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Efflux of γ-Aminobutyric Acid by Synaptic Plasma Membrane Vesicles Isolated from Rat Brain†

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ABSTRACT: Synaptic plasma membrane vesicles isolated from rat brain were "actively" loaded with γ-aminobutyric acid (GABA) by a process driven by a sodium ion as well as a chloride ion gradient (both out > in). Subsequently, dilution-induced efflux from these vesicles was monitored. This efflux was 2-3-fold enhanced by the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone and 4-6-fold by externally added GABA. The ability of GABA to stimulate was most pronounced when both sodium and chloride ions were present in the dilution medium. The dependency of efflux of γ-aminobutyric acid on both internal sodium and chloride ions was demonstrated by three independent types of experiments: (a) preloading of the vesicles with sodium and chloride ions markedly stimulated γ-aminobutyric acid efflux. (b) Conditions presumably enhancing the internal sodium concentration, such as dilution in sodium-containing media in the presence of nigericin, enhanced the efflux about 10-fold. Such stimulation was not observed with vesicles previously loaded with sodium. Efflux into chloride-containing media was only slightly enhanced by triphenyltin chloride; on the other hand, this compound strongly inhibited efflux into chloride-free media. (c) A freeze–thaw technique was used to load GABA passively into the vesicles (thus without the need to introduce the external sodium and chloride required for the active loading). The efflux from such vesicles was dependent on the simultaneous presence of internal sodium and chloride ions. It is concluded that the efflux of γ-aminobutyric acid is in many aspects symmetrical with its influx [Kanner, B. I. (1978) *Biochemistry 17*, 1207-1211]. It appears that in order for γ-aminobutyric acid to interact with the carrier both sodium and chloride have to be present on the same side as the γ-aminobutyric acid. The simplest way to account for these and the previous data is to postulate cotransport of sodium, chloride, and γ-aminobutyric acid through the carrier.

Membrane vesicles isolated from various bacterial and mammalian cells have proved extremely useful for the study of active transport (cf. Kaback, 1974; Aronson & Sacktor, 1974; Hopfer et al., 1973; Colombini & Johnstone, 1974; Lever, 1977; Rudnick, 1977). Some of their advantages include the possibility of using well-defined energy sources and the lack of metabolism and storage in subcellular organelles. Recently, the use of membrane vesicles has been extended to those apparently originating from the synaptic plasma membrane (Kanner, 1980) for the study of sodium-dependent neurotransmitter transport in rat brain (Kanner, 1978; Kanner & Sharon, 1978). These transport systems have been implicated in the termination of transmitter action on postsynaptic receptors (Iversen, 1971). These latter transport studies have provided direct evidence that the general concept that solute accumulation can be achieved by cotransport with ions (Crane, 1965; Riggs et al., 1958; Mitchell, 1963) also applies to brain. 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-glutamic acid

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(Kanner & Sharon, 1978). 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, 1978). The gradients of these ions may also serve as additional driving forces for the above transport systems (Kanner, 1978; Kanner & Sharon, 1978). A similar situation has been reported for serotonin transport in platelet membranes (Rudnick, 1977; Nelson & Rudnick, 1979) and glutamate transport in renal brush border vesicles (Burckhardt et al., 1980).

The absolute ion dependency of a transport process as well as the ability of gradients of these ions to drive accumulation can be considered as strong evidence in favor of cotransport of the solute with these ions. However, since many transport systems are electrogenic, an ion whose gradient may be expected to create a membrane potential with the right polarity to drive transport need not necessarily be translocated through the carrier under study. The chloride gradient (out > in) in the case of GABA may be an example since it is thought to be a rather permeant anion, and a membrane potential (interior negative) enhances GABA transport (Kanner, 1978). This argument would, of course, not apply for sodium. However, chloride has an essential role in transport—even in the presence of both a sodium gradient (out > in) and a membrane potential generated with a potassium gradient (in > out) and valinomycin, chloride is still required (Kanner, 1978). If cotransport with chloride and sodium would be operative, one would expect that for efflux of GABA sodium and chloride are required internally. This is opposed to influx where both are required externally.

