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Sodium-Stimulated Glutamate Transport in Osmotically Shocked Cells and Membrane Vesicles of *Escherichia coli*

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Three phenotypically distinct strains of *Escherichia coli* B were studied: one in which the transport of glutamate was strongly stimulated by sodium, one in which the transport was relatively independent of sodium, and one which did not transport glutamate. Membrane vesicle preparations from the three strains followed the behavior of whole cells with respect to sodium-stimulated transport. Although glutamate-binding material could be released from cells by osmotic shock, its affinity for glutamate was not significantly influenced by sodium. Furthermore, the shocked cells retained sodium-stimulated transport. The accumulated results suggest that the sodium-activated glutamate transport system resides in the cytoplasmic membrane and that releasable binding protein(s) is not intimately involved in its function.

The involvement of inorganic cations in transport processes of microorganisms has begun to receive considerable attention in the last several years (6, 7, 15, 25, 28). Thus, by application of Mitchell's (16) chemiosmotic hypothesis to bacterial systems, it has been suggested that a transmembrane proton gradient, generated in electron transport, provides the energy for transport of sugars (30) and amino acids (10) against a concentration gradient. In animal systems it seems generally accepted (26, 27) that a sodium gradient established by the membrane (Na, K)-adenosine triphosphatase (ATPase) is energetically coupled to the active transport of some organic solutes. Bacterial ATPases have not been shown to have a similar function; yet bacteria, like animal cells, do reveal sodium-stimulated transport of organic solutes. MacLeod and associates (3, 4, 31) demonstrated this interaction in marine pseudomonads which manifest a specific requirement for sodium over and above the general salt requirements for growth. In 1969, Frank and Hopkins (6) showed that the transport of L-glutamate in *Escherichia coli* B is strongly stimulated by sodium. Growth of this organism on glutamate as a sole source of carbon and nitrogen is also strongly stimulated by sodium although there seems to be no general growth requirement for the cation. Since 1969 there have been further reports of sodium-stimulated transport in *E. coli* and other microorganisms (7, 15, 25, 28).

After the extension of transport studies to membrane preparations from bacteria (12), inorganic cation involvement has been scrutinized in these systems. Lombardi and Kaback (13) reported inhibition by sodium of the uptake of proline, serine, and aspartate in membrane vesicles prepared from *E. coli* ML. Rayman, Lo, and Sansal (21) found succinate uptake in membrane vesicles of *E. coli* K-12 to be stimulated by sodium, potassium, and lithium. More closely related to the present report, MacLeod, Thurman, and Rogers (15) have demonstrated sodium-stimulated uptake of L-glutamate, L-alanine, and α-aminoisobutyrate in membrane preparations from *Bacillus licheniformis*.

In this communication we describe experiments whose results are consistent with the notion that the system responsible for the sodium stimulation of glutamate transport in *E. coli* B resides in the cytoplasmic membrane fraction.

MATERIALS AND METHODS

Chemicals. L-[U-^{14}C]glutamate (22.8 mCi/mmol) was obtained from Amersham-Searle and L-[3-^{3}H]-glutamate (16.2 Ci/mmol) was from New England Nuclear Corp. L-[5-^{3}H]proline (35.5 mCi/mmol) was prepared as described previously (5). The antibiotics monensin, nigericin, and dianemycin were obtained from Eli Lilly & Co. Reagents were the best grades obtainable commercially, and doubly-distilled water was used throughout. As estimated by flame-photometry, the various solutions used in this work contained contaminating levels of sodium not greater than 0.1 mM.

