Leonard, W. J., Depper, J. M., Kanehisa, M., Kronke, M., Peffer, N. J., Svetlik, P. B., Sullivan, M., & Greene, W. C. (1985) *Science (Washington, D.C.)* 230, 633–639.
Lerman, M. I., Thayer, R. E., & Singer, M. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3966–3970.
Lorand, L., Losowsky, M. S., & Miloszewski, K. J. M. (1980) *Prog. Hemostasis Thromb. 5*, 245–290.
Lozier, J., Takahashi, N., & Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 3640–3644.
Lurquin, P. F. (1988) *BioTechniques 6*, 942–944.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Morton, N. E., & Burns, G. A. (1987) *Cytogenet. Cell Genet. 46*, 102–130.
Mount, S. M. (1982) *Nucleic Acids Res. 10*, 459–472.
Nussinov, R. (1981) *J. Mol. Biol. 149*, 125–131.
Patthy, L. (1985) *Cell (Cambridge, Mass.) 41*, 657–663.
Perlman, P. S., & Butow, R. A. (1989) *Science (Washington, D.C.) 246*, 1106–1107.
Rodeghiero, F., Morbin, M., & Barbui, T. (1981) *Thromb. Haemostasis 46*, 621–622.
Rodriguez-de-Cordoba, S., Rey-Campos, J., Dykes, D. D., McAlpine, P. J., Wong, P., & Rubinstein, P. (1988) *Immunogenetics 28*, 452–454.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schmid, C. W., & Jelinek, W. R. (1982) *Science (Washington, D.C.) 216*, 1065–1070.
Sharp, P. A. (1981) *Cell (Cambridge, Mass.) 23*, 643–646.
Skowronski, J., Fanning, T. G., & Singer, M. F. (1988) *Mol. Cell. Biol. 8*, 1385–1397.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Sun, L., Paulson, K. E., Schmid, C. W., Kadyk, L., Leinwand, L. (1984) *Nucleic Acids Res. 12*, 2669–2690.
Tabor, S., & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4767–4771.
Takahashi, N., Takahashi, Y., & Putnam, F. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8019–8023.
Thomashow, M. F., Nutter, R., Montoya, A. L., Gordon, M. P., & Nester, E. W. (1980) *Cell (Cambridge, Mass.) 19*, 729–739.
Vik, D. P., Keeney, J. B., Munoz-Canoves, P., Chaplin, D. D., & Tack, B. F. (1988) *J. Biol. Chem. 263*, 16720–16724.
Wang, A. J. J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.
Webb, G. C., Coggan, M., Ichinose, A., & Board, P. G. (1989) *Hum. Genet. 81*, 157–160.
Woo, S. L. C. (1979) *Methods Enzymol. 68*, 389–395.
Yorifuji, H., Anderson, K., Lynch, G. W., Van De Water, L., & McDonagh, J. (1988) *Blood 72*, 1645–1650.
Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W., & Kurachi, K. (1985) *Biochemistry 24*, 3736–3750.

Counterflow of L-Glutamate in Plasma Membrane Vesicles and Reconstituted Preparations from Rat Brain†

Gilia Pines and Baruch I. Kanner*
Department of Biochemistry, Hadassah Medical School, The Hebrew University, P.O. Box 1172, Jerusalem 91010, Israel
Received May 15, 1990; Revised Manuscript Received July 25, 1990

ABSTRACT: Membrane vesicles from rat brain exhibit sodium-dependent uptake of L-[^{3}H]glutamate in the absence of any transmembrane ion gradients. The substrate specificity of the process is identical with (Na^+ + K^+)-coupled L-glutamate accumulation. Although these vesicles are prepared after osmotic shock and are washed repeatedly, they contain about 1.5 nmol/mg of protein endogenous L-glutamate, apparently located inside the vesicles. The affinity of the process ($K_m \approx 1$ μM) is similar to that of (Na^+ + K^+)-dependent accumulation by the L-glutamate transporter. Membrane vesicles have been disrupted by the detergent cholate, and the solubilized proteins have been subsequently reconstituted into liposomes. The reconstituted proteoliposomes also exhibit the above uptake—with the same characteristics—provided they contain entrapped cold L-glutamate. Counterflow is optimal when sodium is present on both sides of the membrane, but partial activity is still observed when sodium is present either on the inside or on the outside. Increasing the L-glutamate concentration above the K_m results in counterflow completely independent of cis sodium. The initial rate of counterflow is 100–200-fold lower than that of net trans potassium dependent flux. The rate of net flux in the presence of trans sodium or lithium is about 10-fold lower than when choline or Tris are used instead. However, the rate of counterflow (no internal potassium present) was not stimulated by replacing internal sodium or lithium by internal choline. Therefore, optimal functioning of the transporter requires internal potassium while internal sodium and lithium are inhibitory. In addition, the membrane vesicles also contain a low-affinity uptake system (K_m about 100 μM) for L-glutamate, which is also dependent on cis sodium and trans potassium. The above data are accommodated in a refined model of the translocation cycle of the (Na^+ + K^+)-coupled L-glutamate transporter.