In this communication, we report our studies on the efflux of GABA. One of the central findings is that this process appears to require sodium as well as chloride ions, both internally.

Experimental Procedures

Methods

Preparation of Membrane Vesicles. Membrane vesicles from 14-day-old female rats were prepared and stored as described (Kanner, 1978). The vesicles were preloaded prior to the transport assays with 0.1 M potassium phosphate +1 mM $MgSO_4$, pH 6.8, unless indicated otherwise in the legends to the figures. Protein was determined according to the Lowry method (Lowry et al., 1951).

Efflux from Actively Loaded Vesicles. Vesicles (5 μL, 4–14 mg/mL) preloaded with the buffer indicated in the figure legends were diluted into 20 μL of 0.1 M NaCl and 1 mM $MgSO_4$ containing 0.25–0.5 μCi of [2,3-^{3}H]GABA (16–40 Ci/mmol) unless indicated otherwise. After the indicated times allowed for influx, the incubation mixtures were diluted 20-fold with the specified efflux solutions. 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. This value was very similar to that obtained by stopping the reaction without addition of the efflux solution. Filtration, washing, and counting of the radioactivity on the filters were done as described (Kanner, 1978).

Efflux from Passively Loaded Vesicles. Vesicles were collected from the liquid air refrigerator, thawed at 37 °C, and diluted to 10–20-fold with an ice-cold solution composed of the desired internal medium, but without radioactive GABA. 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 12–15 μCi of [2,3-^{3}H]GABA (28.2 Ci/mmol) were added per 100–200 μL of the concentrated vesicle suspension, which was subsequently frozen in liquid air. The vesicles were then thawed in an ice–water bath. Efflux was initiated by diluting 5-μL samples into 200 μL of a medium containing 0.1 M potassium phosphate, pH 6.8, 1 mM $MgSO_4$, and 5 μM CCCP. Reactions were terminated by filtration, washed, and counted as described above. Zero-time values were obtained as described above. All values were corrected for by subtracting zero-time values, which were obtained by replacing the 0.15 M NaCl containing stop and wash solution with cold distilled water (to release the internalized counts by osmotic shock).

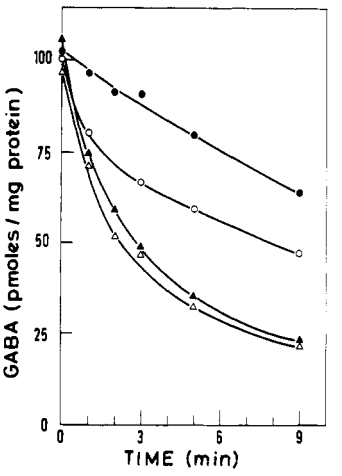

FIGURE 1: Dilution-induced efflux of GABA efflux of GABA from actively loaded membrane vesicles. Membrane vesicles (5 μL, 32.6 μg of protein) were diluted into 20 μL of 0.1 M NaCl + 1 mM $MgSO_4$ containing 0.25 μCi of [^{3}H]GABA (36.9 Ci/mmol). After 5 min of influx, the vesicles were diluted (t = 0 on abscissa) by addition of 0.5 mL of the efflux solution containing 90 mM NaCl + 10 mM NaP_i, pH 6.8, + 1 mM $MgSO_4$. At the times indicated on the abscissa, the efflux reaction was stopped. Additions to the efflux solution: (●) none; (○) 5 μM CCCP; (▲) 20 μM GABA; (△) 5 μM CCCP + 20 μM GABA.

Materials

[2,3-^{3}H]GABA was obtained either from New England Nuclear or from the Nuclear Research Centre, Negev, Israel. Valinomycin and CCCP were from Sigma, and triphenyltin chloride was purchased from Eastman Kodak. Nigericin was a generous gift of Dr. R. J. Hosley from Eli Lilly.

