Active Transport of γ-Aminobutyric Acid by Membrane Vesicles Isolated from Rat Brain†

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ABSTRACT: (1) Membrane vesicles which are probably derived from synaptic plasma membranes have been isolated from rat brains. Active transport of γ-aminobutyric acid into these vesicles has been demonstrated with artificially imposed ion gradients as the sole energy source. This process can be driven either by a Na^+ gradient (out > in) or by a gradient of any of a small number of small monovalent anions (out > in) of which chloride is by far the most effective. Transport of γ-aminobutyric acid was absolutely dependent on the simultaneous presence of both types of ions in the external medium. (2) Transport is inhibited about 50% by the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone and is enhanced up to twofold in those cases, in which K^+-loaded vesicles are diluted into NaCl when valinomycin is present. Stimulations by SCN^- were noted when the main driving force for transport was a Na^+ gradient. This process thus appears to be an electrogenic process, which is stimulated by a membrane potential (interior negative). (3) The transport process, the K_m of which has been determined to be 2.5 μM, is strongly inhibited by ionophores able to collapse sodium gradients, such as nigericin and gramicidin. Also strong inhibition has been noted with chloropromazine, triphenyltin chloride, and *p*-hydroxymercuribenzoate. In contrast transport was not affected by ouabain and only slightly inhibited by arsenate. (4) The results provide direct evidence for Na^+-coupled active γ-aminobutyric acid transport by rat brain membrane vesicles. The anion dependence of the process is discussed.

High affinity, sodium dependent, uptake systems for a variety of neurotransmitters have been detected in brain preparations, such as synaptosomes (Iversen, 1971, 1973; Kuhar, 1973; Bennett et al., 1974). These uptake systems have been implicated in termination of transmitter action on postsynaptic receptors (Iversen, 1971) as well as in maintaining constant levels of transmitters in the neurons (Hedgvist & Stjärne, 1969). This uptake appears to be active and is inhibited by various metabolic poisons such as 2,4-dinitrophenol, cyanide, or ouabain (Iversen & Neal, 1968; White & Keen, 1970). In these experiments glucose has been present as an exogenous energy source. The sensitivity of these transport systems to ouabain and their sodium dependence have suggested that the sodium ion and also the potassium ion gradients across the plasma membranes of presynaptic nerve terminals and/or glial cells may provide the driving forces for active neurotransmitter uptake (Bogdanski, et al., 1968; Martin & Smith, 1972; Martin, 1973; Holtz & Coyle, 1974). This is in accordance with the hypothesis, that solute accumulation can be achieved by cotransport with ions, which move down their electrochemical potential gradient into the cell or cell organelle (Riggs et al., 1958; Crane, 1965; Mitchell, 1963). In the case of mitochondrial, chloroplast, or bacterial membranes, these ions are protons and in these cases the gradients are generated either by coupled electron flow or by the reversible proton translocating ATPase (Mitchell, 1966). In the case of the cellular plasma membranes, the coupling ions are thought to be sodium ions and the device which creates the gradient is the $(Na^+ + K^+)$-ATPase system (Crane, 1965).

Membrane vesicles isolated from various bacterial and mammalian cells have proved extremely useful for the study of active transport (Kaback, 1974; Aronson & Sacktor, 1974; Hopfer et al., 1973; Colombini & Johnstone, 1974; Lever, 1977; Rudnick, 1977). Some of their advantages include the possibility to use well-defined energy sources and the lack of metabolism and storage in subcellular organelles.

This study describes the active transport of the neurotransmitter γ-aminobutyric acid into a membrane vesicle preparation, obtained after osmotic shock of rat brain synaptosomes. Active transport of GABA[1] was measured in the absence of any exogeneous energy source, except for ion gradients. The dependence of the process of the ion gradients and the membrane potential is described.

