Tokyo, for his encouragement and discussion during this work.

Registry No. Glutamic acid, 56-86-0; sodium, 7440-23-5; hydrogen ion, 12408-02-5.

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Mechanism of Glutamate Transport in *Escherichia coli* B. 2. Kinetics of Glutamate Transport Driven by Artificially Imposed Proton and Sodium Ion Gradients across the Cytoplasmic Membrane†

Tsutomu Fujimura, Ichiro Yamato, and Yasuhiro Anraku*

ABSTRACT: Simultaneous imposition of a pH gradient (ΔpH, interior alkaline) and a sodium gradient (ΔpNa, $[Na^+]_{out} > [Na^+]_{in}$) across cytoplasmic membrane vesicles from *Escherichia coli* B led to a several hundred fold accumulation of glutamate. Although less effective, ΔpH (interior alkaline) alone caused accumulation of glutamate in the presence of Na^+. In addition, ΔpNa ($[Na^+]_{out} > [Na^+]_{in}$) alone also drove the transport system, where the maximum level of glutamate accumulation was affected by the pH of the medium. A membrane potential imposed by valinomycin-induced K^+ diffusion (interior negative) enhanced the accumulation, indicating that the system operates in an electrogenic manner. The Michaelis constant of glutamate transport was greatly affected by changes in the concentrations of both Na^+ and H^+ and could be expressed by a linear combination of the reciprocals of the Na^+ and H^+ concentrations in the medium. On the contrary, a membrane potential (interior negative) exerted its effect by increasing the maximum velocity. When membrane vesicles were loaded with glutamate and Na^+, but not with glutamate alone, rapid efflux of glutamate with Na^+ as the cocation down the concentration gradients took place upon dilution. These results indicate that both Na^+ and H^+ are syn-coupled ions of glutamate transport in *E. coli* B and that the carrier/Na^+/H^+/Glu^- complex observed in the binding reaction is an intermediate in the transport.

In the preceding paper (Fujimura et al., 1983), we investigated the binding of glutamate (Glu)[1] to a glutamate carrier in the membrane of *Escherichia coli* B and proposed a binding model (Figure 1): Glutamate can only bind to an Na^+/H^+/carrier complex that is formed by random-order binding of Na^+ and H^+ to the unloaded carrier. Assuming that the

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[1] Abbreviations: Glu, glutamate; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; K_d, dissociation constant; K_t, Michaelis constant of transport; V_{max}, maximum velocity of transport; $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; ΔpH, pH gradient; $\Delta\psi$, membrane potential; ΔpNa, chemical gradient of Na^+.

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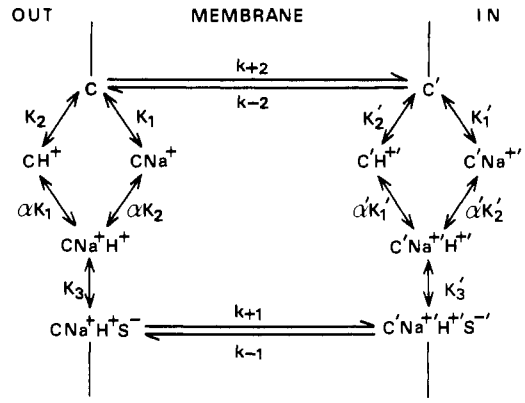

FIGURE 1: Model for glutamate binding and transport in *E. coli* B. This model postulates that binding of glutamate to the carrier occurs symmetrically at the external and internal surfaces of the membrane. K_1, K_2, and K_3 and K_1', K_2', and K_3' are dissociation constants at the external and internal surfaces of the membrane, respectively. k_{+1}, k_{-1} and k_{+2}, k_{-2} are the respective permeability coefficients in the forward and reverse directions of the $CNa^+H^+S^-$ complex and of the unloaded carrier. K_a, an equilibrium constant for the interconversion of the internal and external pools of the unloaded carrier, is defined as k_{+2}/k_{-2}.

$Na^+/H^+/Glu^-$/carrier complex is an intermediate of the syn-coupled transport reaction, here we present a model for active glutamate transport (Figure 1): Glutamate binds to the Na^+/H^+/carrier complex at the external surface of the membrane and then is translocated across the membrane. Glu^-, Na^+, and H^+ dissociate at the internal surface by reverse of the forward reactions, and the resulting unloaded carrier then becomes reorientated to the external surface. From this transport model, the following items are predicted (Fujimura et al., 1983): (1) Both Na^+ and H^+ are coupling ions of glutamate transport. In other words, glutamate transport can be driven by electrochemical gradients of Na^+ and H^+, and the fluxes of both Na^+ and H^+ are syn coupled with glutamate translocation. (2) Glutamate transport may result in transfer of a net positive charge. Therefore, a membrane potential (interior negative) is expected to enhance the reaction. (3) The maximum velocity of transport may not be affected by the concentrations of Na^+ and H^+ in the medium, whereas the Michaelis constant may be a function of the concentrations of both Na^+ and H^+ of the medium, like the dissociation constant of glutamate binding, which has been expressed in terms of a linear combination of the reciprocals of the Na^+ and H^+ concentrations (Fujimura et al., 1983).

This paper reports evidence that these predictions are valid and that the model proposed can explain the mechanism of syn-coupled glutamate transport with Na^+ and H^+ as cosubstrates and coupling energy.

Experimental Procedures

Bacteria and Membranes. The bacteria and membranes used were described in the preceding paper (Fujimura et al., 1983).