The reuptake of neurotransmitters from the synaptic cleft by high-affinity transport appears to play an important role in the overall process of synaptic transmission (Iversen, 1975; Kuhar, 1973). The process is catalyzed by sodium-coupled neurotransmitter transport systems [reviewed in Kanner (1983, 1989) and Kanner and Schuldiner (1987)] located in plasma membranes of nerve endings and glial cells. These transport systems have been investigated in detail by using plasma

† This work was supported by Grant NS-16708 from the National Institute of Neurology and Communicative Disorders and Stroke, and by Grant I-02-006.1187 from the German–Israeli Foundation of Scientific Research and Development.

0006-2960/90/0429-11209$02.50/0

membranes obtained upon osmotic shock of synaptosomes. It appears that these transporters are coupled not only to sodium but also to additional ions like potassium or chloride.

L-Glutamate is considered to be the major excitatory transmitter in brain, and aspartate may have a similar action (Fonnum, 1984; Ottersen & Storm-Mathisen, 1984; Roberts et al., 1986). Its reuptake system is held to be the mechanism by which synaptically released excitatory amino acids are inactivated (Johnston, 1981) and kept below toxic levels in the extracellular space (McBean & Roberts, 1985). The system appears to catalyze electrogenic cotransport of sodium and L-glutamate while potassium is translocated in the opposite direction (Kanner & Sharon, 1978a,b; Kanner & Bendahan, 1982; Gordon & Kanner, 1988). Three sodium ions are translocated with one glutamate anion, and one potassium ion is countertransported (Kanner & Sharon, 1978a; Barbour et al., 1988).

Using efflux and exchange studies (Kanner & Marva, 1982; Kanner & Bendahan, 1982), we have shown that the translocation cycle of this transporter can be divided into two distinct parts: (1) translocation of sodium and L-glutamate and (2) reorientation of the binding sites upon binding and translocation of potassium. In those studies the vesicles were actively loaded with L-[^{3}H]glutamate, prior to efflux and/or exchange, by sodium-dependent accumulation of the labeled amino acid into potassium-loaded vesicles. In order to better characterize the L-glutamate translocation step, we have studied here the transporter in the complete absence of potassium. Surprisingly, it appears that potassium is required for the optimal function of the L-glutamate translocation step as well.

Experimental Procedures

Materials

Soybean phospholipids (Sigma P-5638, type II-s, commercial grade) were partially purified by acetone precipitation. Crude bovine brain lipids were extracted with chloroform–methanol (Folch et al., 1957). Cholic acid (Sigma) was recrystallized from 70% ethanol (Kagawa & Racker, 1971) and neutralized with NaOH to pH 7.4. Sephadex G-50 fine was from Pharmacia. L-[^{3}H]Glutamic acid (20–60 Ci/mmol) was obtained from Amersham or from ARC. Valinomycin was from Sigma. Nigericin was from Calbiochem. One-milliliter disposable syringes were from Becton and Dickinson and Co. (Rutherford, NJ). All other reagents were analytical grade.

Methods

Preparation of Crude Synaptic Plasma Membranes. Three week old rats were killed by decapitation, and the brains were rapidly dissected out. The tissue was homogenized with a Dounce glass homogenizer (type A) in about 10 volumes of ice-cold 0.32 M mannitol containing 1 mM EDTA (pH 7.4) and centrifuged (10 min, 1000*g*, 4 °C). The pellets were washed once by centrifugation as above with the mannitol solution. The combined supernatants were centrifuged again (20 min, 27000*g*, 4 °C), and the pellets were suspended in about 20 volumes of hypotonic buffer (1 mM EDTA, 1 mM Tris-HCl, pH 7.4) and centrifuged (20 min, 27000*g*, 4 °C). The pellets were resuspended in 5–10 volumes of "resuspension buffer" (100 mM NaP_i,[1] pH 7.4, 5 mM Tris–SO_4, 1 mM $MgSO_4$, 0.5 mM EDTA, 1% glycerol) and centrifuged (20 min, 27000*g*, 4 °C). The final pellets were resuspended in about 0.3 volume of resuspension buffer (yielding about 15 mg of protein/mL), frozen in aliquots in liquid nitrogen, and stored at −70 °C for up to 14 months without loss of activity.

Solubilization of the L-Glutamate Transporter. Membranes were rapidly thawed at 37 °C in a water bath, and sequentially 1/8 volume of saturated ammonium sulfate (adjusted with NH_4OH so that a 1:20 dilution gave a pH reading of 7.4) and 1/8 volume of 20% cholate were added. The mixture was incubated 10 min on ice prior to reconstitution. From separate experiments, where the cholate-containing mixture was centrifuged prior to reconstitution, we know that 70–80% of the L-glutamate transporter is solubilized (N. C. Danbolt, and B. I. Kanner, unpublished experiments).

Reconstitution of the Samples. The reconstitution procedure was a modification of the previously described method (Radian & Kanner, 1985).

