# Efflux of L-Glutamate by Synaptic Plasma Membrane Vesicles Isolated from Rat Brain†

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ABSTRACT: Synaptic plasma membrane vesicles isolated from rat brain were loaded with L-glutamate either passively, by using a freeze–thaw technique, or by active transport. Subsequently the ion dependency of glutamate efflux from these vesicles was studied. With each of the types of loading similar results were obtained. Efflux requires the simultaneous presence of internal sodium ions and external potassium ions. The process is also stimulated by chloride ions, but either internal or external chloride ions cause stimulation. Addition of unlabeled L-glutamate stimulates efflux about 2-fold. It is concluded that efflux of L-glutamate is in many aspects symmetrical with its influx [Kanner, B. I., & Sharon, I. (1978) *Biochemistry 17*, 3949–3954]. It appears that in order for L-glutamate to interact with the transporter, sodium has to be present on the same side as L-glutamate whereas potassium has to be simultaneously present on the opposite site. The simplest way to account for these and the previous data is to postulate that the L-glutamate transporter catalyzes sodium and L-glutamate cotransport, while it simultaneously catalyzes antiport of potassium.

Sodium-dependent high-affinity uptake systems for a wide range of neurotransmitters have been detected in brain preparations, such as synaptosomes (Iversen, 1971, 1973; Kuhar, 1973; Bennett et al., 1974). Their probable physiological importance is the termination of transmitter action on postsynaptic receptors (Iversen, 1971) as well as to maintain constant levels of transmitters in the neurons (Hedgvist & Stjarne, 1969). Various observations have led to the proposal that ion gradients, generated by the $(Na^+ + K^+)$ATPase, are the immediate driving force for neurotransmitter accumulation (Bogdanski et al., 1968; Holtz & Coyle, 1974), in line with the general concept that solute accumulation can be achieved by cotransport with ions (Crane, 1965; Riggs et al., 1958; Mitchell, 1963). Direct evidence that this concept applies to sodium-coupled neurotransmitter transport in the brain has been obtained recently by using membrane vesicles. Those vesicles, apparently derived from the synaptic plasma membrane (Kanner, 1980), catalyze neurotransmitter transport under conditions where artificially applied ion gradients are the sole source of energy (Kanner, 1978; Kanner & Sharon, 1978a). Thus it appears that the electrochemical potential gradient of $Na^+$ serves as a direct driving force for the transport of GABA[1] (Kanner, 1978) and L-glutamate (Kanner & Sharon, 1978a). Surprisingly, these studies revealed that neurotransmitter transport is absolutely dependent on additional ions, such as external $Cl^-$ or small monovalent anions in the case of GABA (Kanner, 1978) and internal $K^+$ in the case of L-glutamate (Kanner & Sharon, 1978a). In fact, a chloride gradient (out > in) or a potassium gradient (in > out) has been shown to stimulate influx of GABA (Kanner, 1978) or L-glutamate (Kanner & Sharon, 1978a), respectively. Thus it is possible that these other ions (potassium and/or chloride) are also translocated through the carrier together with sodium and the neurotransmitter. This may also be the case for transport of serotonin in platelet membranes (Rudnick, 1977; Nelson & Rudnick, 1979) and glutamate transport in renal brush border vesicles (Burckhardt et al., 1980; Schneider & Sacktor, 1980).

Measurements of efflux, in addition to influx, can provide evidence on the question if these additional ions are also transported through the carrier—together with sodium and the solute. For instance, it seems that in the case of GABA, cotransport with sodium and chloride is operative, since it can be shown that efflux of GABA requires the simultaneous presence of sodium and chloride, both internally (Kanner & Kifer, 1981), even in the presence of uncouplers, while influx requires both ions on the outside. In this communication we report on studies where this principle is applied to L-glutamate efflux. The central finding is that this efflux requires internal sodium and external potassium. Thus, this transporter is symmetrical with regard to the sodium and potassium requirement for L-glutamate transport.