Assays of Glutamate Uptake. The procedure used for loading cytoplasmic membrane vesicles with a buffer of a desired pH and ionic composition was essentially the same as that described by Hasan & Tsuchiya (1977): Loading was done by dilution of membrane vesicles 20-fold or more with buffer of the pH and ionic composition indicated. After incubation for 30 min at 25 °C, the vesicles were precipitated by centrifugation (200000*g*, 20 min) and washed once with the same buffer. The vesicles were finally suspended in the same buffer (15–20 mg of protein/mL) and used immediately for transport assays.

The transport assay was started by dilution of the loaded vesicles 100-fold or more with assay medium containing buffer of the indicated pH and concentrations of NaCl and KCl, with 10 mM $MgSO_4$ and 1 μM [^{14}C]glutamic acid. For determination of kinetic constants, 0.1–20 μM glutamic acid was used. Other additions, the concentration of buffer, and the ionic composition are indicated in the text. A ΔpH or ΔpNa across the membrane was created by dilution of vesicles loaded with a lower concentration of H^+ or Na^+ with medium of a higher concentration of H^+ or Na^+. Mes adjusted with Tris (Mes–Tris) was used as buffer between pH 5.5 and 7.0, and Tris adjusted with Mes (Tris–Mes) was used between pH 7.5 and 9.0.

A membrane potential was imposed by valinomycin-mediated K^+ efflux. The transport reaction was carried out at 25 °C. After an appropriate incubation time, an aliquot (100 or 200 μL) was filtered on a nitrocellulose filter (0.45 μm) and washed twice with 5 mL of LiCl solution (0.1–0.3 M), the concentration of which was changed depending on the intravesicular cation concentration. The filter was transferred to a vial containing scintillator (ACS II, Amersham), and its radioactivity was counted.

Assay of Effluxes of Glutamate and Na^+. For experiments on glutamate efflux, membrane vesicles (20–25 mg of protein/mL) were loaded with 10 mM Tris–Mes (pH 8.0) and 50 mM KCl as described above. A small volume of a solution of [^{3}H]glutamate (20 mCi/mmol, adjusted to pH 8.0 with KOH) and NaCl, or KCl as a control, was added to the loaded vesicles to give final concentrations of 10 mM glutamate and salt, respectively, and the mixture was incubated at 25 °C for 1 h. Efflux was started by dilution of the loaded vesicles (5 μL) with 900 μL of assay mixture containing 10 mM Tris–Mes (pH 8.0), 50 mM KCl, 10 mM $MgSO_4$, and 10 mM NaCl at 25 °C. In control experiments, NaCl was replaced by KCl. At the indicated time, an aliquot (100 μL) of the mixture was filtered, and its radioactivity was counted. For experiments on Na^+ efflux, final concentrations of 10 mM ^{22}NaCl (10 mCi/mmol) and 10 mM glutamate (adjusted to pH 8.0 with KOH) were added to vesicles that had been loaded with 10 mM Tris–Mes (pH 8.0) and 50 mM LiCl. After incubation for 1 h at 25 °C, efflux was started by dilution of the vesicles (5 μL) with 900 μL of assay mixture containing 10 mM Tris–Mes (pH 8.0), 50 mM LiCl, 10 mM $MgSO_4$, and 10 mM glutamate (pH 8.0), and the rate of efflux was determined as described above. As a control, 20 mM KCl or 10 mM proline plus 10 mM KCl was used in place of 10 mM glutamate. These two control experiments gave the same result. Zero-time values were determined by dilution of the loaded vesicles (2 μL) directly with 0.1 M LiCl, followed promptly by filtration and two washings with 5 mL of 0.1 M LiCl. Values of 22 ± 2.2 nmol of glutamate and NaCl/mg of protein were obtained in separate experiments by using [^{3}H]glutamate-loaded and ^{22}NaCl-loaded vesicles.

Chemicals. L-[U-^{14}C]Glutamic acid and L-[^{3}H]glutamic acid were from the Radiochemical Centre, Amersham, and ^{22}NaCl was from New England Nuclear. Other chemicals were standard commercial products of analytical grade and are described in the preceding paper (Fujimura et al., 1983).

Results

Glutamate Transport Driven by ΔpH and ΔpNa. Cytoplasmic membrane vesicles prepared from *E. coli* B accumulated glutamate on simultaneous imposition of ΔpH and ΔpNa (interior alkaline and $[Na^+]_{out} > [Na^+]_{in}$) (Figure 2). The maximal concentration of glutamate in the vesicles was calculated as 200-fold that in the medium when the internal water

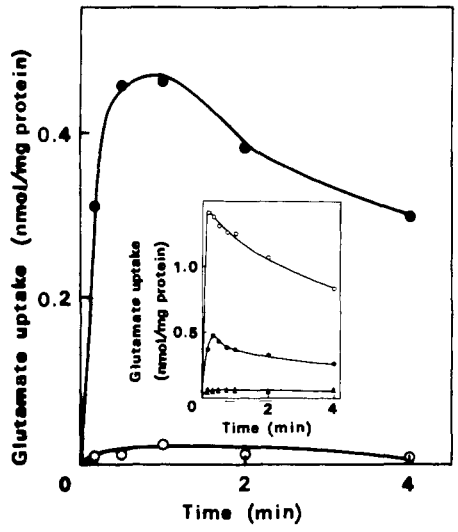

FIGURE 2: Glutamate uptake by membrane vesicles of *E. coli* B (●) or its derivative 36-39 (○) driven by simultaneous imposition of ΔpH and ΔpNa. Vesicles loaded with 50 mM Tris–Mes (pH 9.0) and 50 mM KCl were diluted with assay medium containing 50 mM Mes–Tris (pH 5.75) and 50 mM NaCl. Inset: Effects of uncoupler and ionophores on glutamate uptake. Membrane vesicles of *E. coli* B loaded with 50 mM Tris–Mes (pH 9.0) and 50 mM KCl were diluted with assay medium containing 50 mM Mes–Tris (pH 5.75), 50 mM NaCl, and 1% ethanol. The following indicated inhibitors were added in ethanolic solution: none (●); 5 μM valinomycin (○); 2.5 μM SF6847 (▲); 5 μM monensin (△).