Experimental Procedure

Methods

Preparation of Membrane Vesicles. These vesicles were derived from synaptosomal fractions. The latter were prepared from rat brains in a manner similar to published methods (Abdel-Latif, 1966; Haldar, 1971; Cotman, 1974). In this investigation 14-day-old female animals were used. All steps, subsequent to decapitation and removal of the cortex, were performed at 0–4 °C. The brains of 30 rats were homogenized in ME, 10 mL/brain, using a Dounce all glass homogeniser A (three strokes). After centrifugation of the homogenate at 3000*g* for 10 min, a crude mitochondrial pellet was obtained by centrifugation of the supernatant for 15 min at 27 000*g*. The mitochondrial pellet was resuspended in about 5 mL of ME and layered over three discontinuous gradients, each consisting of five layers of 20%, 16%, 12%, 8%, and 2% Ficoll in ME, respectively. The gradients were then centrifuged in a SW 25.2 rotor at 20 000 rpm for 90 min. The 8–12% interfaces were

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[1] Abbreviations used: ATPase, adenosine triphosphatase; GABA, γ-aminobutyric acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ME, 0.3 M mannitol–1 mM K-EDTA, pH 7.4; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane; I, the solvent system: isopropanol 70% (v/v), ammonia 30% (v/v).

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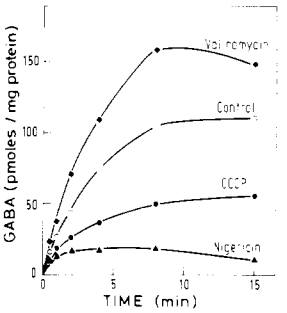

FIGURE 1: Transport of GABA by plasma membrane vesicles. Transport assays were performed as described in Methods. Ionophores: (○—○) none; (♦—♦) valinomycin, 2.5 μM; (●—●) CCCP, 5 μM; (▲—▲) nigericin, 5 μM.

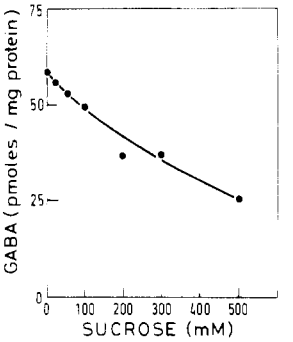

FIGURE 2: Effect of external osmolarity on the extent of GABA transport. Transport was measured as described in Methods. The external solution also contained the indicated amounts of sucrose, given as final concentrations. The extent of transport was determined after 3 and 6 min in order to ensure that the plotted values—those at 6 min—represent the extent of transport.

collected and diluted with approximately 3 volumes of ME. After centrifugation, 20 min at 27 000*g*, the pellet (synaptosomal fraction) was homogenized (three strokes) in a minimal volume of 5 mM Tris-HCl + 1 mM K-EDTA (pH 7.4). Subsequently the volume was adjusted to 40 mL using the same buffer. After stirring for 45 min, the suspension was centrifuged again—20 min at 27 000*g*—and the pellet was resuspended in 0.32 M sucrose, containing 5 mM Tris-SO_4, 1 mM $MgSO_4$, 0.5 mM EDTA (pH 7.4), at a protein concentration of 5–10 mg/mL. Aliquots were stored in liquid air. Under these storage conditions the transport ability of the membrane vesicles was stable for at least 2 months.