(*A*) *Reconstitution Mixture.* The crude brain lipids and partially purified phospholipids (see above) were mixed, dried, lyophilized, and suspended in dialysis buffer (120 mM KP_i, pH 7.8, 5 mM Tris–SO_4, pH 7.4, 1 mM $MgSO_4$, 0.5 mM EDTA, 1% glycerol) to give 13 mg of crude brain lipids (of which 7 mg was phospholipids) and 43 mg of soybean phospholipids per milliliter of dialysis buffer. Two hundred fifty microliters of 3 M NaCl and 45 μL of 20% cholate were added per milliliter of dialysis buffer. Then the mixture was frozen in aliquots. Some hours before use it was thawed and briefly sonicated.

(*B*) *Reconstitution.* Twenty-microliter fractions containing 2% cholate were mixed with 145 μL of the above reconstitution mixture, incubated on ice for 15 min, and passed through "minicolumns", filled with Sephadex G-50 (fine) equilibrated with the desired "in" medium, as previously described (Radian & Kanner, 1985). In order to remove any external L-glutamate (in those cases where internal L-glutamate was desired), proteoliposomes were respun again through the minicolumn, equilibrated with the same "in" medium but without L-glutamate.

Transport Experiments. Membrane vesicles were loaded with the desired "in" medium as described (Kanner, 1978). The loading of the reconstituted proteoliposomes was as done above. "Native" or reconstituted membrane vesicles (10 μL, 100 μg of protein per time point) were diluted in 190 μL of the desired "out" medium at room temperature usually supplemented with 1 μCi of L-[^{3}H]glutamic acid. The compositions of "in" and "out" media for each experiment are given in the legends to the figures and footnotes to the tables. All the solutions were filtered prior to use. Without this filtration, extremely high background values were recorded. Stopping of the reactions—after addition of 2 mL of ice-cold 0.15 M NaCl—was done by membrane filtration, washing, and scintillation counting as described previously (Kanner, 1978; Gordon & Kanner, 1988). Each experiment was repeated at least three times. The absolute values of transport were variable between preparations, but all gave qualitatively similar results. Therefore, representative experiments are shown.

Chemical determination of endogenous L-glutamate was done upon HPLC by Amino-lab, Kiryat Weizmann, Rehovot, Israel, as described (Richards & Andrews, 1981).

Protein. Protein was determined as described (Lowry et al., 1951).

Phosphate. Phosphate was determined as described (Ames et al., 1966).

Results

Counterflow in Reconstituted and Native Membrane Vesicles. In the experiment depicted in Figure 1A membrane

[1] Abbreviations: GABA, γ-aminobutyric acid; NaP_i, sodium phosphate buffer; KP_i, potassium phosphate buffer; TTX, tetrodotoxin; THA, *threo*-3-hydroxyaspartate.

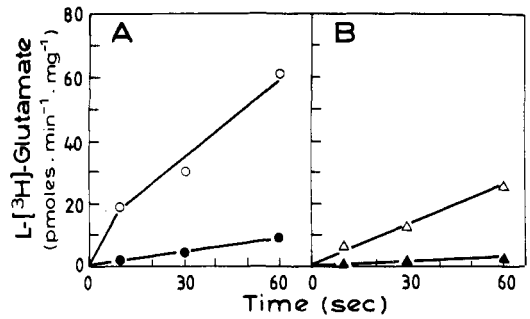

FIGURE 1: Dependence of L-glutamate uptake on external and internal sodium ions. Uptake was performed as described under Experimental Procedures. Membrane vesicles were loaded with 90 mM NaCl + 10 mM NaP_i, pH 7.4 (open symbols), or 90 mM LiCl + 10 mM LiP_i, pH 7.4 (closed symbols). The external medium contained 90 mM NaCl + 10 mM NaP_i, pH 7.4 (A), or 90 mM LiCl + 10 mM LiP_i, pH 7.4 (B), both supplemented with 1 μCi of L-[^{3}H]glutamic acid. Per time point 160 μg of membrane protein was used.

Table I: Specificity of L-[^{3}H]Glutamate Uptake[a]

addition	L-[^{3}H]glutamate uptake [pmol/(min·mg of protein)]
none	28.0
L-glutamate, 10 μM	7.0
L-glutamate, 100 μM	1.5
D-glutamate, 10 μM	19.5
D-glutamate, 100 μM	18.0
GABA, 100 μM	23.0
THA, 10 μM	6.0
THA, 50 μM	3.0
D-aspartate, 50 μM	1.5
NMDA, 50 μM	22.0
kainate, 50 μM	13.0

[a] Membrane vesicles were loaded with 90 mM NaCl + 10 mM NaP_i, pH 7.4. The external medium contained 90 mM NaCl + 10 mM NaP_i, pH 7.4, the indicated concentrations of analogues, and 1 μCi of L-[^{3}]glutamate. Uptake was terminated after 1 min. Per time point 100 μg of protein was used.