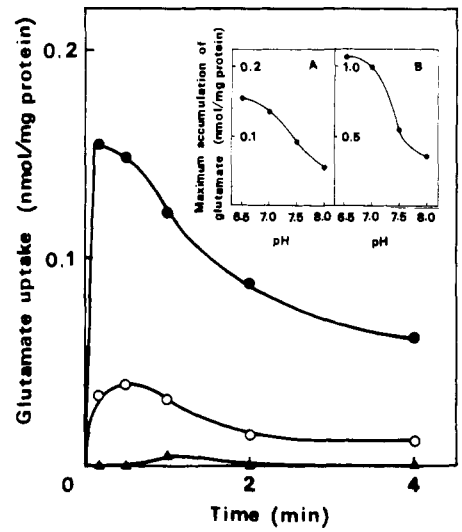

FIGURE 3: Glutamate uptake driven by ΔpNa. Membrane vesicles of *E. coli* B loaded with 50 mM Mes–Tris (pH 6.5) and 250 mM KCl were diluted with assay medium containing 50 mM Mes–Tris (pH 6.5), 250 mM NaCl, and 1% ethanol. Additions: none (●); 5 μM monensin (○); none but NaCl was replaced by KCl (▲). Inset: Effect of pH on the maximum level of glutamate accumulation driven by ΔpNa (A) or ΔpNa plus Δψ (B). Membrane vesicles of *E. coli* B loaded with 250 mM KCl and 50 mM buffer at given pH values were diluted with assay medium containing 50 mM buffer of the same pH, 250 mM NaCl, and 1% ethanol. A membrane potential was created with 5 μM valinomycin.

space of the membrane vesicles (T. Mogi and Y. Anraku, unpublished results) was assumed to be 2.2 μL/mg of protein. On the contrary, vesicles prepared from the mutant strain 36-39, which has no Na^+-dependent transport system for glutamate (Tsuchiya et al., 1977), did not show transport activity of glutamate on artificial imposition of ΔpH plus ΔpNa. The accumulation of glutamate was completely inhibited by addition of the potent uncoupler SF6847 or the sodium ionophore monensin to the assay medium (Figure 2, inset). The effect of concentration gradients of various monovalent cations on glutamate transport in the presence of ΔpH was examined. Vesicles loaded with 50 mM choline chloride and 100 mM Tris–Mes buffer (pH 9.0) were diluted with assay medium containing 50 mM salt in the chloride form and 100 mM Mes–Tris buffer (pH 5.75). Only Na^+ could drive the glutamate transport: Li^+, K^+, and Rb^+ could not replace Na^+ at all. This cation specificity is consistent with that of the glutamate transport in whole cells (unpublished results) and of glutamate binding to a glutamate carrier (Fujimura et al., 1983). These results indicate that the accumulation of glutamate is mediated via the glutamate carrier and suggest that both Na^+ and H^+ participate in glutamate transport as syn-coupling ions.

Effect of a Membrane Potential on Transport. A membrane potential was created by valinomycin-induced K^+ efflux from the vesicles. The membrane potential enhanced glutamate accumulation (Figure 2, inset), and the maximum glutamate concentration in the vesicles reached 700-fold that of the medium, indicating that the transport system operates in an electrogenic manner. Because glutamate predominantly exists in a negatively charged form under the experimental conditions, this result can be explained by assuming that both Na^+ and H^+ are simultaneously transferred along with Glu^- via the glutamate carrier.

Glutamate Transport Driven by either ΔpH or ΔpNa. The data obtained above suggest that both Na^+ and H^+ are syn-coupling ions of glutamate transport. Therefore, it seemed interesting to examine whether ΔpH or ΔpNa alone could drive the system. When vesicles loaded with 250 mM KCl and a buffer of pH 6.5 were diluted with assay medium (pH 6.5) containing 250 mM NaCl (thus creating ΔpNa), glutamate accumulation was observed, as shown in Figure 3. Monensin inhibited the accumulation. Glutamate accumulation driven by ΔpNa alone also exhibited H^+ dependence (Figure 3, inset A). Inset B of Figure 3 shows the stimulatory effect of a membrane potential on glutamate accumulation driven by ΔpNa. This accumulation was also affected by the pH of the assay medium. Thus, this H^+ dependence can be explained by assuming that H^+ is a cosubstrate of a ΔpNa-driven glutamate transport.

The ΔpH alone also drove glutamate transport. When vesicles loaded with 0.15 M Tris–Mes (pH 9.0) and 25 mM NaCl were diluted with assay medium containing 0.15 M Mes–Tris (pH 5.5) and 25 mM NaCl (thus imposing ΔpH), they accumulated glutamate (Figure 4). This accumulation was inhibited by SF6847. Omission of Na^+ from the assay medium also prevented the accumulation, indicating that uptake was mediated via the glutamate carrier. These results indicate that both Na^+ and H^+ are syn-coupling ions of glutamate transport.