Transport Assays. Transport assays were performed essentially as described (Rudnick, 1977). Membrane vesicles were rapidly thawed at 37 °C and loaded (Rudnick, 1977) and were then resuspended in loading solution to a final concentration of 1–2 mg of protein per mL. The composition of the loading solution was 0.1 M potassium phosphate (pH 6.8) and 1 mM $MgSO_4$, unless stated otherwise in the legend to Figure 3 and in Table I. Of the latter suspension, 20 μL was added to 180 μL of external solution, consisting of 0.1 M NaCl + 1 mM $MgSO_4$, and 0.14 μM [2,3-^{3}H]GABA (approximately 4000 cpm/pmol), unless stated otherwise in the figure legends. Inhibitors and ionophores, when present, were usually added to the external solution prior to the addition of the membranes. After incubation for various times at room temperature (23–25 °C), reactions were terminated by the addition of 2 mL of ice-cold 0.15 M NaCl (at 0–4 °C, no GABA transport was detected) and filtration through membrane filters (Schleicher and Schuell, 0.45-μm pore size). The filters were then washed once with 2 mL of 0.15 M NaCl. Stopping the reaction, filtration and washing took about 15 s. The washed filters were dried and counted using liquid scintillation counting. The counting efficiency was about 30%. All experimental values were corrected for by substracting zero-time values; membrane vesicles were added after the stopping solution. It was established that, in the protein concentration range used, the transport activity was proportional to the amount protein added.

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

Total Internal Volume. This was determined as described (Padan & Rottenberg, 1973). [^{14}C]Inulin was used to measure the external osmotic space and ^3H_2O was used to determine the total water. The total intravesicular space determined in this manner was found to be 7.4 ± 1.2 μL/mg of protein.

Internal Volume to Which GABA Has Access. This was determined after incubating concentrated membrane vesicles (5–10 mg of protein/mL) in 90 mM NaCl + 10 mM potassium phosphate (pH 6.8) in a final volume of 0.16 mL with GABA (25 Ci/mmol; 7 × 10^6 cpm at 0–4 °C). At various times the amount of GABA which had entered the membrane vesicles was determined by rapid filtration and washing. Blanks were obtained by diluting parallel samples of vesicles into 20 volumes of incubation mixture at room temperature for various times (efflux was complete in 20–30 min).

The amount of internal GABA was obtained from the difference of the zero-time and the 30-min time points. Under these equilibrium conditions (after 24 h), the the maximal amount taken up by the vesicles was used to calculate the "GABA volume". This was found to be 3.8 ± 0.7 μL/mg of protein. When Na^+ ions were replaced by Li^+ ions, the maximal amount of GABA taken up by the vesicles dropped by at least 80%.

Materials

[2,3-^{3}H]GABA was obtained from New England Nuclear and ^3H_2O and [^{14}C]inulin were from Amersham. Valinomycin, gramicidin, and CCCP were purchased from Sigma Chemical Co. Nigericin was a generous gift of Dr. R. Hosley from Eli Lilly.

Results

The data presented in Figure 1 illustrate the uptake of [2,3-^{3}H]GABA into membrane vesicles derived from rat brain. An artificial sodium ion gradient is created by diluting (at time zero) the membrane vesicles loaded with potassium phosphate tenfold into a NaCl solution containg [2,3-^{3}H]GABA. In this experiment approximately 110 pmol of GABA per mg of protein was taken up. $MgSO_4$ (1 mM) was included because it had a slightly stimulatory effect on transport rates and extents were obtained (data not shown). At longer times the amount of GABA taken up decreases (at 60 min about 50% of maximum, at 2 h less than 20% of maximum)—presumably due to the decay of the artificially imposed ion gradients. The extent of uptake was dependent on the batch of membrane vesicles and ranged from 50 to 180 pmol/mg of protein (using 0.14 μM GABA). The process exhibited saturation kinetics. From Lineweaver-Burk plots, a K_m of 2.5 μM and a V_{max} of 750 pmol/min per mg of protein have been found. This value for K_m agrees well with that published for GABA transport in intact synaptosomes (Martin, 1973).

When the osmolarity of the extravesicular volume is increased, the extent of GABA accumulation is diminished (Figure 2). In addition exposure of the membrane vesicles to water results in the loss of most of the radioactivity. These findings indicate that GABA accumulates in the intravesicular

TABLE I: The Effect of Various Inhibitors and of Internal Ions on GABA Transport.