vesicles, loaded with buffered sodium chloride, are diluted into the same medium containing L-[^{3}H]glutamate (1 μCi, 21 Ci/mmol). This results in sodium-dependent uptake of the labeled amino acid (Figure 1). Replacement of sodium on both sides of the membrane by lithium totally inhibits it (Figure 1B). Intermediate levels of uptake are observed when sodium is replaced by lithium on either the outside or inside (Figure 1, panels A and B, respectively). The extent of the uptake is observed after 3–5 min, reaching an apparent steady state of 150 pmol of L-[^{3}H]glutamate (data not shown). This represents more than 40% of the input counts. The substrate specificity of the uptake process is very reminiscent of that of the ($Na^+ + K^+$)-coupled L-glutamate transporter (Kanner & Sharon, 1978a; Kanner & Bendahan, 1982). It exhibits stereospecificity; L-glutamate potently inhibits the uptake process, whereas the D-isomer has not much of an effect (Table I). GABA and NMDA, which are not substrates of the transporter, do not inhibit. On the other hand, THA and D-aspartate—which are excellent substrates for the L-glutamate transporter—strongly inhibit the uptake process. Kainate is a weak inhibitor in agreement with observations of L-glutamate transport in synaptosomes (Pocock et al., 1988). The sodium dependence and substrate specificity of the uptake process suggest that uptake is catalyzed by the ($Na^+ + K^+$)-coupled L-glutamate transporter. Net flux of L-glutamate by this transporter requires the presence of trans potassium ions (Kanner & Sharon, 1978a,b; Kanner & Bendahan, 1982; Gordon & Kanner, 1988); however, exchange does not. Since no internal potassium is present, the uptake process likely is due to exchange of labeled external glutamate with internal

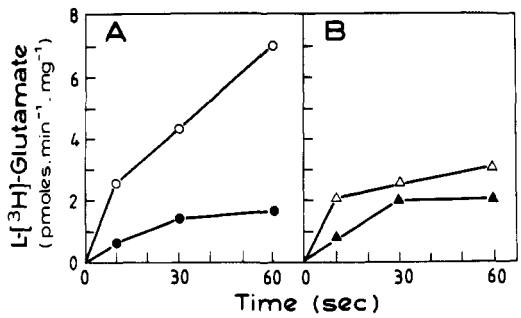

FIGURE 2: Uptake of L-[^{3}H]glutamate in reconstituted vesicles. Solubilization and reconstitution were done as described under Experimental Procedures. Membrane vesicles were loaded with 90 mM NaCl + 10 mM NaP_i, pH 7.4, with (○) or without (●) 100 μM L-glutamate (A), or with 90 mM LiCl + 10 mM LiP_i, pH 7.4, with (△) or without (▲) 100 μM L-glutamate (B). The external medium was the same as the internal medium supplemented with 1 μCi of L-[^{3}H]glutamate. Per time point 13 μg of membrane protein was used.

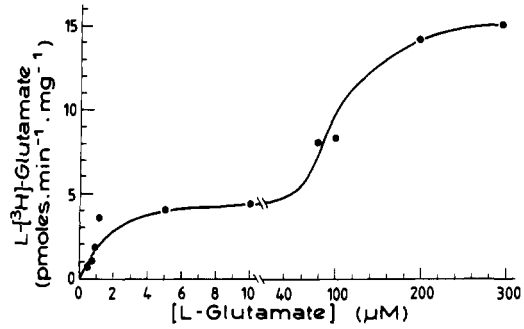

FIGURE 3: Dependence of counterflow on the internal L-glutamate concentration. Reconstituted vesicles were prepared as described under Experimental Procedures. The internal medium was 90 mM NaCl + 10 mM NaP_i, pH 7.4, plus the indicated concentrations of unlabeled L-glutamate. The external medium had the same ionic composition and also contained 1 μCi of L-[^{3}H]glutamate. The amount of membrane protein in each time point was 15 μg. The transport was terminated after 1 min.

(endogenous) L-glutamate. Measurement of endogenous L-glutamate levels of one batch of membrane vesicles revealed about 1.5 nmol/mg of protein. In the experiment depicted in Figure 1, 160 μg of membrane protein was used, corresponding to 240 pmol of endogenous L-glutamate. As indicated below, this probably reflects internal L-glutamate. To the outside was added 47 pmol. A full isotopic equilibrium mediated by exchange of internal and external L-glutamate would yield 84% of the counts taken up. We observed an uptake of 42%. It is of interest to note that these high levels of endogeneous L-glutamate were present, notwithstanding the fact that these membrane vesicles were prepared after subjecting synaptosomes to osmotic shock. Extensive washing does not reduce these levels (data not shown). In order to demonstrate the requirement of the uptake process on internal L-glutamate, we solubilized the transporter and reconstituted it into liposomes (Kanner & Sharon, 1978b; Gordon & Kanner, 1988). Under these conditions any endogenous L-glutamate will be removed. The use of the reconstituted system readily reveals the requirement for internal L-glutamate Figure 2A). Also, in this system the process is dependent on sodium (Figure 2).

