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Sodium-stimulated glutamate uptake in membrane vesicles of *Escherichia coli*: The role of ion gradients

(chemiosmotic hypothesis/Na^+ symport/Na^+–H^+ antiport/protonmotive force)

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ABSTRACT **Membrane vesicles prepared from *Escherichia coli* B/r grown on glutamate as a sole source of carbon and energy require sodium for glutamate accumulation when energized by D-lactate oxidation. Glutamate uptake can also be driven by a prearranged sodium gradient (out to in) in the absence of an energy source or a protonmotive force. Sodium ions are exchanged rapidly in respiring vesicles and the sodium gradient may be large enough under certain conditions to drive glutamate uptake after the protonmotive force is abolished with *m*-chlorocarbonylcyanide phenylhydrazone. Glutamate uptake due to a prearranged sodium gradient or lactate oxidation is inhibited by monensin but not by nigericin. Transport does not occur in response to valinomycin-induced membrane potential. We interpret these results to indicate that glutamate transport is obligately coupled to sodium transport and can only occur when there is a net flux of sodium ions. This flux is driven by a chemical gradient of sodium that is created by the protonmotive force generated by respiration.**

The intracellular accumulation of nutrients and ions against large concentration gradients is a general biological phenomenon that enables most free-living cells to exist in environments much more dilute than their intracellular sap. While less obvious, the ability to accumulate nutrients is an obligate necessity of many cells of highly developed organisms as well, particularly those cells involved in the exchange of nutrients across the gut wall and in the excretion of body wastes. A convincing body of evidence exists (1–11) that links the ability of cells to concentrate substrates to their ability to generate chemical and electrical transmembrane gradients for cations.

The direct coupling of sugar and amino acid transport to proton circulation in both prokaryotes and eukaryotes, and to sodium transport in eukaryotes, is well established (1, 3, 5, 6). Among the prokaryotes, however, sodium-coupled transport has only been demonstrated clearly in membrane vesicles prepared from *Halobacterium halobium* (9–11) (See *Note Added in Proof*). Several reports of a sodium requirement for amino acid or sugar transport have appeared (12–15) but direct coupling through a sodium-symporter has not been demonstrated. This is somewhat surprising because sodium gradients are often observed in bacteria and a mechanism by which sodium is pumped out of cells has been known for several years. In 1972, Harold and Papineau (16) described a mechanism in streptococci for the exchange of intracellular Na^+ for extracellular H^+. Later, West and Mitchell (17) reported experiments that indicated a similar Na^+–H^+ antiport system in *Escherichia coli* cells. More recently, we demonstrated (18) a Na^+–H^+ antiport system in halobacteria membrane vesicles that can generate large sodium gradients in response to light-induced proton motive force. Na^+–H^+ antiport systems also have been reported in several eukaryotic systems (19, 20).

The observation by Miner and Frank (13) that a sodium-stimulated glutamate uptake system was present in *E. coli* vesicles suggested to us that this may be similar to the glutamate uptake system in halobacteria. In this study, we have attempted to demonstrate a direct involvement of a sodium gradient in glutamate uptake by *E. coli* vesicles. This gradient may be prearranged or generated metabolically and appears to effect transport independently of an electrochemical proton gradient which may also be present. Thus, the obligate nature of the sodium requirement in some bacteria is due to a sodium–substrate symport. This clearly shows that the sodium and potassium gradients normally generated in growing prokaryotic cells can serve as energy reservoirs available for the transport of needed nutrients, as is the case in many eukaryotic cells.