Effects of Na^+ and H^+ on the Kinetic Constants of Transport. For determination of the exact effects of Na^+ and H^+ on the rate of transport, glutamate transport was driven by an artificially imposed ΔpH and ΔpNa (interior alkaline, and $[Na^+]_{out} > [Na^+]_{in}$), not by respiratory substrates. Figure 5A shows the effect of the Na^+ concentration of the medium. The K_t values obtained from the intercepts with the x axes showed a linear relation with the reciprocals of the Na^+ concentration (Figure 5A, inset). On the other hand, the maximum velocity of transport [V_{max} = 12 ± 1 nmol min^{-1} (mg of protein)$^{-1}$] was not affected so much by the Na^+ concentration. The effect of H^+ concentration on the transport is shown in Figure 5B. The K_t values were also linearly related to the reciprocals of the H^+ concentration of the medium (Figure 5B, inset), where the same V_{max} value of 12 ± 1 nmol

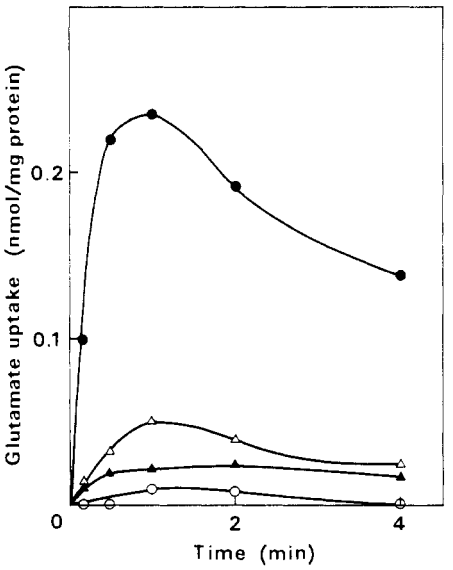

FIGURE 4: Glutamate uptake driven by ΔpH. Membrane vesicles of *E. coli* B loaded with 0.15 M Tris–Mes (pH 9.0), 25 mM NaCl, and 25 mM KCl were diluted with assay medium containing 0.15 M Mes–Tris (pH 5.5), 25 mM NaCl, 25 mM KCl, and 0.5% ethanol. Additions: none (●); 2.5 μM SF6847 (▲); none but NaCl in the assay medium was replaced by KCl (omission of NaCl) (△); none but Mes–Tris (pH 9.0) (no driving force) (○).

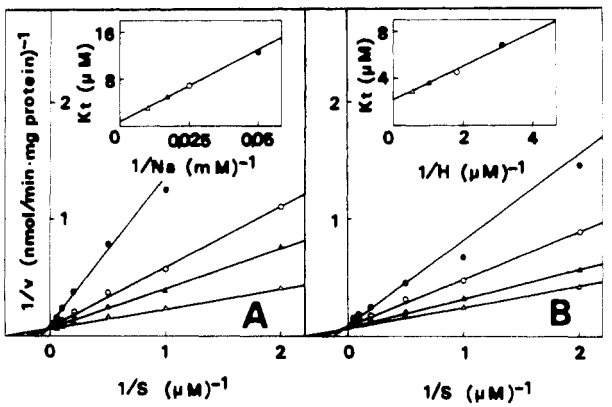

FIGURE 5: Effects of Na^+ and H^+ concentrations on double-reciprocal plots of glutamate uptake driven by ΔpH plus ΔpNa. (A) Membrane vesicles of *E. coli* B loaded with 50 mM Tris–Mes (pH 9.0) and 100 mM KCl were diluted with assay medium containing 50 mM Mes–Tris (pH 5.75) and a mixture of NaCl and KCl (the final concentration of salt was adjusted to 100 mM): 100 mM NaCl (△); 60 mM NaCl (▲); 40 mM NaCl (○); 20 mM NaCl (●). The K_t values obtained were plotted against the reciprocals of Na^+ concentrations in the inset. (B) Membrane vesicles of *E. coli* B loaded with 50 mM Tris–Mes (pH 9.0) and 100 mM KCl were diluted with assay medium containing 50 mM Mes–Tris at the pH indicated and 100 mM NaCl: pH 5.75 (△); pH 6.0 (▲); pH 6.25 (○); pH 6.5 (●). The K_t values obtained were plotted against the reciprocals of the H^+ concentrations in the inset.

min^{-1} (mg of protein)$^{-1}$ was obtained in medium of pH 5.75–6.5. These results will be discussed in consideration of a model for syn-coupled transport.

The effect of a membrane potential (interior negative) on the kinetic parameters was also examined. A membrane potential specifically increased the V_{max} value [10 and 30 nmol min^{-1} (mg of protein)$^{-1}$ in the absence and presence of a membrane potential, respectively] but did not affect the K_t value (4.7 μM). These results indicate that the affinity of the glutamate transport system was controlled by the concentrations of both Na^+ and H^+ in the medium.