## Experimental Procedures

*Preparation of Membrane Vesicles.* Membrane vesicles from 14-day-old female rats were prepared and stored as described (Kanner, 1978). Protein was determined according to the method of Lowry et al. (1951).

*Efflux from Passively Loaded Vesicles.* Loading was done as described (Kanner & Kifer, 1981). Vesicles were collected from the liquid-air refrigerator, rapidly thawed in a water bath at 37 °C, and diluted 10–20-fold with an ice-cold solution composed of the desired internal medium. The diluted vesicles were collected after centrifugation at 4 °C for 20 min at 27000*g* and resuspended in the same medium at a protein concentration of 20–25 mg/mL. Then 30–40 μCi of L-[3,4-$^3$H]glutamic acid (40.3 Ci/mmol) was added per 150–200 μL of the concentrated vesicle suspension, which was subsequently frozen in liquid air. The vesicles were then thawed in an ice–water bath. Subsequently (at $t = 0$ on the abscissa of the figures) efflux was initiated by diluting 5-μL samples into 200 μL of external medium, and efflux was performed as described (Kanner & Kifer, 1981). The composition of the internal and external media is described in the legends to the figures.

*Efflux from Actively Loaded Vesicles.* Membrane vesicles were loaded with 0.1 M $KP_i$, pH 6.8, as described (Kanner, 1978) and were loaded with L-glutamate by active transport (Kanner & Sharon, 1978a). The $KP_i$-loaded vesicles (5 μL,

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[1] Abbreviations: GABA, γ-aminobutyric acid; CCCP, carbonyl cyanide (*m*-chlorophenyl)hydrazone; EDTA, ethylenediaminetetraacetic acid.

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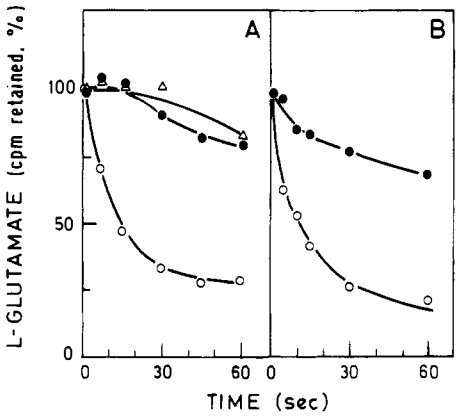


FIGURE 1: Effect of internal sodium and external potassium ions on L-glutamate efflux from passively loaded membrane vesicles. The vesicles were passively loaded with L-[3,4-$^3$H]glutamic acid and the internal media indicated below, by freezing and thawing, and subsequently dilution-induced efflux was measured. Loading and efflux are described in detail under Experimental Procedures. (A) The internal medium was 90 mM sodium chloride and 10 mM sodium phosphate, pH 6.8 (○, △), or 90 mM lithium choride and 10 mM lithium phosphate, pH 6.8 (●). The external medium was 100 mM potassium phosphate, pH 6.8, and 5 μM CCCP (○, ●) or 100 mM lithium phosphate, pH 6.8, and 5 μM CCCP (△). At each time point 134 μg (sodium-loaded vesicles) or 102 μg (lithium-loaded vesicles) of protein was filtered. (B) The internal medium was 90 mM sodium chloride and 10 mM sodium phosphate, pH 6.8 (○), or 100 mM sodium phosphate, pH 6.8 (●). The external medium was 100 mM potassium phosphate, pH 6.8, and 5 μM CCCP.

10–20 mg/mL) were diluted into 45 μL of 0.1 M NaCl containing 0.5 μCi of L-[3,4-$^3$H]glutamic acid (40.3 Ci/mmol), unless indicated otherwise.