METHODS

E. coli B/r was grown on a shaker at 37° in Tris/salts medium (21), with 0.2% potassium glutamate as the energy and carbon source as described by Miner and Frank (13). Membrane vesicles were prepared according to the method of Kaback (22) and stored in liquid nitrogen until needed. Microscopic examination of concentrated samples of these vesicle preparations indicated whole-cell contamination to be <0.01%. Potassium-loaded vesicles were prepared in 50–400 mM potassium phosphate buffer, pH 6.6/10 mM $MgSO_4$. Sodium-loaded vesicles were prepared by suspending potassium-loaded vesicles in 100-fold, or greater, volumes of 50–400 mM sodium phosphate buffer, pH 6.6/10 mM $MgSO_4$. The suspensions were allowed to equilibrate for at least 24 hr at 4° and then were concentrated by centrifugation and resuspended in a small volume of the same buffer. Final vesicle protein concentration was 2–5 mg of protein per ml as determined by the Lowry method (23) with egg white lysozyme as the protein standard.

The sodium dependence of glutamate uptake in whole cells and membrane vesicles was determined as described by Miner and Frank (13) with glycerol as the energy source for whole-cell uptake and lithium D-lactate as the energy source for vesicles. *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride and ascorbate (24) were used as energy sources for these vesicles in some experiments, with similar results. All uptake measurements were carried out at 30°. Cells or vesicles were collected on cellulose nitrate filters (0.45 μm pore size) that had been presoaked in 10 mM potassium glutamate, washed five times with 1 ml of 0.4 M lithium chloride, and dried. The radioactivity retained was determined as described (9). L-[^{3}H]-

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Abbreviations: CCCP, *m*-chlorocarbonylcyanide phenylhydrazone; $TPMP^+$, triphenylmethylphosphonium cation.

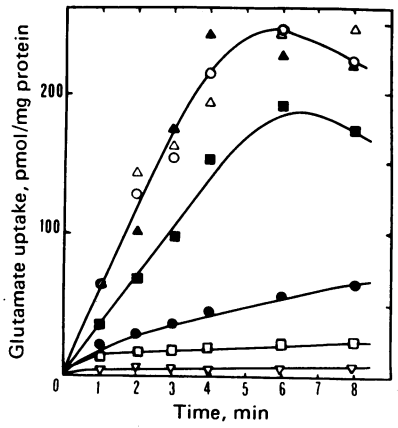

FIG. 1. Sodium-stimulated uptake of L-glutamate by membrane vesicles of *E. coli*. Membrane vesicles were prepared in 50 mM potassium phosphate buffer, pH 6.6/10 mM $MgSO_4$ as described in *Methods*, at a protein concentration of 5.1 mg/ml. Reaction mixture consisted of the above buffer, 600 μl/vesicles, 30 μl/2 M lithium D-lactate, 6 μl/0.1 mM L-[^{3}H]glutamate (99 μCi/ml), 5 μl. Additions: none (●); (10 mM Na phosphate, final concentration) (○); 10 mM Na phosphate and 10 μM nigericin (▲); 10 μM dicyclohexylcarbodiimide (△); 10 mM Na phosphate and 10 mM sodium azide (□); 10 μM CCCP or *p*-trifluoromethoxycarbonylcyanide phenylhydrazone (■); and 10 mM Na Phosphate and 3 μM monensin (▽).