Analytical. Protein was determined by the method of Lowry et al. (14). Bacterial growth was followed by turbidity measurements in a Bausch and Lomb spectrophotometer at 600 nm. Transport assays were performed by incubating the indicated biological preparations with radioactively labeled solute and collecting samples periodically on 0.45-μm membrane filters (Schleicher & Schuell Co.). The filters were dissolved in 10 ml of Aquasol (New England Nuclear Corp.) for radioassay in a Nuclear Chicago Unilux I liquid scintillation counter. Quench corrections were made by reference to previously constructed curves, utilizing the instrument's external standard. Uptake of solute by intact cells and osmotically shocked cells was followed as described previously (6), including the addition of arsenite plus NH_4Cl (to retard metabolism of accumulated glutamate) and chloramphenicol (to inhibit protein synthesis). The inhibitors were omitted in the experiments with whole cells described in Table 2. Uptake by membrane vesicles utilized a reaction mixture containing 50 mM potassium phosphate buffer, pH 6.6, 10 mM $MgSO_4$, and 20 mM Li-D-lactate as an energy source. Where applicable, incubations contained either 10 mM NaCl or, as a control, 10 mM KCl. As with intact cell assays, mixtures were preincubated for 5 min before the addition of radioactive substrate. Filtered samples were washed with 15 ml of 0.1 M LiCl (12). All transport assays were conducted at 25 C. The binding of glutamate to shock-releasable material was measured by the rapid-flow dialysis method of Colowick and Womack (2), as described by Rosen (23). The buffer used was 10 mM potassium phosphate, pH 7.1, containing either 50 mM KCl or NaCl to assess the influence of sodium on binding activity. Initially, the upper chamber of the apparatus (2) contained 0.9 ml of buffer; 1 μliter of L-[3-^{3}H]glutamate (1.1×10^6 counts/min) was added to a concentration of 6×10^{-8} M. Fractions were collected for 4 min before 0.1 ml of protein solution was added. Every 4 min thereafter, increasing concentrations of L-glutamate were added in 5-μliter amounts. In experiments with presumptive inhibitors, the compound was added with the buffer in the upper chamber. Binding assays were carried out at room temperature (23 C).

Biological. Bacteria were grown in B7 salts (6) supplemented with the indicated compounds. This salt medium contains no added sodium. The parent strain used in this work, 29-78, is a derivative of wild-type *E. coli* B selected (6) for rapid and reproducible growth on L-glutamate as carbon and nitrogen source. As described previously (6), strain 29-78 requires sodium both for growth on L-glutamate and the concentrative uptake of this amino acid. Two mutants, 5-83 and 36-6, were derived from the parent strain without use of a mutagen. One of them, strain 5-83, was selected for the inability to grow on L-glutamate as either a carbon or nitrogen source whether or not sodium was added. It was isolated by virtue of resistance (6, 9) to the glutamate analogue, α-methylglutamate: approximately 10^8 cells of strain 29-78 were plated on 0.8% Oxoid ion-agar containing B7 salts supplemented with 10 mM each of glycerol, NH_4Cl, and NaCl, and 200 mM DL-α-methylglutamate. From the colonies which appeared, one with the desired characteristics was selected and purified by reisolation from plates containing the same medium, but lacking the analogue. The other mutant, 36-6, was selected for the ability to grow on glutamate in the presence or absence of added sodium: strain 29-78, initially grown in B7 salts with 10 mM glycerol and 15 mM NH_4Cl, was transferred to B7 salts containing only 3 mM potassium L-glutamate. After a long lag, the culture increased in density and it was then transferred sequentially in the same medium. Finally, the culture was plated on agar containing B7 salts and glutamate, and a single colony was picked and purified, as described above.

Membrane vesicles were prepared as described by Kaback (11).

Osmotic shock was performed by a modification of the method of Neu and Heppel (17), as described by Rosen (22). Shocked cells were recovered by centrifugation and saved for subsequent assay of transport. The supernatant was concentrated in an Amicon Corp. ultrafiltration cell with a UM-10 membrane and then fractionated on Sephadex G-100. The column was eluted with 10 mM potassium phosphate, pH 7.1, containing 50 mM KCl. Glutamate binding activity in the fractions was detected by a filter assay described by Rosen (22). Appropriate fractions were pooled and concentrated to yield a protein concentration of 12 mg/ml.

RESULTS AND DISCUSSION

The growth behavior of the parent strain, 29-78, and the two derived mutants, 5-83 and 36-6, as a function of sodium addition is shown in Table 1. As expected from the isolation history, all three strains grew on glycerol: the growth of 29-78 on glutamate was strongly dependent on sodium addition, whereas that of 36-6 was not; and 5-83 did not grow on glutamate whether or not sodium was added. Transport assays (Fig. 1) confirmed the previously described dependence of glutamate uptake on sodium in the case of 29-78, but 36-6, whose growth rate was independent of sodium, still showed a small but significant stimulation of

TABLE 1. *Effect of Na^+ on growth rate of three strains of E. coli B*

Strain	Growth substrate[a] (doublings/h)			
	L-glutamate		Glycerol	
	+ Na^+	− Na^+	+ Na^+	− Na^+
29-78	0.69	0.08	1.03	1.03
36-6	0.68	0.71		1.00
5-83	0	0		0.86

[a] Potassium L-glutamate (10 mM) or glycerol (10 mM) plus NH_4Cl (15 mM) were added as carbon and nitrogen sources to B7 salts. Where present, NaCl was added at 10 mM.