Inhibitor (μM)[a]	Initial rate (% of control)	Extent (% of control)
Inhibitor (μM)[a]		
Ouabain (100)	100	100
P-hydroxymercuribenzoate (100)	23	1
Triphenyltin chloride (5)	0	0
Gramicidin D (50)	58	27
Chloropromazine (500)	0	0
Loading solution		
K-phosphate	100	100
K-Mes	94	85
K-Mes + K-arsenate	64	65
K-Tricine	157	109
Tris-phosphate	60–100	70–100
Sodium phosphate	25	23
Tris-Cl	27	34
NaCl	0	0

[a] Concentration in μM is in parentheses. Transport was measured as described under Methods. For the initial rate measurements, 30-s time points were taken and for the extent of 6-min time points (inhibitors). When the effects of internal ions were examined, initial rate values were obtained by averaging the rates based on the 30-s and 1-min time points, and the extent was computed by averaging values based on the 4- and 6-min time points. The composition of the loading solutions was as described under Methods (inhibitors) or as indicated in the table. The concentration of these solutions was 0.1 M, except for the arsenate containing solution. In the latter case both components were present at 50 mM. All loading solutions also contained 1 mM $MgSO_4$.

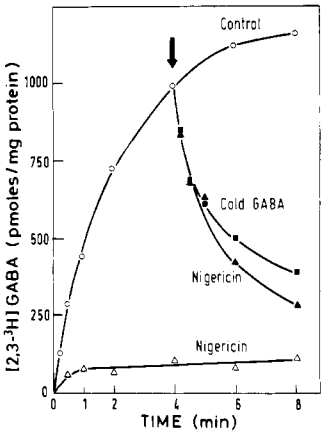

FIGURE 3: Efflux of [2,3-^{3}H]GABA from membrane vesicles induced by either nigericin or unlabeled GABA. Transport assays were performed as described under Methods. Membrane vesicles were diluted at time zero into the external solution, containing GABA, 5.6 μM (218 000 cpm). At 4 min, efflux was started by addition of nigericin, 5 μM final concentration (▲—▲); or by addition of unlabeled GABA, 336 μM final concentration (■—■); no addition (○—○); nigericin (5 μM) added at time zero (△—△).

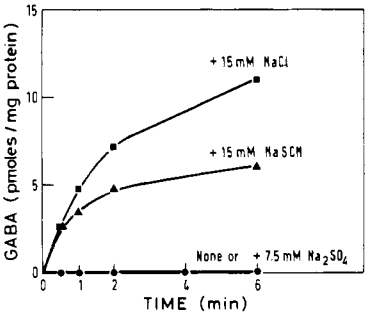

FIGURE 4: Dependency of GABA transport on external small monovalent anions. Transport was measured as described in Methods. The composition of the "loading solution" was 100 mM Tris-phosphate (pH 6.8) containing 1 mM $MgSO_4$. Membrane vesicles were diluted at time zero into 0.18 mL of the "external solution", containing GABA (28 pmol; 562 000 cpm). The composition of the external solution was 100 mM sodium phosphate (pH 6.8) containing 1 mM $MgSO_4$. The following additions to the external solution were made: none or 7.5 mM Na_2SO_4 (●—●); 15 mM NaSCN (▲—▲); 15 mM NaCl (■—■).

space as opposed to binding to sites on the membrane surface. That the accumulated radioactivity represents unmodified GABA is supported by the fact that GABA accumulated in the vesicles (released with acid) has been found to cochromatograph with authentic GABA using thin layer chromatography (solvent system I). Using the determined value of the total intravesicular volume of 7.4 μL/mg of protein, the minimal calculated internal GABA concentration for the experiment described in Figure 1 is 14.2 μM. This thus represents a concentration gradient of at least 100-fold. Similar concentration gradients have been observed for 5-hydroxytryptamine in platelet membrane vesicles (Rudnick, 1977).