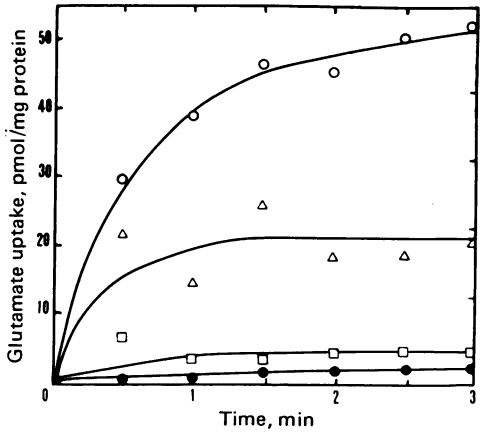

FIG. 2. Uptake of glutamate in response to a prearranged sodium gradient. Vesicles loaded with 0.4 M potassium phosphate, pH 6.6/10 mM $MgSO_4$ as described in *Methods* were added to 0.4 M sodium phosphate, pH 6.6/10 mM $MgSO_4$ containing 1 μM L-[^{3}H]glutamate and 5 μM CCCP (○). Vesicles loaded with 0.4 M sodium phosphate, pH 6.6/10 mM $MgSO_4$ were added to 0.4 M potassium phosphate, pH 6.6/10 mM $MgSO_4$ or to 0.4 M sodium phosphate, pH 6.6/10 mM $MgSO_4$ containing 1 μM [^{3}H]glutamate and 5 μM CCCP (■). Vesicles loaded with 0.4 M sodium phosphate, pH 8.0/10 mM $MgSO_4$ were added to 0.4 M sodium phosphate/10 mM $MgSO_4$/10 mM sodium azide/1 μM [^{3}H]glutamate (●). Same conditions as (○) except that 3 μM monensin was also present (△).

Glutamate (New England Nuclear Corp.) was used at a final concentration of 1 μM (specific activity, 90 mCi/mmol) unless otherwise indicated. ^{22}NaCl was obtained from New England Nuclear Corp. Lithium D(−)-lactate, valinomycin, tetraphenylboron, and *m*-chlorocarbonylcyanide phenylhyrazone (CCCP) were obtained from Sigma Chemical Co.; monensin was a gift from E. L. Potter, Lilly Research Laboratories; nigericin was a gift from Robert Hosley, Lilly Research Laboratories; and [^{3}H]triphenylmethylphosphonium ($TPMP^+$) was a gift from H. R. Kaback, Roche Institute of Molecular Biology. Unlabeled $TPMP^+$ was obtained from Pfaltz and Bauer, Inc., and *p*-trifluoromethoxycarbonylcyanide phenylhydrazone was obtained from Pierce Chemical Co. All other chemicals were reagent grade.

RESULTS

Fig. 1 shows the effect of several compounds reported to inhibit membrane transport (1, 25, 26) on glutamate uptake in *E. coli* membrane vesicles. CCCP or *p*-trifluoromethoxycarbonylcyanide phenylhydrazone, uncouplers which permit the rapid exchange of H^+ across membranes and thus the discharge of both pH and electrical gradients, completely abolished the D-lactate-energized uptake. Sodium azide, a potent inhibitor of D-lactate oxidation, also blocked uptake of glutamate, but dicyclohexylcarbodiimide, which inhibits membrane-bound ATPase (25), had no significant effect. Nigericin, an antibiotic that exchanges H^+ for K^+ across membranes and thus discharges a pH (but not an electrical) gradient, had no effect on glutamate uptake. On the other hand, monensin, an antibiotic that exchanges H^+ for Na^+ across membranes without affecting the electrical gradient, significantly reduced both the rate and extent of glutamate uptake. Removal of sodium from the reaction mixture also greatly reduced the ability of vesicles to accumulate this amino acid.

These observations suggest that D-lactate-energized uptake of glutamate is dependent on the transmembrane electrical potential that is generated by the oxidation of D-lactate (27). The inhibitory effect of monensin and the requirement for Na^+ in the reaction mixture further suggest that in this system the transport of glutamate might be coupled directly to the transport of Na^+, as has been demonstrated in other prokaryotic (9–12, 28) and eukaryotic (5–8) systems. If this were the case, it would be expected that accumulation of glutamate could be induced by the presence of an excess of Na^+ on the outside of the vesicle membrane, in the absence of any other source of energy. This possibility was examined by adding a small volume of vesicles loaded with potassium phosphate to a much larger volume of sodium phosphate of the same molarity containing L-[^{3}H]glutamate and 10 μM CCCP (to suppress endogenous respiration and to discharge any preexisting protonmotive force). Fig. 2 shows that under these conditions a 10- to 20-fold concentration of glutamate took place, whereas no measurable uptake took place if the vesicles were preloaded with sodium phosphate and added to equimolar potassium phosphate or if potassium-loaded vesicles were added to equimolar potassium phosphate. No glutamate uptake could be detected if an artificial pH gradient was created by adding vesicles, equilibrated at pH 8.5 in sodium phosphate, to a large volume of the same solution at pH 5.5 (containing 10 mM sodium azide to supress endogenous respiration). Ramos *et al.* (26) have shown that *E. coli* vesicles are able to generate a protonmotive force in this pH range if energized with phenazine methosulfate/ascorbate. Thus, the failure of vesicles to accumulate glutamate in response to a prearranged pH gradient is probably not due to the destruction of the integrity of the membrane. In a control experiment (not shown), sodium azide had no effect on the sodium gradient-driven glutamate uptake but the possibility exists that sodium azide also acts as a proton-specific ionophore.