Syn Coupling of Glutamate and Na^+ Effluxes via the Glutamate Carrier. The data so far obtained strongly suggest

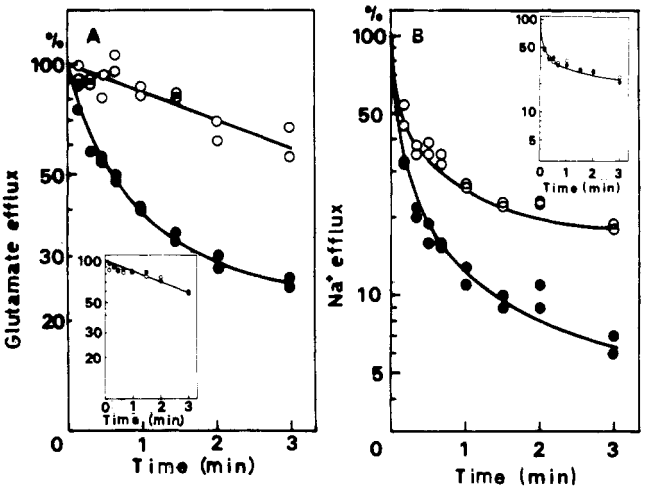

FIGURE 6: (A) Effect of Na^+ on glutamate efflux from membrane vesicles of *E. coli* B or of its derivative 36-39 (inset). Vesicles loaded with 10 mM [^{3}H]glutamate and 10 mM NaCl (●) were diluted with assay medium (180-fold dilution) containing 10 mM NaCl. As a control, vesicles loaded with [^{3}H]glutamate and 10 mM KCl were diluted with assay medium containing K^+ in place of Na^+ (○). Data are expressed as percentages of glutamate retained by using zero-time values for normalization. See text for details. (B) Effect of glutamate on Na^+ efflux from membrane vesicles of *E. coli* B or of its derivative 36-39 (inset). Vesicles loaded with 10 mM ^{22}NaCl and 10 mM glutamate (●) were diluted with assay medium (180-fold dilution) containing 10 mM glutamate. As a control, vesicles loaded with 10 mM ^{22}NaCl and 10 mM proline were diluted with assay medium containing proline in place of glutamate (○). Data are expressed as percentages of Na^+ retained by using zero-time values for normalization.

that both Na^+ and H^+ are syn-coupling ions of glutamate transport. This was confirmed by measuring the syn-coupled efflux of glutamate with Na^+ via the glutamate carrier. When vesicles of strain B loaded with [^{3}H]glutamate were diluted 180-fold with medium without added glutamate, glutamate efflux was stimulated by Na^+ loaded in the vesicles (Figure 6A). On the other hand, when vesicles of strain 36-39 were used, Na^+ did not stimulate glutamate efflux (Figure 6A, inset). The rate of glutamate efflux from vesicles of strain 36-39 and from vesicles of strain B in the absence of Na^+ showed a first-order reaction, and 60% of the loaded glutamate was retained by these vesicles after incubation for 3 min. These results indicate that the Na^+-stimulated efflux of glutamate from vesicles of strain B was mediated via the glutamate carrier and that glutamate efflux from vesicles of strain B in the absence of Na^+ was due to nonspecific simple diffusion.

Next, Na^+ efflux with glutamate from the vesicles was measured by using ^{22}NaCl. *E. coli* cells possess an Na^+/H^+ antiporter (West & Mitchell, 1974), and this system can use Li^+ as substrate as well as Na^+ (Schuldiner & Fishkes, 1978; Beck & Rosen, 1979). Since Li^+ did not replace Na^+ in the glutamate/Na^+ symport (see text) or inhibited it (unpublished results), efflux experiments were performed under conditions with excess Li^+. Although nonspecific efflux of Na^+ from the vesicles was more rapid than that of glutamte, stimulation of Na^+ efflux by glutamate was observed (Figure 6B). When vesicles of strain 36-39 were used, the Na^+ efflux was not stimulated by glutamate (Figure 6B, inset), and the pattern of efflux was the same as that obtained when vesicles of strain B in the absence of glutamate were used. These results indicate that stimulation of Na^+ efflux by glutamate loaded in the vesicles was mediated via the glutamate carrier. Therefore, we concluded that the fluxes of Na^+ and glutamate are both tightly coupled via the function of the glutamate carrier.

The stoichiometry of the initial rates of efflux of Na^+ and

glutamate via the glutamate carrier was examined. Glutamate efflux stimulated by Na^+ via the carrier in the initial 10 s of incubation can be calculated by subtracting the amount of glutamate retained in the presence of Na^+ from that in the absence of Na^+. The Na^+ efflux stimulated by glutamate via the carrier can be obtained in a similar way. The results indicate that the amounts of syn-coupled glutamate and Na^+ transport via the glutamate carrier show a stoichiometry of 1:1.

We also attempted to observe coupling of the fluxes of glutamate and H^+ via the glutamate carrier in intact cells, as demonstrated in the lactose transport system (West, 1970; West & Mitchell, 1973). In the presence of 10 mM potassium arsenite and 10 mM NH_4Cl to prevent glutamate metabolism (Frank & Hopkins, 1969), the pH change of the medium due to concomitant movement of H^+ with glutamate was monitored with a pH electrode under anaerobic conditions. We could measure lactose-induced H^+ uptake by strain ML308-225 cells as a control under the same conditions (not shown) but not glutamate-induced H^+ uptake by strain B in the presence of Na^+.

Discussion

From quantitative analyses of glutamate binding to its carrier (Fujimura et al., 1983), we postulated that the mechanism of glutamate/Na^+/H^+ symport was formation of an $Na^+/H^+/Glu^-$/carrier complex on the external surface of the membrane, translocation of the complex across the membrane, dissociation of the complex at the internal surface, and relocation of the unloaded carrier, as shown in Figure 1. In this paper, we examined the kinetics of the overall transport reaction of glutamate in cytoplasmic membrane vesicles with special consideration of the effects of ΔpH and ΔpNa on syn-coupled transport and accumulation of ligands.