Ionophores able to collapse the Na^+ and K^+ gradients such as nigericin (Figure 1) or gramicidin (Table I) strongly inhibit GABA uptake. With the ionophore valinomycin present, an approximately 50% stimulation is noted (Figure 1). It should be mentioned that this stimulation has been found to be somewhat variable from one batch of vesicles to the other (but consistent within a given batch). Stimulation ranged from 15 to 100% in the various batches tested. Valinomycin under the experimental conditions ($[K^+]_{in} > [K^+]_{out}$) is expected to create (or enhance the magnitude of) a membrane potential (interior negative). In line with this is the inhibitory effect of the proton conductor CCCP (Figure 1) which will diminish the membrane potential. Upon addition of nigericin to membrane vesicles, which have previously been allowed to accumulate GABA, a rapid release of GABA is observed (Figure 3). A similar release is also affected by the addition of a 60-fold excess of unlabeled GABA (Figure 3). It is of interest to note that ouabain at 10^{-4} M has no effect on GABA transport (Table I). Moreover, only a slight inhibition (25%) of transport is detected with 50 mM arsenate present (Table I). Thus the possibility that the Na^+, K^+-dependent ATPase plays a role under those experimental conditions, for instance, by synthesis of ATP utilizing the artificially imposed ion gradients via this ATPase, appears unlikely. Sulfhydryl reagents and chloropromazine (Iversen & Johnston, 1971) have been found to inhibit GABA uptake in synaptosomes. These reagents are also inhibitors of GABA transport in isolated membrane vesicles (Table I). The difference in inhibition by parahydroxymercuribenzoate on initial rate and extent seems to indicate that this inhibition is not instantaneous.

Transport of GABA has been found completely dependent on external Na^+ ions. The other cations tested (Li^+, NH_4^+, $Tris^+$, and K^+) cannot replace the Na^+ ions (data not shown). The transport process is also completely dependent on the presence of small external monovalent anions, of which chloride is by far the most effective. In the presence of small monovalent anions such as thiocyanate or nitrate, both rate and extent are 20–35% of those in the presence of chloride, whereas glucuronate, phosphate, and sulfate are completely ineffective (data not shown). This absolute requirement of small monovalent anions is further exemplified in Figure 4. When vesicles loaded with Tris-phosphate are diluted into a sodium phos-

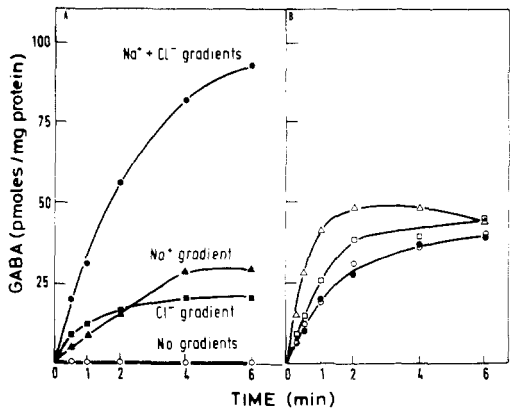

FIGURE 5 (A): Dependency of GABA transport on transmembraneous sodium and chloride ion gradients. Transport was measured as described under Methods. $Na^+ + Cl^-$ gradient (●—●): the "loading solution" consisted of 100 mM Tris-phosphate (pH 6.8) containing 1 mM $MgSO_4$. Cl^- gradient (■—■): membrane vesicles were equilibrated with 100 mM sodium phosphate (pH 6.8) containing 1 mM $MgSO_4$ × Na^+ gradient (▲—▲): the composition of the leading solution was 100 mM Tris-Cl (pH 6.8) + 1 mM $MgSO_4$. No gradients (○—○): membrane vesicles were equilibrated with 100 mM NaCl containing 1 mM $MgSO_4$. In those experiments in which the loading solution contained either Tris-Cl or NaCl virtually identical results were obtained when the internal osmolarity was adjusted (with 50 mM sucrose) to that of the other two experiments, or when 10 mM Tris-phosphate (pH 6.8) was added in the system (both in "loading" and "external solutions") to provide more buffering power. (B) Stimulation by small monovalent anions of GABA transport, driven by an artificially imposed sodium gradient. Transport was measured as described under Methods. The loading solution consisted of 100 mM Tris-Cl + 1 mM $MgSO_4$. Additions: (●—●) none; (○—○) 7.5 mM Na_2SO_4; (□—□) 15 mM NaCl; (△—△) 15 mM NaSCN.