These experiments do not completely rule out the involvement of an electrical gradient because a significant membrane potential may be formed by the outward diffusion of potassium, if the membrane is more permeable to K^+ than to other ions. However, when membrane vesicles were loaded with 0.2 M potassium phosphate and 0.2 M sodium phosphate and suspended in 0.4 M sodium phosphate, the addition of valinomycin, which has been shown to permit the formation of a significant potassium diffusion potential in *E. coli* membrane

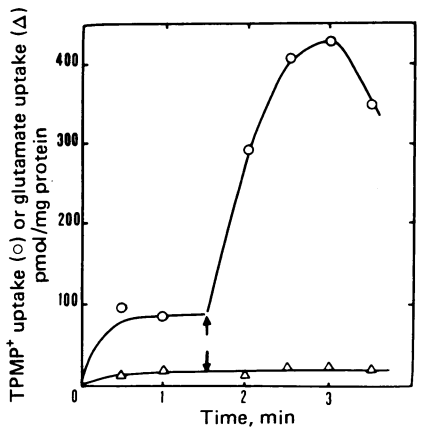

FIG. 3. Uptake of glutamate and [^{3}H]TPMP$^+$ in response to a valinomycin-induced membrane potential. Vesicles loaded with 0.2 M potassium phosphate/0.2 M sodium phosphate, pH 6.6/10 mM $MgSO_4$ were added to a solution containing 0.4 M sodium phosphate, pH 6.6/10 mM $MgSO_4$/10 mM Na azide/0.05 mM tetraphenylboron. To equal aliquots, we added 1 μM [^{3}H]glutamate (△) and 0.5 μM [^{3}H]TPMP$^+$ (○); 1.5 min later, 10 μM valinomycin was added (*arrow*).

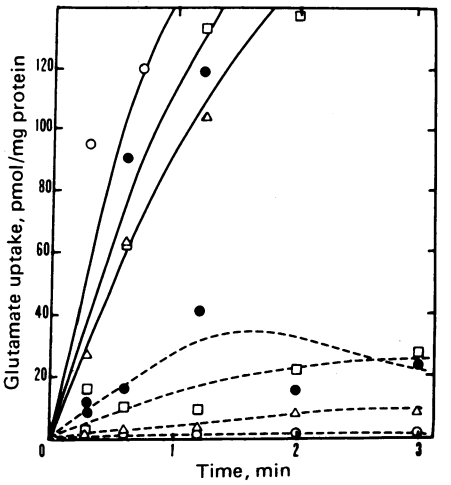

FIG. 4. CCCP-insensitive uptake of glutamate in D-lactate-energized membrane vesicles. Vesicles loaded with 0.2 M potassium phosphate, pH 6.6/0.2 M sodium phosphate, pH 6.6/10 mM $MgSO_4$ were suspended in the same buffer to a final protein concentration of 70 $\mu g/ml^{-1}$. D-Lactate (20 mM) was added to start the experiment and 1 μM [^{3}H]glutamate (solid lines) or 1 μM [^{3}H]glutamate and 5 μM CCCP (dashed lines) were added at the following time intervals: 0 min (○); 4 min (△); 16 min (□); 34 min (●). All concentrations are final concentrations.

vesicles (27), did not stimulate glutamate uptake at all (Fig. 3). Under the same conditions, in the presence of tetraphenylboron (27), a 10-fold or greater concentration of [^{3}H]TPMP$^+$ took place.