We found that when ΔpH and ΔpNa (interior alkaline, and $[Na^+]_{out} > [Na^+]_{in}$) were simultaneously provided as a driving force, membrane vesicles prepared from strain B accumulated glutamate at 200 times higher concentration than in the medium (Figure 2). Moreover, ΔpNa or ΔpH alone, as an energy source, could drive the system (Figures 3 and 4) in the presence of the other ion. These findings strongly suggest that Na^+ and H^+ are both cosubstrates and coupling ions for glutamate transport.

Direct evidence for syn coupling of Na^+ flux to glutamate transport was obtained in efflux experiments (Figure 6). The Na^+ efflux from the vesicles via the glutamate carrier was specifically stimulated by the presence of glutamate, and vice versa, with a stoichiometry of Na^+ to glutamate of 1 to 1. These observations support the model presented in Figure 1. However, we could not measure the coupling of H^+ movement to glutamate influx with a pH electrode, although concomitant uptake of H^+ induced by lactose influx was detected.

Two reasons for this are conceivable: One is because the maximum velocities of glutamate and lactose transport are different. The V_{max} value of glutamate transport by cells of strain B was only 8 nmol min^{-1} (mg of protein)$^{-1}$ at pH 7.0 with potassium succinate as the energy source (unpublished results), and this value is one-twentieth of that of lactose transport by cells of strain ML308-225 (Winkler & Wilson, 1966). Second, metabolism of glutamate within the cell may interfere with the pH change monitored with a pH electrode. We have noticed that when lactose was added to cells of strain B grown on lactose, no alkalinization but rapid acidification of the assay medium took place, suggesting that lactose metabolism is initiated actively by β-galactosidase induced under the conditions employed. Therefore, release of acid(s) resulting from glutamate metabolism in the cells might counteract the effect of H^+ movement concomitant with glutamate influx, even in the presence of potassium arsenite. This problem needs further study with use of a proper strain and suitable experimental conditions.

Thus, evidence that a proton is cotransported with glutamate and Na^+ is still indirect and is supported only by observations that (1) glutamate transport was driven by ΔpH in the presence of Na^+ (Figure 4) and (2) a ΔpNa-driven glutamate transport showed marked pH dependence (Figure 3, inset). However, theoretical analysis of glutamate transport based on the assumption that both Na^+ and H^+ are syn-coupling ions as shown in Figure 1 could best explain an empirical equation representing the K_t for glutamate transport as discussed below. This finding is consistent with an idea that a proton is cotransported with glutamate and Na^+ via a glutamate carrier.

With regard to the role of a proton in the process of glutamate/Na^+/H^+ symport, another possibility is that the ΔpH is converted into an electrochemical gradient of Na^+ via an Na^+/H^+ antiporter, which in turn drives the transport via Na^+/glutamate syn coupling. However, this possibility seems unlikely for three reasons: (1) Glutamate carrier absolutely requires H^+ to bind glutamate (Fujimura et al., 1983). (2) Although effluxes of Na^+ and glutamate via the glutamate carrier showed a 1:1 stoichiometry, a membrane potential enhanced glutamate accumulation (see text). Since glutamate exists predominantly in the negatively charged form under the experimental conditions used and the glutamate carrier binds the glutamate anion, the stimulatory effect of $\Delta\psi$ indicates the participation of another coupling cation in addition to Na^+ in glutamate transport. (3) When the system was driven by ΔpNa or ΔpNa plus $\Delta\psi$, the steady-state level of accumulation was affected by the H^+ concentration of the medium (Figure 3, inset). Since the energy provided was the same in each set of experiments, this H^+ dependence cannot simply be explained by the existence of an Na^+/H^+ antiporter as an energy converter but rather suggests the direct interaction of H^+ with the carrier, possibly as one of its substrates. Therefore, it is most conceivable that both H^+ and Na^+ are syn-coupling ions for glutamate transport.

Theoretical considerations of the thermodynamics of active transport have been attempted in model systems (Kotyk, 1973; Delaage, 1975; Lagarde, 1976). Here, we will present some quantitative considerations on the effects of syn-coupling ions and membrane potential on glutamate transport. If Goldman's constant field assumption (Goldman, 1943) is employed, i.e., $d\psi/dx = -\Delta\psi/(\Delta x)$, and if an unloaded carrier is electrically neutral, the fluxes of the unloaded carrier and the $CNa^+H^+S^-$ complex, J_c and J_s, respectively, can be expressed as follows:

$$J_c = k_{+2}[C] - k_{-2}[C'] \quad (1)$$

$$J_s = a\{k_{+1}[CNa^+H^+S^-]e^{F\Delta\psi/(RT)} - k_{-1}[C'Na^{+\prime}H^{+\prime}S^{-\prime}]\} \quad (2)$$

where

$$a = \frac{F\Delta\psi}{(e^{F\Delta\psi/(RT)} - 1)RT}$$

k_{+2}, k_{-2} and k_{+1}, k_{-1} are permeability coefficients in the forward and reverse directions of the unloaded carrier and the $CNa^+H^+S^-$ complex, respectively. F and R are the Faraday constant and the gas constant, respectively. When the steady state is attained, $J_c + J_s = 0$. Therefore

$$k_{+2}[C] + ak_{+1}[CNa^+H^+S^-]e^{F\Delta\psi/(RT)} = k_{-2}[C'] + ak_{-1}[C'Na^{+\prime}H^{+\prime}S^{-\prime}] \equiv X \quad (3)$$