phate containing medium no transport is observed. While addition of 7.5 mM sodium sulfate is without effect, transport of GABA is noted upon addition of the sodium salts of several small monovalent anions (Figure 4). It is of interest to note that this requirement persists even in those conditions where both a sodium gradient as well as a membrane potential are present. For example, with internal potassium phosphate and external sodium phosphate no GABA accumulation was detected, even in the presence of valinomycin (data not shown). As shown in Table I, the requirement for internal ions is by far less stringent. Thus internal potassium ions can be replaced by Tris ions and even in the presence of internal sodium transport occurs (provided that the proper anion is present, Table I). Also, internal phosphate can be substituted for by a variety of anions such as Mes or Tricine (Table I).

Artificially imposed ion gradients provide a driving force for active GABA transport. The simultaneous presence of two gradients across the membrane appears to be required for high rates of GABA uptake: (1) of a Na^+ concentration gradient ($[Na^+]_{out} > [Na^+]_{in}$); and (2) a Cl^- concentration gradient ($[Cl^-]_{out} > [Cl^-]_{in}$) (Table I and Figure 5A). When NaCl is present on both sides of the membranes, no uptake at all can be detected. In the absence of a sodium gradient, but when a chloride gradient is present (external NaCl; internal sodium phosphate), significant GABA uptake is observed. Similar uptake of GABA is observed when only a sodium gradient is present (external NaCl, internal Tris-Cl). With both ion gradients present, much higher levels of GABA are obtained than with either ion gradient alone (Table 1 and Figure 5A). The strong inhibition of GABA transport by triphenyltin chloride (Table I) seems to be in apparent harmony with the requirement of a transmembraneous chloride ion gradient, since this compound is able to collapse such gradients (Selwyn et al., 1970). However, its addition to membrane vesicles, which had previously been allowed to accumulate GABA, does not result in a rapid efflux (data not shown). Therefore it is possible that inhibition of GABA transport is caused by an interaction of this compound with the GABA carrier protein. This also might explain why in the presence of this inhibitor there is no GABA uptake, although the sodium ion gradient is not abolished (Figure 5A).

The importance of anion gradients is emphasized further in Figure 5B. With a sodium gradient as the main driving force, addition of the highly permeable thiocyanate anion results in a marked stimulation of GABA uptake especially the initial rate). Additional chloride results in a lesser stimulation and sulfate has no significant effect. These observations suggest that the stimulatory effects of anion gradients may be in part explained by their ability to impose a membrane potential across the membranes (interior negative).

Discussion

GABA transport in membrane vesicles of rat brain has many similar features to that measured in less fractionated preparations such as brain slices or synaptosomes. These features include sodium ion dependence, K_m for GABA, and inhibitor sensitivity. In the latter type of preparations the (Na^+, K^+)-ATPase plays an indispensable role and glucose is added as an exogenous energy source. In the transport studies described here, no exogenous energy source has been present except for artificially imposed ion gradients. The observations that transport is unaffected by ouabain and only slightly inhibited by 50 mM arsenate indicate that both the (Na^+, K^+)-ATPase as well as ATP are not required for active transport of GABA in membrane vesicles.

The method of preparation of the membrane vesicles follows essentially published procedures for the isolation of purified synaptosomal fractions (Abdel-Latif, 1966; Haldar, 1971; Cotman, 1974) and the subsequent isolation of shocked "synaptic plasma membranes" (Cotman, 1974). Moreover when the GABA transport activity of shocked membrane fractions obtained from the various gradient fractions is assayed for, those fractions considered to be mainly of mitochondrial origin (the lower two fractions) are almost completely devoid of this activity (data not shown). It is therefore likely that the activity is associated with synaptic plasma membranes.