Nigericin had no effect on glutamate uptake driven by a sodium gradient (data not shown); therefore, K^+–glutamate antiport or H^+–glutamate symport is unlikely. Monensin, on the other hand, caused an 80% decrease in the rate of glutamate uptake.

If glutamate uptake is driven only by a sodium gradient, then a mechanism must be present in the vesicles to create such a gradient. This proved to be very difficult to demonstrate directly because of the large amount of ^{22}Na that binds to vesicles and to filters. However, by loading vesicles with ^{22}NaCl, sodium exit can be followed. Thus, vesicles were equilibrated with 250 mM potassium phosphate and 50 mM sodium phosphate for 48 hr at 4° in the presence of ^{22}NaCl (250 μCi/ml; final specific activity, 0.22 mCi/mmol). The exit was followed by diluting this preparation into $^{22}Na^+$-free buffer of the same molarity of sodium and potassium phosphate but containing 20 mM D-lactate for energized exit or 10 μM CCCP for unenergized exit. Under these conditions, the k_{exit} for $^{22}Na^+$ was 0.25 min^{-1} in energized vesicles and 0.08 min^{-1} in unenergized vesicles. A possible interpretation of these observations is that the increased ^{22}Na exit rate represents an increased exchange of sodium, because of the electrochemical sodium gradient present under energized conditions, rather than the net efflux of sodium due to a sodium pump. This cannot be entirely ruled out and, in fact, it is possible that sodium–glutamate symport can be coupled to either a chemical or an electrical sodium gradient as is the case for most amino acid transport systems in halobacteria vesicles (28). The following experiments, however, provide some supporting evidence for the direct formation of a chemical sodium gradient.

If vesicles loaded with 0.2 M potassium and 0.2 M sodium phosphate and energized with D-lactate were allowed to accumulate L-[^{3}H]glutamate, it would take up to 20 min to reach a maximal glutamate concentration. In contrast, glutamate uptake in all potassium vesicles reached the maximum in about 5 min (Fig. 1). Addition of an excess of unlabeled glutamate to the reaction mixture resulted in a first-order exit of L-[^{3}H]-glutamate, indicating that exchange was constantly taking place. However, if the exit rate was measured at regular intervals before the final equilibration, a 60–70% decrease during the initial 20-min-uptake period was observed. This could be explained if the intravesicle sodium concentration is decreasing with time as suggested in the previous experiment and, therefore, glutamate exit, which also requires sodium, is inhibited.

If the above interpretation is correct, it should be possible to create a chemical sodium gradient with respiration that would drive glutamate uptake after the protonmotive force is abolished. Vesicles containing 0.2 M potassium and 0.2 M sodium phosphate were energized with D-lactate. At increasing time intervals either L-[^{3}H]glutamate or L-[^{3}H]glutamate plus 5 μM CCCP were added and subsequent glutamate uptake was measured. Fig. 4 shows that a progressive increase in the initial CCCP-insensitive uptake rate of glutamate occurred although the uptake rate and the net accumulation were quite low. Repeating the experiment with much longer preincubation times gave essentially the same results. The low rate of transport may be due to the rapid decay of the chemical gradient, especially if the vesicles are quite leaky to Na^+. To test this, vesicles loaded with 0.4 M potassium phosphate were added to 0.4 M sodium phosphate buffer and the ability of the vesicles to accumulate glutamate was determined at short intervals thereafter. The uptake rate for glutamate decreased by 50% in 1 min and by 80% after 4 min, thus indicating that even large prearranged gradients relax quite rapidly.

Formation of the CCCP-insensitive driving force for uptake is not affected by the presence of 1 μM dicyclohexylcarbodiimide or 10 mM sodium arsenate, suggesting that it does not involve an ATPase. On the other hand, it is partially abolished by 2 μM monensin but not by μM nigericin. This suggests that the CCCP-insensitive component of respiring vesicles which drives glutamate uptake is due to a chemical gradient of sodium.