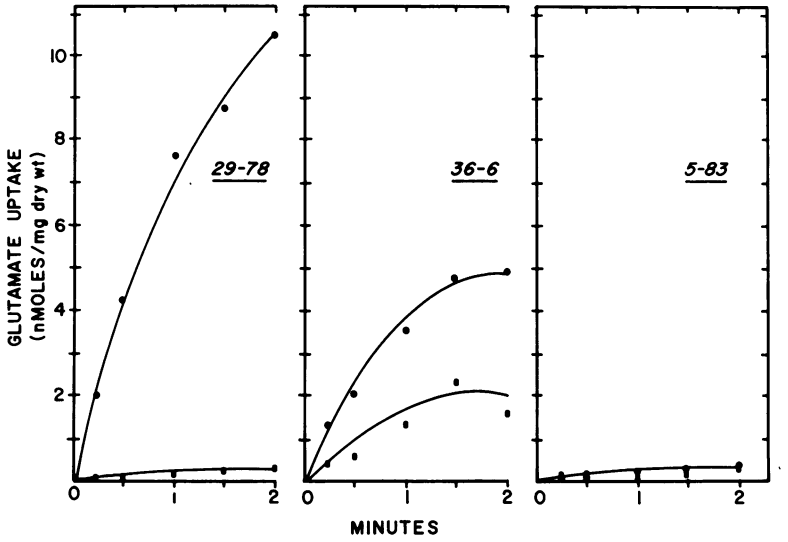

FIG. 1. *Effect of Na^+ on the transport of glutamate by intact cells of strains 29-78, 36-6, and 5-83. The assay was conducted as described under Materials and Methods with glycerol as energy source and L-[U-^{14}C]glutamate at 1.1 μM; bacterial concentration was 10 to 20 μg (dry weight)/ml. Symbols: ●, in the presence of 10 mM NaCl; ○, in the presence of 10 mM KCl.*

glutamate transport by this cation. (We have been unsuccessful in deriving a strain whose uptake of glutamate is totally independent of sodium.) Strain 5-83 did not transport glutamate effectively whether or not sodium was added (Fig. 1). Thus, in a qualitative sense, the parent strain and the two derived mutants manifested transport behavior which followed growth behavior with respect to glutamate and sodium.

Having these strains in hand, we could investigate membrane vesicle preparations from the point of view of glutamate transport and its dependence on sodium. We found that vesicles prepared from the three strains showed transport properties which paralleled those of the intact cells. The accumulation of glutamate by vesicles from strain 29-78 was strongly stimulated by sodium (Fig. 2), although less so than the intact cells (Fig. 1). Vesicles from strain 36-6 also took up glutamate actively with a rate which was much less dependent on the presence of sodium. Finally, vesicles from strain 5-83, which hardly transported glutamate at all, were essentially inactive in glutamate uptake. There is no reason to believe that the inactivity of 5-83 vesicles reflected general damage to the system because these vesicles accumulated proline as well as those prepared from the other strains (Table 2).

That these results were due to the properties of membrane vesicles and not to contaminating intact cells is suggested by several observations. The viable count of the vesicle preparation was far too low to yield measurable transport. Accumulation of glutamate and proline by the vesicle preparations was dependent on the addition of D-lactate. And whereas intact cell transport of glutamate was unaffected by the ionophoric antibiotic dianemycin, the vesicle preparation was markedly sensitive to it as well as to monensin and nigericin. Each of these ionophores almost abolished glutamate transport in vesicles prepared from strain 29-78. These antibiotics are monocarboxylic acids which are thought to dissipate cation concentration gradients across membranes (20); each has affinity for sodium ion. It is of interest that, although these compounds strongly inhibited glutamate uptake, they effected only a slight reduction in the rate of transport of lysine, a process which is not affected by sodium in membrane vesicles (13).

The results with vesicles strongly suggest that the glutamate transport system in *E. coli* B and its interaction with sodium are properties resident in the membrane. This conclusion raised the question of the possible participation of shock-releasable binding proteins in our system in view of the report by Barash and Halpern (1) that a releasable binding protein is intimately involved in glutamate transport by *E. coli* K-12. Consequently, we sought to determine whether a sodium-responsive glutamate-binding protein was released from our cells during osmotic shock.