Kinetics of Counterflow. The kinetic parameters of the counterflow process have been determined to be $K_m = 0.6$ μM and $V_{max} = 70$ pmol/(min·mg of protein). The value of K_m is in good agreement with that of the influx process (Kanner & Sharon, 1978a). The V_{max} is about 150–200-fold lower than that of influx, efflux, and exchange. This issue will be addressed below and under Discussion. Because of the difficulties in varying the internal L-glutamate concentrations, we could

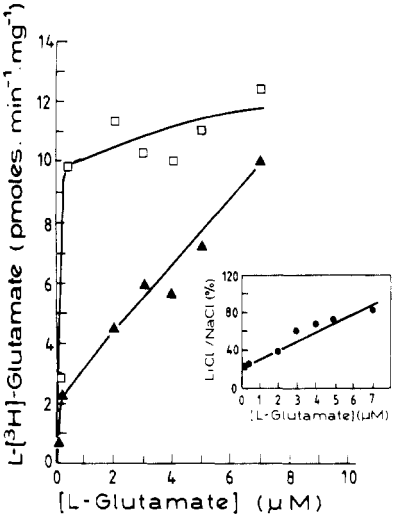

FIGURE 4: Effect of external L-glutamate concentration on the sodium dependence of counterflow. Membrane vesicles were loaded with 90 mM NaCl + 10 mM NaP_i, pH 7.4. The external medium was 90 mM NaCl + 10 mM NaP_i, pH 7.4 (□), or 90 mM LiCl + 10 mM LiP_i, pH 7.4 (▲), both supplemented with the indicated concentrations of L-glutamate. The specific activity ranged from 8 to 60 Ci/mmol. For each time point, 100 μg of membrane protein was used. The transport was terminated after 1 min. The ratio of counterflow in the absence of external sodium to that in its presence is plotted in the inset.

determine its effect on counterflow only by using the reconstituted system. As shown in Figure 3, a double saturation curve is observed. The higher apparent affinity—half-maximal effect at about 1 μM—is in good agreement with the apparent K_m for counterflow and influx (Kanner & Sharon, 1978a). The "native" membrane vesicles are predominantly in the "right-side-out" orientation (Kanner, 1980). Therefore, the internal high-affinity site for L-glutamate probably reflects transporter molecules reconstituted in the "inside-out" orientation. The lower apparent affinity—about 100 μM—possibly reflects the cytoplasmic L-glutamate binding site of the transporter, i.e., transporters reconstituted in the "right-side-out" orientation. However, alternative explanations are possible (see Discussion).

Effect of Ions. As already shown in Figure 1A, some counterflow still remains when sodium is taken away from the outside, provided it is still present on the inside. This fractional counterflow can be dramatically increased by raising the external L-glutamate concentration. At L-glutamate levels above the K_m of the high-affinity system (Figure 4) counterflow becomes almost independent of external sodium. This concentration dependence—half-maximal effect at about 3.0 μM L-glutamate—is reasonably close to that of counterflow. Also, when in the reconstituted system the internal L-glutamate concentration is increased, the process becomes almost independent of internal sodium (Figure 5). This dependence on the internal concentration of L-glutamate is very similar to that observed in Figure 3, second phase.

It is of interest to note that the counterflow process was optimal in the presence of chloride. When chloride was fully replaced by phosphate—either on the inside or on the outside—the rate was decreased by about 50% (data not shown).

As mentioned before, the V_{max} of the counterflow process is very low, especially when it is compared with net flux and exchange in the presence of internal potassium (Kanner & Sharon, 1978a; Kanner & Bendahan, 1982). Replacement of 10–50% of the sodium by potassium did not change the velocity of the process (data not shown). Therefore, it appears

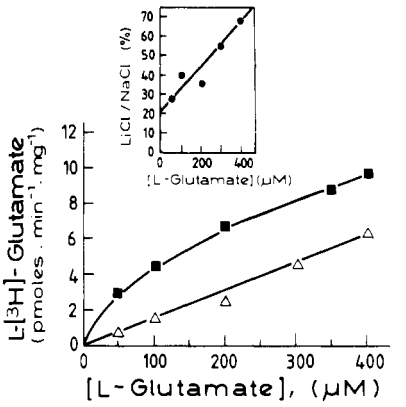

FIGURE 5: Effect of internal L-glutamate concentration on the sodium dependence of counterflow. Crude membranes were solubilized and reconstituted into liposomes as described under Experimental Procedures. The internal medium was 90 mM NaCl + 10 mM NaP_i, pH 7.4 (■), or 90 mM LiCl + 10 M LiP_i, pH 7.4 (△), plus the indicated concentrations of L-glutamate. The external medium was 90 mM NaCl + 10 mM NaP_i, pH 7.4. One microcurie of L-[^{3}H]-glutamate was used for each time point. The transport was terminated after 1 min. The ratio of counterflow in the absence of internal sodium to that in its presence is plotted in the inset.

