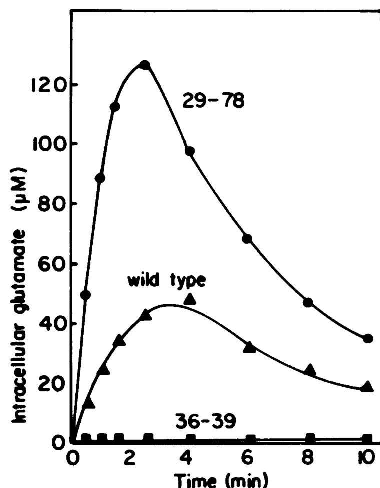

FIG. 5. *Glutamate transport energized by an electrochemical Na^+ gradient in wild-type E. coli B and strain 29-78. Both a membrane potential (inside negative) and a chemical potential for Na^+ were imposed across the plasma membrane in each experiment. The membrane potential was produced by a proton diffusion potential in the presence of CCFP.*

mechanism of stimulation was not elucidated in most cases. Drapeau and MacLeod (4), for example, reported that Na^+ stimulated α-aminoisobutyric acid transport in a marine pseudomonad. Later, Thompson and MacLeod (21) concluded that the presence of Na^+ was required, but an Na^+ gradient was not the driving force for transport. The first clear indication of Na^+-coupled symport of nutrient in a microbial system came from the work of MacDonald and Lanyi (14). They found that amino acid transport in membrane vesicles of *H. halobium* was dependent on Na^+. They showed the uptake of leucine by imposing an artificial chemical gradient of Na^+ across the membrane in the presence of a proton conductor (CCFP). Later they also showed that glutamate was accumulated by membrane vesicles when Na^+ gradient was imposed (13). Other Na^+-dependent transport systems for amino acids have been reported in *Brevibacterium flavum* (19), in *Mycobacterium phlei* (10), and in an alkalophillic bacillus (12). In *Salmonella typhimurium* Stock and Roseman (20) reported that Na^+ (or Li^+) greatly stimulated methyl-β-D-thiogalactopyranoside (TMG) transport by the melibiose transport system and suggested that this system represented Na^+-TMG cotransport. Tokuda and Kaback (22) confirmed these observations and reported that the stoichiometry of Na^+-TMG was approximately 1:1 in membrane vesicles of *S. typhimurium*. Recently we have presented evidence for Na^+-TMG cotransport mediated by the melibiose transport system in *E. coli* (23).

This communication presents evidence that the driving force for glutamate accumulation in *E. coli* B and its derivative 29-78 is an electrochemical potential difference of Na^+ across the plasma membrane. To distinguish between H^+ and Na^+ as the critical cation in this transport process, an experiment was performed in which these two ions moved in opposite directions. A pH gradient was established (inside acid), and the outward movement of protons was facilitated by the addition of CCFP. In the same experiment an inward movement of sodium was assured by adding Na^+ externally to cells containing very little Na^+. The outward movement of protons via CCFP would be expected to produce a membrane potential (inside negative) that would provide an additional driving force for inward migration of Na^+. In this experiment, only the Na^+ would be available for inward cotransport with glutamate.

The transport system for glutamic acid appears to be electrogenic. A membrane potential (interior negative) alone was able to drive glutamate into the cell against a considerable concentration gradient. In one experiment (Fig. 3) the membrane potential was provided by a proton gradient in the presence of CCFP; in the second experiment (Fig. 4) it was induced by the addition of SCN^-. Very recently Ramos and Kaback (17) reported that glutamate was taken up by membrane vesicles of *E. coli* ML308-225 when energized by ascorbate-phenazinemethosulfate at pH 7.5. Under these conditions a large membrane potential (interior negative) and no pH gradient are generated. Although it is not known whether glutamate transport in the ML strain is an H^+-coupled one or an Na^+-coupled one, their results suggest that glutamate transport in vesicles of ML 308-225 is electrogenic.

In vivo the operation of the respiratory chain or energy-transducing adenosine triphosphatase results in outward pumping of protons resulting in a pH gradient (outside acid) and a membrane potential (inside negative). The pH gradient provides the driving force for Na^+ extrusion from the cell via the Na^+-H^+ exchange system described by West and Mitchell (24). These ionic events result in a large inwardly directed electrochemical potential difference for Na^+. Evidence presented in the paper suggests that the energy stored in this Na^+ gradient may be utilized for transport of glutamic acid.

It is quite likely that several different transport systems for glutamate are present in cells of *E. coli*. In mutant 36-39, which lacks the

sodium-stimulated glutamate uptake system, a residual transport of 80-fold accumulation is observed (Fig. 1). The driving force for the other system(s) is not known. It is of interest that *S. aureus* appears to possess an H^+-glutamate cotransport system (16). It will be of considerable interest to further characterize the several transport systems of glutamate.

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