Results

Figure 1 illustrates a typical efflux.experiment. Membrane vesicles previously loaded with potassium phosphate are diluted into a medium containing sodium chloride and [2,3-^{3}H]GABA and incubated for 5 min. Under these conditions, active transport of GABA occurs until the steady-state plateau level is reached (Kanner, 1978). At this time, the reaction mixture is diluted 20-fold with buffered NaCl, resulting in a slow efflux (Figure 1). Since GABA transport is an electrogenic process (Kanner, 1978), lack of charge compensation might be one reason for the slow efflux rate. Indeed, the presence of 5 μM of the proton conductor CCCP, which is expected to prevent the build up of any membrane potential, enhances the rate of efflux severalfold (Figure 1). Unlabeled GABA at 20 μM, which is 8-fold the K_m (Kanner, 1978), when added to the

[1] Abbreviations used: GABA, γ-aminobutyric acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DTT, dithiothreitol.

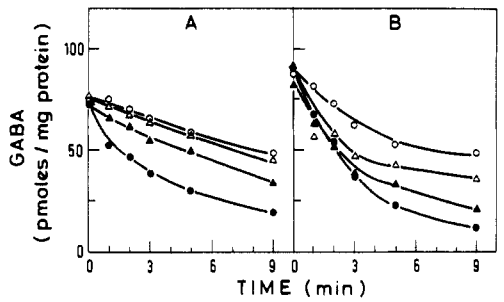

FIGURE 2: Effect of external sodium and chloride ions on the stimulation of efflux by GABA. Influx (5 min) was performed exactly as described above by using 34.5 μg (panel A) or 31.4 μg (panel B) of protein. The composition of the efflux media: (A) (○) 90 mM NaCl + 10 mM NaP_i, pH 6.8, + 1 mM $MgSO_4$; (●) the same + 20 μM GABA; (△) 90 mM LiCl + 10 mM LiP_i, pH 6.8, + 1 mM $MgSO_4$; (▲) the same + 20 μM GABA. (B) (○) 90 mM NaCl + 10 mM NaP_i, pH 6.8, + 1 mM $MgSO_4$; (●) the same + 20 μM GABA; (△) 100 mM NaP_i + 1 mM $MgSO_4$; (▲) the same + 20 μM GABA.

efflux medium converts efflux into exchange which, of course, is electroneutral. This results in an even larger stimulation of loss of radioactive GABA from the vesicles (Figure 1). This observation may be explained as follows. Efflux consists of at least four steps: (a) binding of substrate to the carrier on the inner surface of the membrane, (b) translocation of the loaded carrier across the membrane, (c) release of substrate on the outside, and (d) return of the unloaded carrier. If under the given conditions the return of the unloaded carrier—the step by which efflux and exchange differ—is rate limiting, external GABA would be expected to give a larger stimulation than CCCP. Consistent with this is the observation that the effects of GABA and CCCP are not additive (Figure 1). The ability of external GABA to stimulate the efflux process is dependent on the ion composition of the external medium. Maximal effects are obtained when both sodium and chloride ions are present (Figure 2). In the absence of either external sodium (Figure 2A) or chloride ions (Figure 2B) (thus, with either $[Na^+]_{in} > [Na^+]_{out}$ or $[Cl^-]_{in} > [Cl^-]_{out}$, the efflux rate of GABA is faster than when both ions are present in the external medium. This would be expected if GABA, sodium ions, and chloride ions are cotransported. On the other hand, exchange is fastest when both ions are present in the external medium (Figure 2). It is of interest to note, however, that when the efflux solution lacks either external sodium (Figure 2A) or external chloride (Figure 2B) some stimulation of efflux by external GABA is also observed.