After a 1-min influx period, the incubation mixtures were diluted 20-fold with the specified efflux solutions ($t$ = 0 on the abscissa of the figures). After a further incubation at room temperature for various times, reactions were terminated by the addition of 2 mL of ice-cold 0.15 M NaCl. The zero-time value was obtained by adding the cold stop solution prior to the efflux solution. Only in case of very fast efflux rates was it occasionally up to 25% lower, probably due to a limited amount of efflux during the termination of the reaction (cf. Figure 5). Filtration, washing, and counting of the radioactivity on the filters were performed as described (Kanner, 1978). Each experiment was repeated at least 3 times with different vesicle preparations. Almost every time point was done in duplicate. Averages of these duplicates are plotted in the figures. The variation in these duplicates was very small for the experiments where active loading was performed, usually within the 5% range. The variation was somewhat larger (up to 10%) in the experiments where passive loading was performed (Figures 1 and 2). This is due to lower sensitivity with the passive method (no accumulation but only equilibration of the glutamate radioactivity). The absolute values when experiments were repeated were quite variable from day to day, but qualitatively always the same result was obtained. In view of this the data from representative experiments are shown.

*Materials.* L-[3,4-$^3$H]Glutamic acid was obtained from New England Nuclear. Valinomycin and CCCP were from Sigma, and nigericin was a generous gift of Dr. R. J. Hosley from Eli Lilly.

## Results

The freeze–thaw technique to load synaptic plasma membrane vesicles has been successfully applied to the study on the ion dependency of efflux of GABA (Kanner & Kifer, 1981). This loading technique has been applied to L-glutamate

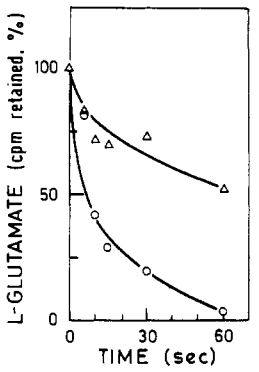


FIGURE 2: Effect of external chloride ions on L-glutamate efflux from passively loaded membrane vesicles. The vesicles were loaded passively with 0.1 M $NaP_i$, pH 6.8, and L-[$^3$H]glutamate, and subsequently efflux was measured, both as described above. For each time point 122 μg of protein was used. The efflux solutions contained (△) 100 mM $KP_i$, pH 6.8, and 5 μM CCCP or (○) 90 mM KCl and 10 mM $KP_i$, pH 6.8, and 5 μM CCCP.

in the experiments depicted in Figures 1 and 2. Vesicles loaded with buffered sodium chloride and L-[3,4-$^3$H]glutamate display dilution-induced efflux of L-glutamate, which is much faster into potassium phosphate than into lithium phosphate medium (Figure 1A). Since the transport of L-glutamate in these vesicles appears to be electrogenic (Kanner & Sharon, 1978a), the proton ionophore CCCP is included in the efflux media in order to prevent any buildup of charge. Thus the trivial explanation for the effects seen in Figure 1A, namely, potassium ions being the most permeable ion in nerves (Baker et al., 1962), appears to be extremely unlikely. The efflux also requires internal sodium ions (Figure 1A). The efflux in this and the subsequent experiments is not linear with time; although the reason for this is not known, the efflux does not display first-order kinetics. Figure 1B shows that efflux of L-glutamate is faster when internal chloride is present rather than internal phosphate. With internal phosphate rather than chloride, the efflux also requires both internal sodium and external potassium (data not shown). Although the influx of glutamate was shown to be absolutely dependent on internal potassium and external sodium, there was only a partial requirement for chloride in this process (Kanner & Sharon, 1978a). It is of interest to note that the stimulation by chloride is also observed with external chloride (Figure 2). Thus the possibility that the effect of chloride is electrical is very unlikely. If this were so, internal chloride would indeed be stimulatory ($[Cl^-]_{in} > [Cl^-_{out}$, Figure 1B) while external chloride would be inhibitory ($[Cl^-]_{out} > [Cl^-]_{in}$, Figure 2).