Both the D-lactate-driven uptake (Fig. 1) and the sodium gradient-driven uptake (Fig. 2) are saturable. The $k_{0.5\ min}$, the half-maximal rate constant calculated for the first 0.5 min of uptake, for glutamate uptake due to respiration is 0.7 μM (V_{max},

323 pmol min^{-1} and for uptake due to a sodium gradient is 0.6μM (V_{max}, 82 pmol min^{-1}).

DISCUSSION

Miner and Frank (13) reported that *E. coli* grown on glutamate as the sole source of carbon and energy and membrane vesicles made from these cells were dependent on sodium for the accumulation of glutamate. They showed that this uptake was inhibited in vesicle preparations by dianemycin, monensin, and nigericin, and although they noted that these antibiotics were thought to discharge ion gradients, they did not pursue this line of reasoning.

We show that glutamate may be concentrated by *E. coli* membrane vesicles in direct response to a chemical gradient of sodium. In previous studies (9–11, 28) with *H. halobium* membrane vesicles, we have shown that glutamate and other amino acids are accumulated via Na^+-symport. The present findings are consistent with those in *H. halobium* and may be explained in a similar manner. Thus, the uptake of L-glutamate by membrane vesicles of *E. coli* is coupled to the influx of Na^+. The circulation of the Na^+ is driven by the protonmotive force generated by the oxidation of D-lactate (26, 27) via the H^+–Na^+ antiporter (17) which causes the re-ejection of Na^+. Glutamate concentration is directly dependent on the flux of Na^+ and indirectly dependent on the size of the protonmotive force.

Monensin inhibits glutamate uptake due to respiration less than that due to a prearranged sodium gradient. This is to be expected if the actual sodium gradient generated in the former case is small but is sustained by the rapid recirculation of Na^+ in response to protonmotive force. On the other hand, the degree to which monensin can discharge a prearranged sodium gradient will be dependent on the size of the pH gradient developed in the opposite direction.

Kahane *et al.* (14) showed that Na^+ added to the outside of potassium-loaded vesicles strongly inhibited the exit of glutamate and suggested that this indicated that sodium may act by inhibiting the binding of glutamate to the carrier on the inside of the vesicle. Our data suggest that this observation may be more simply explained by the stimulatory effect of the sodium gradient on the *reentry* of the glutamate. Because the internal concentration of sodium was low in their experiment, the rate of exit of glutamate was also low and served to accentuate the inhibition.

Niven and Hamilton (29) were unable to demonstrate valinomycin-induced uptake of glutamate in *Staphylococcus aureus* cells and suggested that the translocation of glutamate did not involve the net movement of a positive charge but took place by H^+–glutamate symport in response to a pH gradient. MacDonald and Lanyi (28) have shown that other amino acids are accumulated in halobacteria vesicles in response to a valinomycin-induced potassium diffusion potential if sodium is present, but glutamate is not accumulated under these conditions (30). This may be rationalized by assuming that under the conditions of the experiments a sodium gradient, which alone will drive glutamate uptake, would not arise because the capacity of the diffusion potential is insufficient to sustain it. Thus, transport in response to metabolic energy may occur because sodium is continuously ejected at a rate sufficient to maintain a sodium gradient against a significant inward sodium flux.

We believe that these results clearly indicate that glutamate uptake in *E. coli* is coupled to metabolism through the formation of a sodium gradient as it is in *H. halobium*. Furthermore, we have recently demonstrated a similar mechanism for the uptake of proline in *Mycobacterium phlei* vesicles (unpublished data) and suggest that sodium may play a more important role in membrane transport in prokaryotes than previously recognized (31).

Note Added in Proof. Since submission of this manuscript we have learned that Dr. Barry P. Rosen and colleagues (personal communication) have clearly demonstrated sodium-stimulated glutamate uptake in *E. coli* vesicles and cells using methods very similar to ours. Also others (32, 33) have since reported the cotransport of Na^+ and methyl 1-thio-β-D-galactopyranoside in *Salmonella typhimurium* and *E. coli* vesicles, respectively.

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