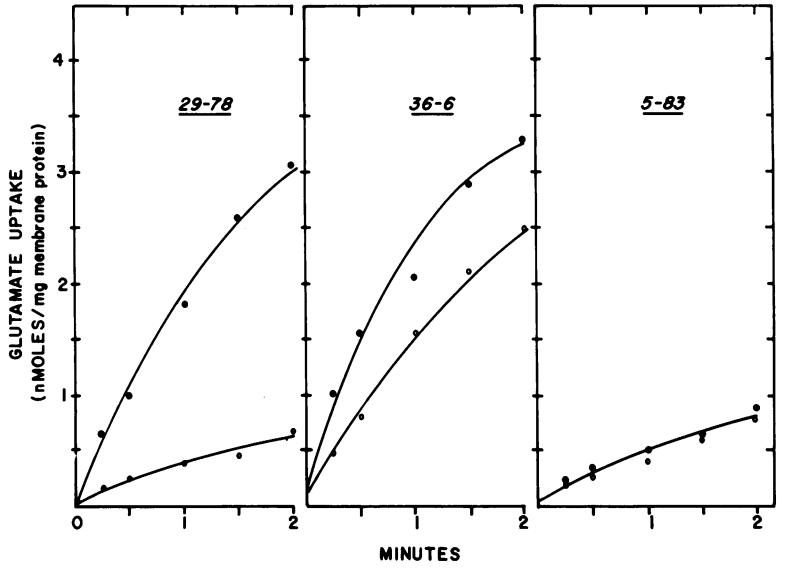

FIG. 2. *Effect of Na^+ on the transport of glutamate by membrane vesicles of strains 29-78, 36-6, and 5-83. Vesicle concentration was 80 to 120 μg of membrane protein per ml; L-[U-^{14}C]glutamate was present at 5.3 μM. Symbols: ●, in the presence of 10 mM NaCl; ○, in the presence of 10 mM KCl. Other experimental conditions are described under Materials and Methods.*

TABLE 2. *Uptake of glutamate and proline by cells and membrane vesicles of three strains of E. coli B. Effect of Na^+ on glutamate uptake*

Strain	Initial rate of solute uptake[a] (nmol/min)				
	L-Glutamate[b]		Na^+	L-Proline[c]	
	Cells	Vesicles		Cells	Vesicles
29-78	6.4	2.0	+	1.6	0.2
	0.1	0.3	−		
36-6	2.8	2.4	+	1.6	0.1
	1.6	1.6	−		
5-83	0.2	0.4	+	1.5	0.1
	0.2	0.4	−		

[a] For whole cells, the rate is per milligram (dry wt) and for vesicles, per milligram of membrane protein.

[b] For cells of strains 29-78 and 36-6, L-[U-^{14}C]glutamate was supplied at 5.3×10^{-7} M; for cells of strain 5-83, it was used at 1.1×10^{-6} M. Bacterial concentration was 0.02 mg (dry wt)/ml. For vesicles, L-[U-^{14}C]glutamate was used at 5.3×10^{-6} M. Vesicle concentration was 0.1 mg of membrane protein per ml.

[c] Proline uptake was assayed without added Na^+ or K^+. For cells, L-[5-^{3}H]proline was used at 4.2×10^{-6} M and, for vesicles, at 2.1×10^{-5} M.

E. coli 29-78 was grown in B7 salts supplemented with 10 mM glycerol and 15 mM NH_4Cl. The culture was subjected to osmotic shock, and binding activity was isolated as described under Materials and Methods. Indeed, as reported by Barash and Halpern for *E. coli* K-12, the shock fluid contained glutamate-binding material. But the binding activity was little affected by sodium (Fig. 3): the K_d for glutamate in the presence of NaCl was 1.1×10^{-7} M and 1.4×10^{-7} M in its absence (with equimolar KCl as a control). Thus, we have no reason to believe that this shock-releasable activity contributes to the locale of sodium action in our system. In fact, we have no convincing evidence that the material is involved in the overall process of glutamate transport; rather, some other observations tend to argue against its participation. Although D-glutamate is a competitive inhibitor of L-glutamate transport in our cells (unpublished data), as it is in *E. coli* K-12 (8), this isomer did not affect binding of L-glutamate to the shock-released material (Table 3). On the other hand, L-aspartate did inhibit L-glutamate binding (Table 3), whereas it did not inhibit L-glutamate uptake by cells even when added in 100-fold excess. Finally, the K_d for glutamate binding to the released material was 60- to 70-fold lower than the K_m for glutamate transport in whole cells. In contrast to these observations, Barash and Halpern (1) presented rather convincing evidence that their glutamate-binding protein does indeed have the properties of a component of the transport system.