Since the ion requirements for glutamate efflux are opposite to those for its influx, the possiblity of inversion of the vesicles during the freeze–thaw cycle employed for the loading needs to be excluded. In control experiments vesicles were loaded by freezing and thawing with either potassium phosphate or lithium phosphate. Such vesicles showed accumulation of L-glutamate that was dependent on internal potassium (data not shown). Still, it can be argued that only a proportion of the vesicles is inverted during the freeze–thaw cycle and that we are looking at different vesicle populations in the influx and efflux experiments. In order to eliminate this possiblity, we have performed the following series of experiments where the vesicles are not subjected to the additional freeze–thaw cycle but are loaded actively, i.e., by active transport (Kanner & Sharon, 1978a). Subsequently efflux was measured from these same vesicles.

In the experiment depicted in Figure 3, the effect of external potassium ions on dilution-induced efflux of L-glutamate—

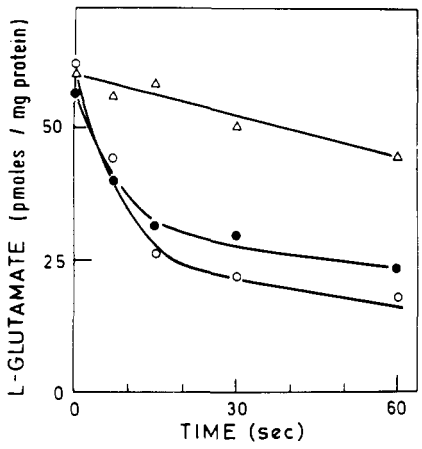


FIGURE 3: Effect of external potassium and CCCP on L-glutamate efflux from actively loaded vesicles. Membrane vesicles (5 $\mu$L, 56 $\mu$g of protein) were diluted into 45 $\mu$L of 0.1 M NaCl containing 0.5 $\mu$Ci of L-[$^3$H]glutamate (40.3 Ci/mmol). After 1 min of influx, the vesicles were diluted ($t$ = 0 on abscissa) by adding 1 mL of efflux solution of the following composition: (○) 0.1 M $KP_i$, pH 6.8, and 5 $\mu$M CCCP; (●) the same without CCCP; (△) 0.1 M $LiP_i$, pH 6.8, and 5 $\mu$M CCCP. The reactions were stopped at the times indicated.

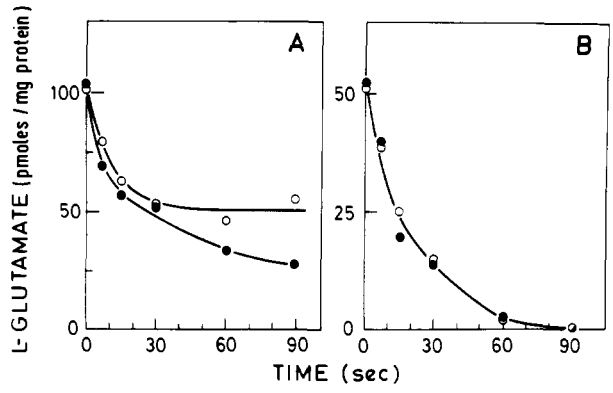


FIGURE 4: Effect of valinomycin plus CCCP on L-glutamate efflux from actively loaded vesicles. Influx was performed exactly as described above by using 80.5 or 102.5 $\mu$g of protein for panels A and B, respectively. The efflux media contained 90 mM LiCl and 10 mM $KP_i$, pH 6.8 (panel A), or 90 mM KCl and 10 mM $KP_i$, pH 6.8 (panel B), without (○) or with (●) 5 $\mu$M CCCP and 2.5 $\mu$M valinomycin.