Another possible reason for the slow efflux rates shown in Figure 1 is that the internal concentration of sodium and/or chloride ions is limiting for the efflux process. Some sodium and chloride are likely to be present in the internal space at the time of the initiation of efflux because it takes time (5 min in these experiments) to load the vesicles with GABA (for which these ions are required). In the experiments depicted in Figure 3, the ionophore nigericin, which exchanges both potassium and sodium ions with protons, is used to manipulate the internal sodium ion concentration during the efflux process. Efflux of GABA from vesicles originally loaded with potassium phosphate into a sodium chloride containing solution is slow, but is very strongly—some 10-fold—enhanced by nigericin (Figure 3A). This is not observed when the dilution is done into lithium chloride (Figure 3A). The latter condition would not lead to an increase of the internal sodium concentration. The effect of nigericin cannot be explained by removal of the internal potassium since the same results are obtained with vesicles preloaded with lithium phosphate (Figure 3B). When

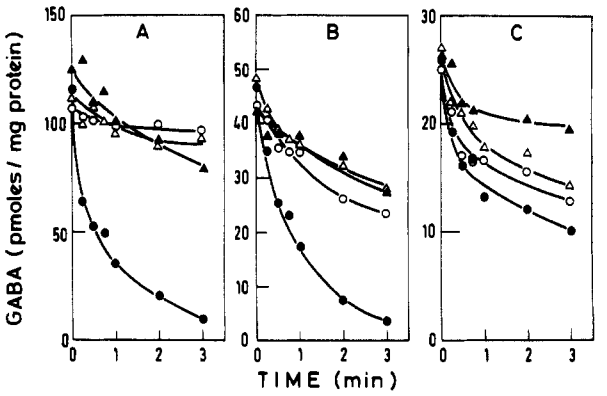

FIGURE 3: Effect of nigericin on dilution-induced GABA efflux from actively loaded membrane vesicles. After 5 min of efflux, the vesicles were diluted into the following: (○) 90 mM NaCl + 10 mM NaP_i, pH 6.8, + 1 mM $MgSO_4$; (●) the same + 5 μM nigericin; (△) 90 mM LiCl + 10 mM LiP_i, pH 6.8, + 1 mM $MgSO_4$; (▲) the same + 5 μM nigericin. The vesicles were preloaded with (A) 0.1 M KP_i + 1 mM $MgSO_4$; (B) 0.1 M LiP_i + 1 mM $MgSO_4$; (C) 0.1 M NaP_i + 1 mM $MgSO_4$. The amounts of protein used per assay were 18.0, 22.3, and 18.7 μg, respectively.

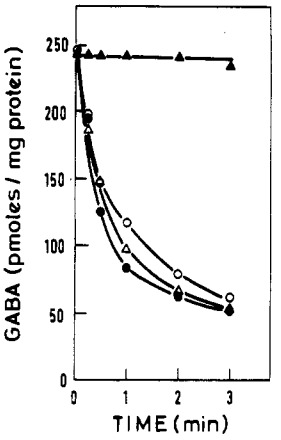

FIGURE 4: Effect of triphenyltin chloride on GABA efflux. Membrane vesicles (5 μL, 72 μg of protein) were actively loaded by diluting into 45 μL of 0.1 M NaCl + 1 mM $MgSO_4$ containing 0.5 μCi of [^{3}H]GABA (18.4 Ci/mmol). After 5 min of influx, efflux was started by diluting in 1 mL of the efflux media containing 5 μM nigericin. When triphenyltin chloride was present, nigericin was added 30 s after dilution. On the abscissa, $t = 0$ in all cases is the moment when the vesicles were exposed to nigericin. (○) 90 mM NaCl + 10 mM NaP_i, pH 6.8, + 1 mM $MgSO_4$ + 5 μM nigericin; (●) the same + 5 μM triphenyltin chloride; (△) 0.1 M NaP_i, pH 6.8, + 1 mM $MgSO_4$ + 5 μM nigericin; (▲) the same + 5 μM triphenyltin chloride.

the vesicles are previously already loaded with sodium phosphate, the effect of nigericin upon dilution into a sodium chloride solution is very minor, probably since the vesicles contain already sufficient sodium (Figure 3C). On the other hand, if these vesicles are diluted into a lithium chloride solution, the effect of nigericin is to slow efflux (Figure 3C), presumably because of the removal of internal sodium by nigericin.