GABA transport appears to be active since under optimal conditions concentration gradients of at least 100-fold were obtained. This number is based on the total internal osmotic space (7.4 μL/mg of protein). However, it is likely that not all membrane vesicles in the population have the ability to accumulate GABA. In fact the intravesicular space to which GABA has access has been determined to be 3.8 μL/mg of protein.

The concept of cotransport of sodium ions and GABA has been suggested in studies, using relatively complicated systems with various possible energy sources (Martin & Smith, 1972; Martin, 1973). The role of the $(Na^+ + K^+)$-ATPase according to this concept is indirect, namely, it creates a transmembraneous sodium gradient. The observation that a Na^+ gradient ($[Na]_{out} > [Na^+]_{in}$) drives active transport in membrane vesicles, as described here, supports the concept of cotransport of sodium ions and GABA. Further experimental evidence for this is the inhibition of GABA transport by ionophores able to collapse transmembraneous sodium ion gradients (Table I and Figures 1 and 3) as well as the ability of such ionophores to cause efflux of previously accumulated GABA from the membrane vesicles (Figure 3).

In studies with intact synaptosomes, a potassium requirement was reported (Martin & Smith, 1972; Martin, 1976).

Since potassium ions are not required for GABA transport in membrane vesicles (Table I), these results suggest that potassium ions are required in the process by which the sodium gradient is created (see also Martin, 1976), but not in the translocation of GABA.

Various experimental observations, such as the effects of valinomycin and CCCP (Figure 1), leave little doubt that this GABA transport is electrogenic. When external potassium is added to membrane vesicles loaded with $Tris^+$, inhibition of GABA transport results and this inhibition is enhanced by valinomycin (data not shown). This latter set of conditions would tend to create a membrane potential with the polarity opposite to that required for GABA transport. Since in this type of experiment KSCN is the least inhibitory of all potassium salts tested (data not shown), it would appear that also in the vesicles involved in GABA transport thiocyanate is an extremely permeant anion and more permeable than chloride. Under conditions where the sodium ion concentration gradient is the major driving force for active uptake, the addition of a small amount of thiocyanate ions results in a twofold stimulation of the initial rate of GABA uptake (Figure 5B). In addition, very recently, experiments with synaptosomes were reported consistent with an involvement of the membrane potential in GABA transport (Blaustein & King, 1976).

One of the less expected results is the absolute dependence of the process on chloride ions (or some other small monovalent anions). Moreover, GABA transport can be driven by a chloride ion gradient alone (Figure 5A and Table I). These and other observations are very similar to those on 5-hydroxytryptamine transport by plasma membrane vesicles isolated from human blood platelets (Rudnick, 1977). Although it appears that all these ions are quite permeable and therefore are able to generate a membrane potential with the right polarity for GABA transport, this is not sufficient to explain the requirement for these anions. When both an inward sodium ion concentration gradient and an outward potassium gradient are applied in the presence of valinomycin, there is no GABA transport unless any of a number of small monovalent anions are present—of which chloride is the most effective one (Figure 4). One possibility is that chloride ions have a dual role in this process—one is to create a membrane potential of the right size, the other is to occupy an external chloride binding site on the carrier in order to activate it. Alternatively, chloride ions may be translocated across the membrane together with GABA and sodium ions with a stoichiometry of $n Na^+$:$m Cl^-$:GABA ($n > m$ to explain the electrogenic phenomena). This alternative would be in harmony with a suggestion that 2 or more sodium ions are translocated per GABA molecule (Martin, 1973, 1976; Blaustein & King, 1976).

Direct flux measurements are required to decide between these two possibilities. Purification of the detergent solubilized carrier and its subsequent incorporation into artificial lipid vesicles may be necessary to be able to perform these measurements.

Acknowledgments

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