from membrane vesicles that had previously been allowed to actively take up this solute—is examined. It can be seen that this efflux, also under these conditons, is potassium dependent (Figure 3). Addition of 1 mM of EDTA in all media (preloading, influx, and efflux media) does not affect the process (data not illustrated). Thus efflux is not dependent on divalent cations. It is also of interest to note that omission of the proton ionophore CCCP has not much of an effect (Figure 3). This is compatible with observations on intact nerve preparations (Baker et al., 1962) that suggest that the permeability of the nerve membrane is sufficient that potassium ions, present on the outside, can compensate for electrical effects during glutamate efflux. Movement of potassium through the transporter in the opposite direction of L-glutamate explains the dependence of L-glutamate efflux on external potassium. However, it is also to be expected that internal potassium will be inhibitory. This prediction is tested in the experiment depicted in Figure 4. When dilution-induced efflux is performed into a medium containing mainly lithium phosphate ($[K^+]_{in} > [K^+]_{out}$), it can be observed that when valinomycin and CCCP are included, a progressive stimulation of efflux of L-glutamate ensues (Figure 4A). Under these conditions a drop of the

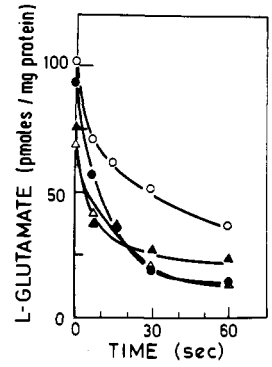


FIGURE 5: Effect of nigericin on dilution-induced L-glutamate efflux from actively loaded membrane vesicles. Active loading was performed as above. After 1 min of influx, the vesicles were diluted into the following media: (○) 50 mM KCl and 50 mM $NaP_i$ pH 6.8; (●) the same plus 5 $\mu$M nigericin; (△) 50 mM KCl and 50 mM $LiP_i$, pH 6.8; (▲) the same plus 5 $\mu$M nigericin. For each time point 59.5 $\mu$g of membrane protein was used.

internal potassium concentration is expected, because potassium will move down its concentration gradient via valinomycin and this movement is electrically compensated by an inward flux of protons via CCCP. When the dilution medium is composed of potassium phosphate, the stimulation by valinomycin plus CCCP is not observed. In this case the internal potassium is not expected to be depleted. It is of interest to note that efflux into the lithium medium, containing only about 15 mg of $K^+$/L, is almost as fast as that into the potassium medium. This is in agreement with separate experiments that indicate that the potassium ion concentration that half-maximally stimulates efflux is well below 10 mM (data not shown).

The dependence of L-glutamate efflux on internal sodium for actively loaded vesicles is harder to test, since when the vesicles are preloaded with potassium and sodium (no sodium gradient), a much lower glutamate accumulation will ensue than in the absence of internal sodium (Kanner & Sharon, 1978a). Also, the fact that part of the sodium will enter the vesicles anyhow during the influx stage is complicating. However, the following indirect experiment serves to indicate that also with active loading, internal sodium is required for glutamate efflux. The exchange ionophore nigericin, which exchanges potassium ions for sodium ions or for protons, is used to manipulate the internal sodium ion concentration during efflux. When the efflux medium is composed of sodium (and potassium of course), addition of nigericin is expected to increase the internal sodium concentration, and a big stimulation of L-glutamate efflux is observed (Figure 5). If on the other hand the efflux medium is devoid of sodium ions (replaced by lithium ions), the stimulation by nigericin is not observed (Figure 5). Actually a small but consistent inhibition at later times is observed by the ionophore, probably because of removal of sodium, which entered during the influx stage (Figure 5). This removal of sodium by nigericin to such a low level to substantially inhibit glutamate efflux is indeed expected to take some time. Thus under the standard conditions we measure suboptimal L-glutamate efflux, because of limitation by internal sodium.

It is of interest to note that the stimulation of L-glutamate efflux by external chloride (Figure 2) is also seen with actively loaded vesicles (data not shown). The effect of chloride will be discussed below.

Another interesting observation is the effect of unlabeled L-glutamate on the efflux of glutamate from the vesicles. This actually represents a comparison between net efflux and exchange. Addition of 50 $\mu$M L-glutamate, which is 17-fold the

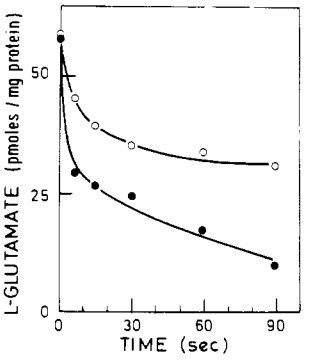


FIGURE 6: Trans stimulation of L-glutamate efflux from actively loaded vesicles. Influx (1 min) was performed as above by using 58.6 μg of protein. The composition of the efflux medium was (○) 50 mM $KP_i$, pH 6.8, and 50 mM $NaP_i$, pH 6.8, or (●) the same plus 50 μM L-glutamate.