If it is assumed that translocation of the unloaded carrier or $CNa^+H^+S^-$ complex is rate limiting and that the binding reaction is in local equilibrium

$$[CNa^+H^+S^-] = \frac{[C][H^+][Na^+][S^-]}{\alpha K_1 K_2 K_3} \quad (4)$$

From eq 3 and 4, we get

$$[C] = \frac{\alpha K_1 K_2 K_3 X}{\Delta} \quad (5)$$

where $\Delta = k_{+2}\alpha K_1 K_2 K_3 + ak_{+1}[H^+][Na^+][S^-]e^{F\Delta\psi/(RT)}$. Therefore

$$[CNa^+H^+S^-] = \frac{X[Na^+][H^+][S^-]}{\Delta} \quad (6)$$

Similarly

$$[C'] = \frac{\alpha' K_1' K_2' K_3' X}{\Delta'} \quad (7)$$

$$\Delta' = k_{-2}\alpha' K_1' K_2' K_3' + ak_{-1}[H^{+\prime}][Na^{+\prime}][S^{-\prime}] \quad (8)$$

$$[C'Na^{+\prime}H^{+\prime}S^{-\prime}] = \frac{X[Na^{+\prime}][H^{+\prime}][S^{-\prime}]}{\Delta'} \quad (9)$$

Inserting eq 6 and 9 into eq 2, we obtain

$$J_s = \frac{aX}{\Delta\Delta'}\{k_{+1}k_{-2}\alpha' K_1' K_2' K_3'[Na^+][H^+][S^-]e^{F\Delta\psi/(RT)} - k_{-1}k_{+2}\alpha K_1 K_2 K_3[Na^{+\prime}][H^{+\prime}][S^{-\prime}]\} \quad (10)$$

We can get the initial velocity (v) from eq 10 by assuming that the internal concentration of glutamate is negligibly low:

$$v = \frac{ae^{F\Delta\psi/(RT)}k_{+1}X[Na^+][H^+][S^-]}{\Delta} \quad (11)$$

When we assume as we did in the preceding paper (Fujimura et al., 1983) that the concentration of the unloaded carrier is low (this assumption implies that translocation of the unloaded carrier is not rate limiting) and that the dissociation constant K_3 is negligibly small, and when we can further assume that the internal concentrations of Na^+ and H^+ are negligibly low, X is related to the total amount of the carrier (C_t) as follows:

$$X = \frac{\Delta C_t}{\alpha K_2 K_3[Na^+] + \alpha K_1 K_3[H^+] + [H^+][Na^+][S^-]} \quad (12)$$

By inserting eq 12 into eq 11, we obtained eq 13.

$$v = \frac{\frac{F\Delta\psi e^{F\Delta\psi/(RT)}}{(e^{F\Delta\psi/(RT)} - 1)RT}k_{+1}C_t[S^-]}{\alpha K_2 K_3/[H^+] + \alpha K_1 K_3/[Na^+] + [S^-]} \quad (13)$$

When $\Delta\psi$ is zero, eq 13 can be simplified to eq 14.

$$v = \frac{k_{+1}C_t[S^-]}{\alpha K_2 K_3/[H^+] + \alpha K_1 K_3/[Na^+] + [S^-]} \quad (14)$$

Equation 13 explains the results of kinetic studies on glutamate transport.

First, the Michaelis constant K_t ($\alpha K_2 K_3/[H^+] + \alpha K_1 K_3/[Na^+]$) is expressed by a linear combination of the reciprocals of the external concentrations of Na^+ and H^+. In fact, from eq 13 and the insets of panels A and B of Figure 5, we could obtain an empirical equation representing the K_t for glutamate transport as follows:

$$K_t = \frac{1.5}{[H^+]} + \frac{220}{[Na^+]}$$

where the dimensions of molarity for K_t, H^+, and Na^+ are micromolar, micromolar, and millimolar, respectively. In the preceding paper (Fujimura et al., 1983), we showed a similar dependence of K_d on Na^+ and H^+ concentrations, i.e., $K_d = 0.32/[H^+] + 40/[Na^+]$.

Theoretically, the K_t value should be close to the K_d value, but the K_t value obtained is about 5 times larger than the K_d value. We think that this inconsistency is due partly to leakiness of the membrane vesicles and partly to the restrictive assumptions that we used in estimating the amount of unloaded carrier and the internal concentrations of H^+ and Na^+. In order to resolve this discrepancy, we may need to study the glutamate transport carrier in a purer reconstitution system showing tighter coupling to substrate ligands than that of the membrane vesicle system conventionally used. Second, the external concentrations of Na^+ and H^+ do not affect the maximum velocity. Third, imposition of a membrane potential (interior negative) specifically enhances the maximal velocity without affecting the Michaelis constant, indicating that the carrier does not undergo a conformational change, which results in an increase in affinity for glutamate, on imposition of $\Delta\psi$.