With regard to effects of internal chloride, all experiments (Figure 4) were performed with sodium-containing efflux media in the presence of nigericin, so that internal sodium would not be limiting. Triphenyltin chloride, which exchanges chloride with hydroxyl ions (Selwyn et al., 1970), has been used. Triphenyltin chloride does not markedly enhance GABA efflux into a sodium chloride containing solution, possibly because enough chloride may enter during the influx stage. On the other hand, when the efflux medium contains sodium phosphate, addition of triphenyltin chloride results in a total inhibition of GABA efflux. Under these conditions, internal

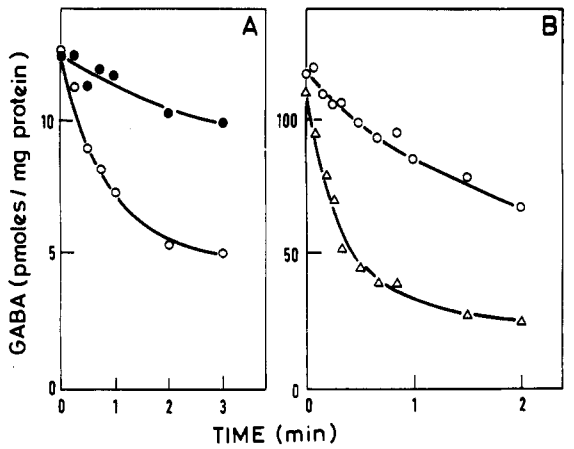

FIGURE 5: Effect of internal ions on GABA efflux. (A) Effect of internal sodium ions on GABA efflux. Vesicles were loaded with (○) 0.1 M NaP_i, pH 6.8, + 1 mM $MgSO_4$ or (●) 0.1 M LiP_i, pH 6.8, + 1 mM $MgSO_4$. Influx was carried out as described under Experimental Procedures for 1 min. The influx medium contained 100 mM NaCl + 1 mM $MgSO_4$ for the sodium-loaded vesicles and 40 mM NaCl + 60 mM LiCl + 1 mM $MgSO_4$ for the lithium-loaded vesicles. Both media contained 0.25 μCi of [^{3}H]GABA (16.8 Ci/mmol); 31.7 μg of sodium-loaded or 30.7 μg lithium-loaded vesicles were used per assay. The dilution medium contained 90 mM NaCl + 10 mM NaP_i + 1 mM $MgSO_4$. (B) Effect of internal chloride ions on GABA efflux. Vesicles were loaded with (○) 0.1 M KP_i, pH 6.8, + 1 mM $MgSO_4$ or (△) 90 mM KCl + 10 mM KP_i, pH 6.8, + 1 mM $MgSO_4$. Influx was carried out for 1 min in the media 35 mM NaCl + 65 mM sodium glucuronate + 1 mM $MgSO_4$ (phosphate-loaded vesicles) or 100 mM NaCl + 1 mM $MgSO_4$ (chloride-loaded vesicles), in both cases containing 0.5 μCi of [^{3}H]GABA (16.8 Ci/mmol). The efflux media contained in both cases 0.1 M NaP_i, pH 6.8, + 1 mM $MgSO_4$ + 5 μM nigericin + 5 μM CCCP.

chloride would be depleted. However, DTT which can destroy triphenyltin chloride (B. Raveh and B. Kanner, unpublished results) can partially restore this total inhibition, indicating that the effect of triphenyltin chloride is more complex. Indeed, it appears that triphenyltin chloride has direct effects on the carrier (B. Raveh and B. Kanner, unpublished results). Therefore, additional experiments have been performed to clarify the chloride requirement and to provide additional support for the sodium requirement for GABA efflux.