$K_m$ (Kanner & Sharon, 1978a), results in a marked stimulation of L-glutamate efflux (Figure 6). Mechanistically, efflux and exchange differ by one step, which occurs under net efflux conditions, namely, the return of the unloaded carrier. The results are thus consistent with the idea that this step is rate limiting. The alternative explanation, namely, that charge compensation is limiting under efflux conditions (does not play a role during exchange, which should be electroneutral), is ruled out by the experiment depicted in Figure 3. The concentration of L-glutamate that stimulates half-maximally is in the range of 2–3 μM (data not shown), which is very similar to the $K_m$ of L-glutamate influx (Kanner & Sharon, 1978a). The stimulation by glutamate is restricted to the L stereoisomer only. Unlabeled D-glutamate, which does not inhibit influx, also does not enhance efflux of L-glutamate from the membrane vesicles (data not illustrated).

## Discussion

The requirement of the efflux process for internal sodium ions and external potassium ions strongly suggests that efflux is taking place via the reverse operation of the transporter of L-glutamate (as compared to influx). Further evidence for this contention is the stereospecific stimulation of this process by external L-glutamate. The concentration giving rise to half-maximal stimulation is very similar to the $K_m$ for L-glutamate influx.

Dilution-induced efflux does not display simple first-order kinetics. The reason for this is not known, but one of the following possiblities may be considered as an explanation. Since efflux is crucially dependent on the internal and external ion concentrations, changes of these ion concentrations—which are likely to occur upon dilution of the vesicles—will give rise to complex kinetics. This is especially true for the internal ion composition, since under the conditions of the efflux the internal volume is very small as compared to the external one. Preliminary experiments indicate that both $K_m$ and $V_{max}$ for L-glutamate for the influx process are affected by the external sodium and internal potassium concentration. Thus during the efflux we may obtain a drift in $K_m$ and/or $V_{max}$. Moreover another complicating factor might be the existence of additional efflux mechanisms of L-glutamate in the vesicle population. Whatever their nature, such processes would be dependent on external potassium and internal sodium. This efflux is not dependent on calcium—it proceeds normally in the presence of 1 mM EDTA on both faces of the membrane—and thus has nothing to do with the physiological release mechanism. Sodium-dependent influx of L-glutamate occurs mainly into neuronal elements of the vesicle population (Kanner, 1980) and displays a single $K_m$ of about 3 μM (Kanner & Sharon, 1978a). The very fast initial rates of efflux did not enable us to determine if the efflux process displays a single $K_m$ as well. Thus, although not very likely, involvement of additional efflux processes cannot be ruled out. Another process, which may play a role, is leakage of L-glutamate through the membrane or the transporter. This might be the explanation for the slow potassium-independent efflux observed when external lithium is used instead of external potassium. It is of interest to note that efflux of L-glutamate from renal brush border membrane vesicles, driven by sodium and potassium ion gradients, does follow first-order kinetics (Sacktor et al., 1981).

The central point of this paper is that the L-glutamate transporter is symmetrical with regard to the sodium and potassium requirement of L-glutamate transport. The following appear apparent: (a) The transporter requires the simultaneous presence of external sodium and internal potassium for influx (Kanner & Sharon, 1978a). (b) Internal sodium (Figures 1 and 5) and external potassium (Figures 1, 3, and 4) are required for efflux, while external sodium (Figures 3 and 6) or internal potassium (Figure 4) are clearly not required and are even inhibitory. (c) The level of L-glutamate accumulation is determined by the gradients of sodium (out > in) and potassium (in > out) (Kanner & Sharon, 1978a). (d) Transport is electrogenic—positive charge(s) moving in the direction of L-glutamate flow (Kanner & Sharon, 1978a). (e) The ion dependence of the carrier is not a result of charge compensation. These data strongly suggest that L-glutamate is translocated through the transporter with sodium ions in one direction, while it simultaneously translocates $K^+$ in other direction.