Our model of glutamate transport (Figure 1) postulates implicitly the tight coupling of glutamate transport with syn fluxes of Na^+ and H^+ via the glutamate carrier. The stoichiometric association of glutamate efflux with Na^+ efflux is positive evidence for this model. The maximum concentration of glutamate within the vesicles was only 200 times that in the medium (Figure 2) when concentration differences of Na^+ and H^+ of about 1000-fold or more were initially imposed on the membrane vesicles. This low level of accumulation indicates leakiness of the vesicles for Na^+ (Figure 6B) and H^+,[2] and this leakiness imposes an unavoidable limitation on studies of the regulatory mechanism of the carrier for the syn-coupled transport reaction.

Lagarde (1976) analyzed active transport with respect to nonequilibrium thermodynamics. It has been generally shown that the degree of coupling between the fluxes of a substrate and its coupling ion mediated by the same carrier is completely governed by the dissociation constants and external concentrations of the species. Thus, when the external concentration of the substrate becomes very large at a fixed external concentration of the coupling ion, a short circuit of substrate flux by the carrier can be expected. Lagarde stated that this self-uncoupling property of the carrier provides the cell with the ability to save energy. However, when it is considered that an intrinsic property of a carrier is bidirectional translocation of substrate, this short circuit may cause leakage of the amino acid in question, since it is unusually present at high concentrations (in the order of millimolar or more) in the cell. Therefore, the self-uncoupling is rather disadvantageous to the cell.

In studies on the glutamate transport system discussed above and on the lactose transport system (West & Mitchell, 1973; Booth et al., 1979; Zilberstein et al., 1979), syn coupling of the fluxes of the substrate and its cosubstrate ion has been demonstrated. Therefore, these results led us to consider that the tight coupling of the fluxes may be a characteristic of glutamate/Na^+/H^+ symport and lactose/H^+ symport, as typical secondary chemiosmotic pumps. Carrier molecules of

[2] Cytoplasmic membrane vesicles were extensively washed with a solution of 3 mM EDTA–10% (w/w) sucrose, pH 7.2. This treatment causes partial release of the F_1 proteins of H^+-ATPase from the membranes (Yamato et al., 1975; Bragg & Hou, 1972). Therefore, the vesicles should be more permeable to protons than native membranes.

this kind may undergo some conformational change(s) to prevent a short circuit.

Acknowledgments

We are most grateful to Professor K. Aida, University of Tokyo, for his encouragement and discussion during this work.

Registry No. Glutamic acid, 56-86-0; sodium, 7440-23-5; hydrogen ion, 12408-02-5.

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Modulation of Membrane Transport by Free Fatty Acids: Inhibition of Synaptosomal Sodium-Dependent Amino Acid Uptake†

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ABSTRACT: High-affinity, Na^+-dependent synaptosomal amino acid uptake systems are strongly stimulated by proteins which are known to bind free fatty acids. The rate of uptake as well as the overall level of accumulation is increased by such proteins as bovine serum albumin, hepatic fatty acid binding protein, β-lactoglobulin, and fetuin. Such a stimulation is not observed with proteins which do not bind fatty acids. The transport activity of synaptosomal preparations can be directly correlated with the free fatty acid content of the preparation. Thus, incubation with albumin reduces the free fatty acid content of synaptosomal preparations, suggesting that the stimulatory effects of the proteins are related to their removal of inhibitory fatty acids formed by hydrolysis of membrane lipids during incubation. Inhibition of amino acid uptake is seen with most cis-unsaturated long chain fatty acids while saturated and trans-unsaturated fatty acids have relatively little or no effect. Under conditions in which the ionophore gramicidin D causes an increase of ^{22}Na flux into synaptosomes, oleic acid (50 μM) has no effect on the influx. These data are consistent with the hypothesis proposed earlier by us [Rhoads, D. E., Peterson, N. A., & Raghupathy, E. (1982) *Biochemistry 21*, 4782] that Na^+-dependent amino acid transport carrier proteins reside in a relatively fluid lipid domain in the synaptosomal membrane and that the effects of cis-unsaturated fatty acids are mediated by interactions with such domains.

Isolated nerve ending particles (synaptosomes) contain high-affinity, Na^+-dependent amino acid uptake systems which are generally considered to be specific for neurotransmitter amino acids (Peterson & Raghupathy, 1972; Bennett et al., 1973; Snyder et al., 1973). It has been demonstrated that such Na^+-dependent uptake systems are sensitive to the depolarizing agent veratridine (Rhoads et al., 1982b) and thus appear to be linked energetically to sodium ion gradients, a conclusion supported by recent studies on amino acid uptake into plasma membrane vesicles prepared from synaptosomes (Kanner, 1978; Kanner & Sharon, 1978; Mayor et al., 1981; Rhoads et al., 1982c). It was also shown that these Na^+-dependent, veratridine-sensitive transport systems are strongly stimulated by bovine serum albumin (Peterson et al., 1979; Rhoads et al., 1982b). In the case of the transport of one of these amino acids, viz., proline, the portion of the albumin molecule that is responsible for the stimulation was shown to be the region comprising amino acid residues 377–504 (Raghupathy et al., 1978). The same sequence has been previously implicated in the binding of long-chain fatty acids by albumin (Reed et al., 1975). This suggested that the stimulatory effect of serum albumin was related to its ability to bind free (unesterified) fatty acids. Na^+-dependent, synaptosomal amino acid uptake systems were subsequently shown to be specifically inhibited by low concentrations of unsaturated free fatty acids (Rhoads et al., 1982d). Saturated fatty acids had negligible effect on the Na^+-dependent transport systems for proline, glutamic acid, and γ-aminobutyric acid (GABA),[1] while neither satu-

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0006-2960/83/0422-1965$01.50/0