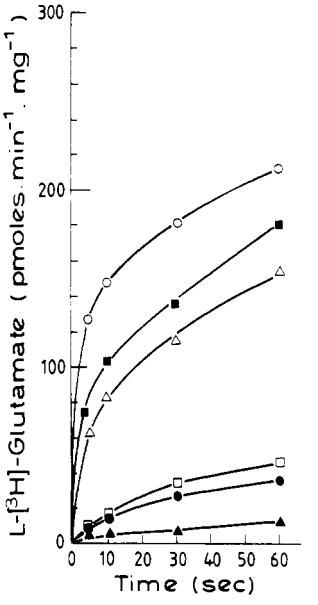

FIGURE 6: Inhibition of the transport by sodium ions. Membrane vesicles were loaded with 90 mM NaCl + 10 mM KP_i, pH 7.4 (●), 90 mM ChCl + 10 mM KP_i, pH 7.4 (△), 90 mM LiCl + 10 mM KP_i, pH 7.4 (▲), 90 mM NaCl + 10 mM NaP_i, pH 7.4 (□), 90 mM Tris-HCl + 10 mM KP_i, pH 7.4 (■), or 100 mM KP_i, pH 7.4 (○). The external medium was 90 mM NaCl + 10 mM NaP_i, pH 7.4. One microcurie of L-[^{3}H]glutamate and 100 μg of membrane protein were used for each time point.

that internal sodium and also internal lithium (Figure 1) are inhibiting the transporter. This would predict that, in the presence of "inert" internal cations, transport will be stimulated. As shown in Figure 6, a dramatic increase in the rate of transport is observed when, together with the obligatory potassium, choline or Tris is present as compared with sodium or lithium. The rate of counterflow (no internal potassium) was not stimulated by replacing internal sodium by choline or Tris (data not shown).

Low-Affinity Transport in Membrane Vesicles. The low-affinity L-glutamate site observed in the reconstituted system (Figure 3) could reflect a separate low-affinity transporter and/or the high-affinity transporter in the "inside-out" conformation. As shown in Figure 7, membrane vesicles indeed exhibit a low-affinity uptake (K_m = 87 μM) in addition to the

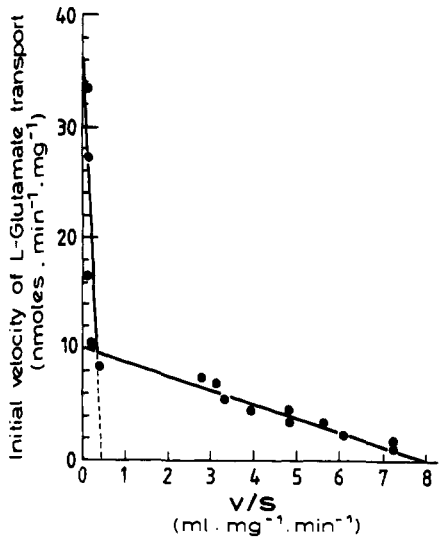

FIGURE 7: High-affinity and low-affinity transport. Membrane vesicles were loaded with 100 mM KP_i, pH 7.4. The external medium was 150 mM NaCl plus the indicated concentrations of unlabeled L-glutamate. For each time point we used 2 μCi of L-[^{3}H]glutamate and 100 μg of membrane protein. The transport was terminated after 1 min.

Table II: Effects of Sodium, Potassium, and Sodium Channel Ligands on Low-Affinity L-Glutamate Transport[a]

condition	L-[^{3}H]glutamate transport (nmol/mg of protein)
control	13.5
–internal K^+	0.9
–external Na^+	0
veratridine, 50 μM	7.5
TTX, 1 μM	15.0
veratridine, 50 μM, + TTX, 1 μM	12.6

[a] Membrane vesicles were loaded with 100 mM KP_i, pH 7.4, or 100 mM LiP_i, pH 7.4 (–internal K^+). The external medium was 0.15 M NaCl or 0.15 M LiCl (–external Na^+) supplemented with 10 mM L-glutamate and 2 μCi of L-[^{3}-]glutamate. The membranes were diluted in the "out" medium—but without L-glutamate—and incubated for 4 min at room temperature. Then transport was initiated by addition of L-glutamate. Reactions were terminated after 5 min. For each time point, 100 μg of membrane protein was used.

well-established high-affinity process (K_m = 1.2 μM). Also, the low-affinity L-glutamate uptake is dependent on external sodium and internal potassium (Table II). Furthermore, it is inhibited by the membrane-permeant sodium channel opener veratridine. This inhibition is reversed by the impermeant blocker TTX (Table II). This suggests that the low-affinity process in membrane vesicles is catalyzed by a transporter which is in the "right-side-out" conformation.