The experiments described in Figure 5 are designed to examine the effect of the internal ion composition on GABA efflux directly. The vesicles are loaded with solutions of varying ion composition and the influx period is shortened to 1 min, as opposed to the previous experiments where the influx stage was allowed to proceed for 5 min, in order to minimize entrance of sodium and/or chloride which needs to be present in this stage. Moreover, the external ion composition of the influx medium is manipulated by decreasing sodium or chloride to obtain similar GABA levels in the various types of vesicles after 1 min, the time when efflux is initiated. It can be seen that under such conditions efflux of GABA from vesicles loaded with sodium phosphate into buffered sodium chloride is much faster than that from vesicles loaded with lithium phosphate (Figure 5A). Similarly, GABA efflux from vesicles loaded with buffered potassium chloride into sodium phosphate is much faster than that from vesicles loaded with potassium phosphate (Figure 5B). In this experiment, the proton ionophore CCCP was included in order to prevent buildup of a membrane potential. Moreover, nigericin has been included in this experiment to prevent limitation by sodium. It should be stressed that the use of short times for the influx stage is of prime importance. The requirement of internal sodium and chloride by this type of experiment could not be shown when the influx stage was 5 min (data not shown).

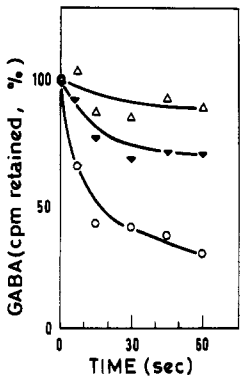

FIGURE 6: Effect of internal ions on GABA influx from passively loaded membrane vesicles. The vesicles were passively loaded by freezing and thawing, and the efflux was measured, all as described under Experimental Procedures. The ion composition of the loading media of the vesicles was (○) 90 mM NaCl + 10 mM NaP_i, pH 6.8, + 1 mM $MgSO_4$ – 109 μg/assay; (△) 0.1 M NaP_i, pH 6.8, + 1 mM $MgSO_4$ – 116.5 μg/assay; (▼) 90 mM LiCl + 10 mM LiP_i, pH 6.8, + 1 mM $MgSO_4$ – 100.5 μg/assay.

Although the above experiments strongly suggest that in order for GABA efflux to occur both sodium and chloride have to be present internally, another type of experiment has been performed in whch the stage of influx (requiring sodium and chloride) is circumvented altogether. Passive equilibration of membrane vesicles with GABA could not be used since in the absence of sodium (Kanner, 1978) or chloride (B. Kanner, unpublished results) much lower levels of GABA were obtained than in the presence of both ions, even after incubation overnight. However, recently it has been shown that membrane vesicles from kidney can be loaded by freezing and thawing (Van Dommelen & De Jonge, 1980). This finding was applied to the synaptic plasma membrane vesicles. It can be seen in Figure 6 that vesicles passively loaded in such a manner with radioactive GABA and both sodium and chloride when diluted into potassium phosphate in the presence of CCCP display a rapid efflux of GABA. When either sodium or chloride is omitted, a marked reduction of efflux occurs (Figure 6). It is of interest to note that in the absence of internal chloride, efflux of GABA is even slower than in the absence of internal sodium. Similar results were obtained by using a sodium chloride containing efflux medium. Moreover, vesicles loaded in such a manner with lithium phosphate or potassium phosphate exhibit GABA influx which is absolutely dependent on the presence of both sodium and chloride ions in the external medium, even in the presence of CCCP or the potassium ionophore valinomycin (data not shown). Thus, also in the vesicles exposed to the additional freeze–thaw cycle, the flux of GABA in either direction is dependent on the presence of both sodium and chloride on the same side of the carrier together with GABA.

Discussion

It appears that efflux can be limited by the buildup of a membrane potential (Figure 1). This also, at least in part, explains why exchange, which is electroneutral, is much faster than efflux (Figure 1). Since exchange is also considerably faster than efflux in the presence of CCCP (Figure 1), it is possible that the return of the unloaded carrier is limiting under these conditions. Similar implications have also been made for the lactose carrier of *Escherichia coli* (Kaczorowski & Kaback, 1979) and the 5-hydroxytryptamine carrier of platelets (Nelson & Rudnick, 1979).