At least qualitatively, assay of sodium-

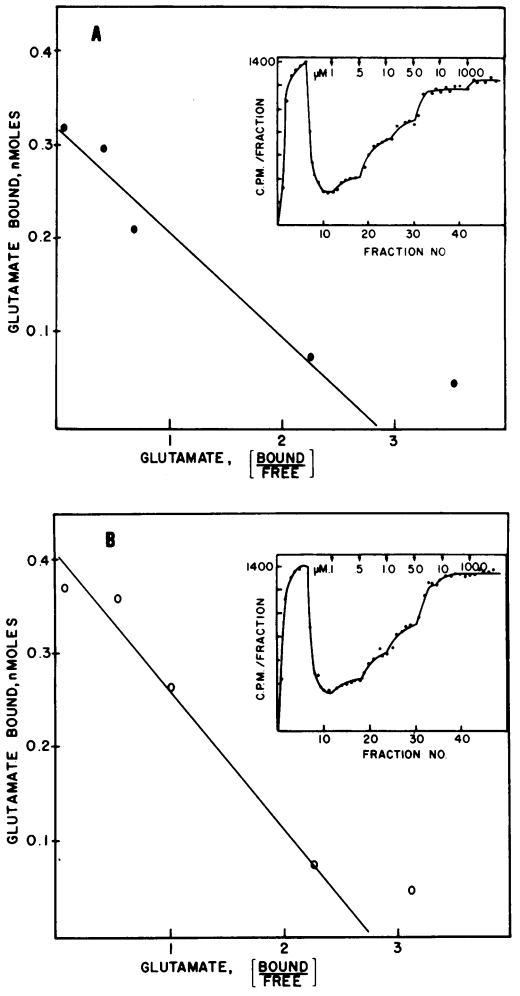

FIG. 3. *Rapid-flow dialysis measurement of glutamate binding to shock-released material. Experimental details are given under Materials and Methods. The figure shows the primary data in the insets and the derived data as Scatchard plots. A) Binding assay carried out in the presence of 50 mM NaCl; B) binding assay carried out in the presence of 50 mM KCl. The lines were fitted by least squares, with the outlying point rejected in each case.*

stimulated transport in the shocked cells supports the notion that shock-released material does not participate in a fundamental way in our system because the shocked cells retained sodium-stimulated glutamate uptake (Fig. 4). However, approximately half of the activity was lost during the shock procedure even though proline uptake, which is resistant to osmotic disruption (19), was hardly affected. The simplest interpretation of these results is that our cells possess a sodium independent system with a shock-releasable component in addition to the sodium-stimulated glutamate transport. If this

TABLE 3. *L-glutamate binding to shock-released protein. Effect of inhibitors*[a]

Inhibitor	Concentration ($M \times 10^6$)	Apparent K_d ($M \times 10^7$)
None		1.1
D-Glutamate	100	1.5
D-Glutamate	10	1.7
L-Aspartate	10	5.6
L-Aspartate	1	1.7

[a] Experimental details are given under Materials and Methods.

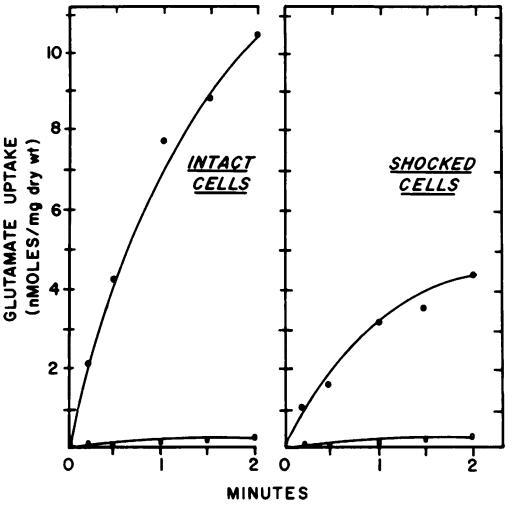

FIG. 4. *Effect of Na^+ on the transport of glutamate by intact and osmotically shocked cells of strain 29-78. The assay was conducted as described under Materials and Methods with glycerol as energy source and L-[U-^{14}C]glutamate at 1.1 μM; cell concentration was 10 to 70 μg (dry weight)/ml. Symbols: ●, in the presence of 10 mM NaCl; ○, in the presence of 10 mM KCl.*

is the case, the auxiliary system seems not to have much significance for the growth of cells on glutamate, nor is it revealed in kinetic analysis of glutamate uptake by whole cells (unpublished data).

Further work is needed to clarify the similarities and differences between glutamate transport in strains K-12 and B of *E. coli*. We have not investigated mutant strains of K-12 which have the ability to transport glutamate. But the recent demonstration by Halpern et al. (7) of sodium-stimulated glutamate transport in a strain of K-12 suggests that our observations may not be unique to *E. coli* B.

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