Discussion

The results described in this paper and previous ones (Kanner & Sharon, 1978a; Kanner & Marva, 1982; Kanner & Bendahan, 1982) can be explained by the model depicted in Figure 8. From the outside the sodium ions (probably n = 3; Kanner & Sharon, 1978; Barbour et al., 1988) bind first (step 1) followed by L-glutamate (step 2). This results in the formation of the translocation complex [$C(Na^+)_n$glut]. After the translocation (step 3), L-glutamate and sodium debind (steps 4 and 5), yielding the unloaded transporter. Subsequently potassium binds to the inside (step 6), and upon translocation of the potassium-loaded transporter (step 7) and debinding of the potassium (step 8), the transporter is ready for a new influx cycle. While net influx proceeds in the counterclockwise direction, net efflux occurs via the same steps,

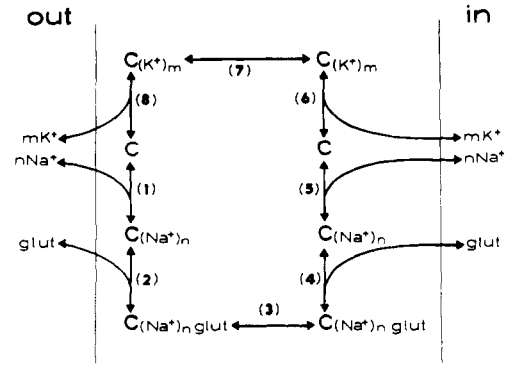

FIGURE 8: A model for the mechanism of L-glutamic acid translocation. The details are provided in the text. As indicated, it is likely that n = 3 and m = 1.

but in the clockwise direction. Exchange is visualized to utilize only some of the steps, namely, steps 1 or 2 through step 5, or steps 4 or 5 in the reverse direction through step 1. At present, the possibility that the binding–debinding order is random on the inside cannot be excluded (see below). This model is an extension of the model published previously (Kanner & Bendahan, 1982). A key feature—the existence of two translocation complexes, $C(Na^+)_n$glut and $C(K^+)_m$—remains unchanged. This is based on the observations (Kanner & Bendahan, 1982) that radioactive L-glutamate previously accumulated by the membrane vesicles can be released either by L-glutamate or by potassium (both external). In the previous study internal potassium was present in order to enable the accumulation of labeled L-glutamate by influx (steps 1–8) prior to the initiation of dilution-induced efflux or exchange. In the present study the technique of counterflow is used, and this permits evaluation of exchange reactions catalyzed by the transporter in the complete absence of potassium. This has permitted a substantial refinement of the model.

In the membrane vesicles, the high-affinity uptake system is oriented in the "right-side-out" orientation. We know this since in the same membrane vesicles also action-potential sodium channels are present (Kanner, 1980), and the side of the membrane to which specific ligands for it bind is known. Thus, the alkaloid veratridine opens the channels and thereby reduces the artificially imposed sodium gradient across the membrane. Since this is the major driving force for L-glutamate uptake (Kanner & Sharon, 1978a,b), it inhibits transport. TTX blocks the channel, even in the presence of veratridine. Therefore, it prevents the inhibition by the alkaloid. Since TTX acts from the outside of the nerve cell, not only the sodium channel but also the high-affinity L-glutamate uptake system has to be in the "right-side-out" orientation (Kanner, 1980). The K_m of counterflow is very similar to that of high-affinity transport, and thus, at least when working in the micromolar range, those transporters in the "right-side-out" orientation are monitored.

Counterflow proceeds, at least partly, also in the absence of external sodium (Figure 1A) as long as sodium and glutamate are present on the inside (Figure 1B). Thus, upon binding of sodium and L-glutamate on the inside (steps 4 and 5) and reorientation of the transporter (step 3), the unlabeled L-glutamate is released (step 2, clockwise) and labeled L-glutamate can rebind (step 2, counterclockwise) before the sodium dissociates. The possibility that, on the outside, glutamate binds before sodium (in other words, binding is random) is rendered highly unlikely by the experiment depicted in Figure 4. If external L-glutamate is used at saturating levels (note: in Figure 1 its concentration is about $^1/_{10}K_m$), the

counterflow becomes totally independent of external sodium. If there is an equal probability that L-glutamate binds before sodium, the initial rate of counterflow should still be dependent on sodium (2-fold) even at saturating L-glutamate levels.

Counterflow is still operative, albeit at a lower rate, in the absence of internal sodium, as long as sodium is present on the outside (Figure 1B). Therefore, for the same reasons as for the binding order on the outside, L-glutamate is released on the inside before sodium. On the other hand, since we cannot manipulate the internal L-glutamate concentration in the membrane vesicle system, we cannot rule out that on the inside the binding/debinding is random. The only possibility to attempt this is with the reconstituted system (Kanner & Sharon, 1978b; Gordon & Kanner, 1988). However, upon reconstitution scrambling may occur. The data of Figure 3 indicate a double saturation curve for internal L-glutamate, one with high and one with low affinity. When using high levels of internal L-glutamate to saturate the low-affinity site, we observed that indeed the process became almost independent of internal sodium (Figure 5). If the low-affinity site would reflect the cytoplasmic face of the transporter, just as in the case of the GABA transporter (Mabjeesh & Kanner, 1989), this would exclude a random order on the inside. However, also in the "native" membrane vesicles there is a low-affinity L-glutamate transporter (Figure 7) which is "right side out" (Table II). This makes it difficult to rule out a random mechanism on the inside for the high-affinity ("right-side-out") transporter. The low-affinity process in membrane vesicles is in harmony with the detection of a low-affinity L-glutamate uptake in synaptosomes (Wood & Sidhu, 1987). However, no information on its ion dependence was reported. It is of interest to note that the low-affinity process we observe here with the membrane vesicles has striking similarities with its high-affinity counterpart, i.e., dependence of the influx on external sodium and internal potassium. It is possible that this low-affinity transporter is related to the high-affinity form, by covalent modification such as phosphorylation. This could even represent a physiological regulatory mechanism of L-glutamate reuptake from the synapse.