The influx of GABA is absolutely dependent on the presence of external sodium and chloride (Kanner, 1978). The ability of external GABA to stimulate efflux of radioactive GABA

is dependent on the presence of external sodium and chloride as well. However, this dependency is not absolute (Figure 2). It is not clear if the small stimulation by GABA in either lithium chloride (Figure 2A) or sodium phosphate (Figure 2B) solutions is due to carry-over of the sodium chloride containing influx solution. Another possibility is the following: The sodium, chloride, and GABA containing carrier translocates from the inside to the outside, discharges the labeled GABA, and rebinds the unlabeled GABA before it can discharge its sodium and chloride. This allows the carrier to return to the inside in its loaded form. This movement of the carrier is presumably faster than its movement in the unloaded form.

The central point in this article is the observation that efflux of GABA requires both internal sodium and chloride. This conclusion has been reached by using several experiments in which the internal sodium and chloride content is manipulated by ionophores (Figures 3 and 4) or the loading conditions both in the case of active (Figure 5A,B) or passive (Figure 6) loading of the carrier. As stated already, the effect of triphenyltin chloride (Figure 4) is more complex than causing the removal of internal chloride which in turn results in decreased efflux. The experiment may be alternatively explained by assuming that triphenyltin chloride is an inhibitor of the GABA carrier and that the inhibition is much stronger in chloride-deficient media than in chloride-containing ones. Indeed, DTT, which is able to destroy the ionophore, can, albeit partly, reverse its inhibition. That reversal cannot be explained by removal of chloride. However, the important role of internal chloride for efflux is unambiguously shown by the experiments shown in Figures 5B and 6. The two methods used to load the vesicles with GABA—actively or passively—have different advantages and drawbacks for the study of efflux. Active loading results in high sensitivity since GABA is transported against its concentration gradient (Kanner, 1978) and high levels of internal radioactivity are reached. On the other hand, sodium and chloride have to be present in the influx stage, and the amount of these ions taken up by the vesicles is not known. However, the problem can be dealt with by using suitable combinations of ionophores and efflux media (Figures 3 and 4) or by shortening the duration of the influx stage (Figure 5). Passive loading has the advantage of circumventing the need to introduce sodium and chloride ions altogether. On the other hand, the sensitivity is lower. Notwithstanding the differences in both methods, both give rise to the conclusion that both internal sodium and chloride are required for efflux of GABA.

It is of interest to note that a large proportion of the vesicles passively loaded with GABA lose GABA in a manner which is dependent on both internal sodium and chloride. This implies that a large proportion of the vesicles contain a functional GABA carrier. The biological implication of this finding is not clear. One possibility is that most nerves (not only the GABA-ergic ones) have GABA carriers, although only the GABA-ergic 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.

It appears that (a) the carrier requires the presence of both sodium and chloride on the outside for influx (Kanner, 1978), (b) the carrier requires both ions internally for efflux, (c) the level of GABA accumulation is determined by the gradient of sodium and chloride ions (both out > in) (Kanner, 1978), (d) the transport is electrogenic—positive charge(s) moving in the direction of GABA flow (Kanner, 1978), and (e) the ion dependence of the carrier is not a result of charge compensation of the translocation cycle. These data strongly suggest that GABA is translocated through the carrier with sodium as well as with chloride ions. Assuming that it is the zwitterionic form of GABA which is translocated, the stoichiometry will be $n\mathrm{Na}^+$:$m\mathrm{Cl}^-$:GABA ($n > m$). It should be mentioned that conclusive proof for such a model will be the demonstration of GABA-dependent sodium and chloride transport. The membrane vesicles are too heterogeneous for this purpose and apparently contain a population which is rather leaky to sodium (Kanner, 1980) and presumably also to chloride ions. The V_{max} for sodium transport was found to be in the same order of magnitude as that through the sodium channel (B. Kanner, unpublished results), which is some 2 orders of magnitude larger than the V_{max} for GABA transport (Kanner, 1978, 1980). These experiments will only have a chance of being successful with proteoliposomes inlaid with the purified GABA carrier. The latter is, at present, being isolated in our laboratory (Agmon & Kanner, 1980).

It should be stressed that although the carrier is symmetrical with regard to the sodium and chloride requirement of GABA transport, this does by no means mean that the transmembrane orientation of the carrier is symmetrical. Studies with the purified carrier will hopefully shed more light on this problem.

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