On the other hand, chloride does not appear to be involved directly in the translocation cycle. It appears that (a) there is a partial requirement for external chloride for glutamate influx (Kanner & Sharon, 1978a), (b) this requirement for chloride exists also for efflux but it is not important if the chloride is present internally or externally, and (c) the level of L-glutamate accumulation is independent on the chloride gradient (out > in). This chloride requirement differs from that reported for the GABA transporter, where the chloride dependency is absolute and the chloride has to be present on the same side of the transporter as GABA (Kanner & Kifer, 1981). In that case chloride does most likely participate in the translocation cycle of the transporter.

The partial requirement for chloride in the case of L-glutamate transport is probably similar to that reported for proline. (Kanner & Sharon, 1980) and possibly is a general feature for many transporters of the synaptic plasma membrane (Kuhar & Zarbin, 1978). The involvement of potassium in addition to sodium in the translocation cycle of the L-glutamate carrier does not stand by itself. Another example of a carrier where additional ions to sodium are part of the translocation cycle is the GABA transporter of synaptic plasma membrane vesicles. It appears that GABA is cotransported with both sodium and chloride ions (Kanner, 1978; Kanner & Kifer, 1981). The serotonin transporter from platelet membranes (Rudnick, 1977; Nelson & Rudnick, 1979) and the glutamate transporter from renal brush border vesicles (Burckhardt et al., 1980; Schneider & Sacktor, 1980) are other examples.

It has been shown recently that L-glutamate efflux in renal brush border vesicles is stimulated by external potassium (Sacktor et al., 1981). Furthermore, serotonin efflux from platelet plasma membranes is stimulated by external potassium

(Nelson & Rudnick, 1979) and by internal chloride (G. Rudnick, personal communication).

No conclusions with regard to the stoichiometry of the L-glutamate transporter can be drawn at present. If the glutamate anion is transported, the stoichiometry will be $n$N$^+$:$m$K$^+$:glutamate—with $n - 1 > m$ to account for the electrogenicity of the process (Kanner & Sharon, 1978a). If the transported species is without net charge, it follows that $n > m$. As discussed previously (Kanner & Kifer, 1981), this membrane vesicle preparation cannot be used for stoichiometry measurements. Those experiments will have to await purification of the L-glutamate transporter, which we have already been able to reconstitute (Kanner & Sharon, 1978b).

Efflux of L-glutamate is similar to that observed with many other transporters, in the sense that the return of the unloaded carrier seems to be limiting. Similar implications have been made for the lactose carrier of *Escherichia coli* (Kaczorowski & Kaback, 1979), the 5-hydroxytryptamine carrier of the platelet plasma membrane (Nelson & Rudnick, 1979), and the GABA carrier from the synaptic plasma membrane of rat brain (Kanner & Kifer, 1981).

Finally, it is of interest to note that a large proportion of the vesicles passively loaded with L-glutamate loses this solute in a manner which is dependent on both internal sodium and external potassium (Figure 1). This implies that a large proportion of the vesicles contain a functional L-glutamate carrier. As discussed previously (Kanner & Kifer, 1981), the biological implication of this finding is not clear. One possibility is that most nerves (not only the glutaminergic ones) have L-glutamate carriers, although only the glutaminergic nerves use the carriers for termination of synaptic transmission. Alternatively, the finding may be the result of an artifact, the possible formation of "hybrid" membranes during the preparation procedure. In any case since a similar situation has been encountered with GABA by using this very vesicle preparation, it seems probable that both transporters are sitting in the same vesicle. This possiblity is currently under investigation.

Acknowledgments

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