One of the surprising findings documented here is that the observed V_{max} for the partial reaction—counterflow—is about 0.5% of that for the full-cycle net influx (Kanner & Sharon, 1978a) and dilution induced efflux upon active loading (Kanner & Bendahan, 1982). One of the differences is that here no internal potassium was present, while it was in all the other types of measurement. Also, high concentrations of internal sodium or lithium were present on the inside in this study. Thus, it is possible that high potassium on the inside and/or low internal sodium are required for optimal functioning of the transporter. Indeed, it has been found that internal sodium or lithium is highly inhibitory to L-glutamate transport as compared with other "inert" ions such as choline or Tris (Figure 6). Yet, in the presence of internal choline and the complete absence of potassium, no consistent increase in the rate of counterflow was observed. This leads us to conclude that the low rates observed during counterflow are due to a requirement of the transporter for internal potassium—even under conditions where translocation of this ion is not required. The strong inhibitory effect of high levels of internal sodium or lithium also contributed to the low rates. The effect of internal potassium on the activity of the transporter could be due to binding to an allosteric site, thereby increasing its activity. Thus, under physiological conditions—high potassium and low sodium inside the cell—the transporter functions optimally.

Registry No. THA, 16417-36-0; L-Glu, 56-86-0; D-Asp, 1783-96-6; Na, 7440-23-5; K, 7440-09-7; Li, 7439-93-2; Cl, 16887-00-6.

REFERENCES

Ames, B. N. (1966) *Methods Enzymol. 8*, 115–118.

Barbour, B., Brew, H., & Attwell, D. (1988) *Nature 335*, 433–435.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Fonnum, F. (1984) *J. Neurochem. 42*, 1–11.

Gordon, A. M., & Kanner, B. I. (1988) *Biochim. Biophys. Acta 944*, 90–96.

Iversen, L. L. (1975) in *Handbook of Psychopharmacology* (Iversen, L. L., Ed.) Vol. 2, pp 381–442, Plenum, New York.

Johnston, G. A. R. (1981) in *Glutamate: Transmitter in the Central Nervous System* (Roberts, P. J., Storm-Mathisen, J., & Johnston, G. A. R., Eds.) pp 77–87, John Wiley and Sons, Chichester, New York, Brisbane, and Toronto.

Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.

Kanner, B. I. (1978) *Biochemistry 17*, 1207–1211.

Kanner, B. I. (1980) *Biochemistry 19*, 692–697.

Kanner, B. I. (1983) *Biochim. Biophys. Acta 726*, 293–316.

Kanner, B. I. (1989) *Current Opinion in Cell Biology 1*, 735–738.

Kanner, B. I., & Sharon, I. (1978a) *Biochemistry 17*, 3949–3953.

Kanner, B. I., & Sharon, I. (1978b) *FEBS Lett. 94*, 245–248.

Kanner, B. I., & Bendahan, A. (1982) *Biochemistry 21*, 6327–6330.

Kanner, B. I., & Marva, E. (1982) *Biochemistry 21*, 3143–3147.

Kanner, B. I., & Schuldiner, S. (1987) *CRC Crit. Rev. Biochem. 22*, 1–39.

Kuhar, J. M. (1973) *Life Sci. 13*, 1623–1634.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mabjeesh, N., & Kanner, B. I. (1989) *Biochemistry 28*, 7694–7699.

McBean, G. J., & Roberts, P. J. (1985) *J. Neurochem. 44*, 247–254.

Ottersen, O. P., & Storm-Mathisen, J. (1984) in *Handbook of Chemical Neuroanatomy, Volume 3: Classical Transmitters and Transmitter Receptors in the CNS, Part II* (Bjorkland, A., Hokfelt, T., & Kuhar, M. J., Eds.) pp 141–246, Elsevier Science Publishers B.V., Amsterdam.

Pocock, J. M., Murphie, H. M., & Nicholls, D. G. (1988) *J. Neurochem. 56*, 745–751.

Radian, R., & Kanner, B. I. (1985) *J. Biol. Chem. 260*, 11859–11865.

Richards, P., & Andrews, J. (1981) *Protein Chemistry Notes 25*, 1–4.

Roberts, P. J., Storm-Mathisen, J., & Bradford, H. F., Eds. (1986) in *Excitatory Amino Acids*, Macmillan, London.