# MECHANISM OF TRANSPORT AND STORAGE OF NEUROTRANSMITTERS

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## I. ROLE OF NEUROTRANSMITTER TRANSPORT PROCESSES IN SYNAPTIC TRANSMISSION

Neurotransmitter transport plays an important role in the process of synaptic transmission. The latter overall process may be divided into three stages (Figure 1): (1) release of transmitter into the synaptic cleft; (2) its interaction with postsynaptic receptors, and (3) removal of the transmitter from the synapse.

Release occurs upon arrival of an action potential at the nerve terminal. The local depolarization brings about an increase of intraterminal calcium. This in turn seems to trigger release, possibly by mediating fusion of neurotransmitter-containing storage vesicles and the synaptic plasma membrane. The released neurotransmitters cross the synaptical cleft by diffusion and then interact with receptors located at the postsynaptic membrane. This brings about changes in ion permeability of the latter membrane, either directly or via a cascade of events. Termination of the signal occurs in most cases through sodium-coupled reuptake of the transmitter to either the presynaptic terminal or into glial elements in a sodium-dependent process.[3-6] The noted exception is acetylcholine which is hydrolyzed into acetate and choline by the acetylcholine esterase, but even in this case the choline moiety is removed by re-uptake.[7] The reuptake is an active process which assures both constant and high levels of neurotransmitters in the neuron[8] and low concentrations in the cleft. It has also been suggested that during depolarization — a condition which favors reversal of the sodium-dependent uptake process (see also Sections IV.A and B.) — extra release may occur via the transporters.[9,10]

An additional class of transport systems is located in the membrane of storage organelles in the presynaptic terminal (Figure 1) (step 4). This is most clearly illustrated in the case of the biogenic amines. After uptake from the synaptic cleft into the cytoplasm by the tricyclic antidepressant-sensitive, sodium-dependent system, a substantial proportion of the amine is further concentrated into a specialized organelle by a reserpine-sensitive process. The latter process, which appears to be proton coupled,[1] is not dependent on sodium. This has a number of consequences. First, it protects the accumulated amine from loss by either leakage or intraneuronal metabolism. Second, the material which is recaptured after its release from the nerve terminal is reincorporated into the storage pool, from which it may subsequently be released again. Third, the removal of intraneuronal amines into the storage system effectively lowers the concentration gradient across the neuronal membrane and may thus act as an amplification stage for the overall process of uptake. Fourth, the acid pH of the interior of the vesicle serves to stabilize the neurotransmitter. In the case of neurosecretory

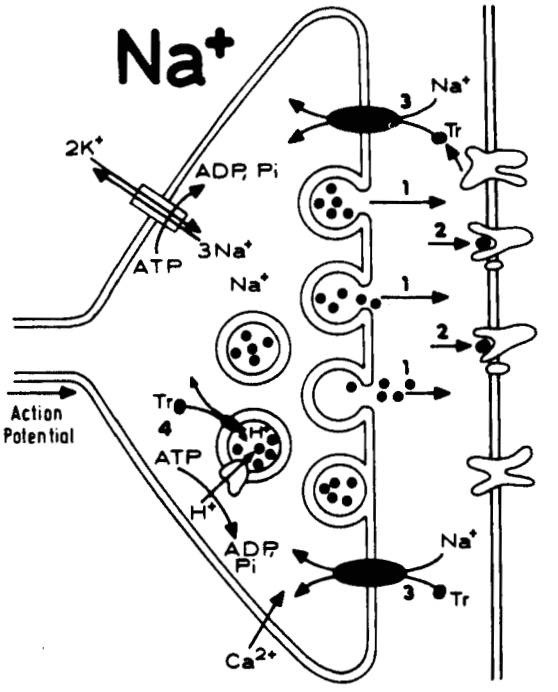


FIGURE 1. An overview of synaptic transmission. As described, following the arrival of an action potential at the nerve terminal and $Ca^{2+}$ entry, release of the transmitter occurs (1). Upon diffusion of transmitter in the cleft, it interacts at the postsynaptic site with its receptors, resulting in signal transmission (2). The last step is reuptake of the transmitter via sodium-coupled transport systems (3), here depicted in the membrane of the terminal. Subsequently, the transmitter is taken up by storage organelles.

vesicles, the internal acid pH promotes the maintenance of the integrity of the storage complex.[11] Sodium-independent systems for acetylcholine[12] and l-glutamate[13] uptake into storage organelles have also been described, while for other neurotransmitters there is no strong evidence as yet for such uptake.

The neurotransmitter transport systems seem to be very well equipped to achieve high concentration gradients by utilization of preexisting ion gradients, created and maintained by ion pumps (see Section III). Both types of transport systems are able to maintain gradients in the order of $10^4$.[2,14] This occurs because it appears that the transporters operate at an effective stoichiometry of several coupling ions per neurotransmitter. In the case of sodium-coupled systems it appears that more than one sodium ion (Sections IV.A to IV.C) plus additional other coupling ions (Sections IV.A to IV.D) participate in a single translocation cycle. In the case of biogenic amine transport into storage organelles, effectively two protons move out per molecule (Section V).

## II. PREPARATIONS USED TO STUDY NEUROTRANSMITTER TRANSPORT

Sodium-dependent neurotransmitter transport has been studied initially in relatively intact preparations[3-6] such as brain slices or synaptosomes. The latter are pinched-off terminals obtained after homogenization of brain in isotonic sucrose solutions and are purified by differential and density gradient centrifugation.[15,16] These isolated terminals behave similarly

to intact nerve-endings, they respire and synthesize ATP,[17] extrude sodium,[18] and retain potassium,[17,19] contain functional action potential sodium channels,[20-22] maintain interior-negative membrane potentials,[23] and release neurotransmitters by calcium-dependent mechanisms under conditions expected to promote depolarization.[24-29]

Significant progress has been achieved following a shift from the relatively complex synaptosomes to plasma membrane vesicles ("ghosts") obtained after osmotic lysis of synaptosomes.[30-34] Such isolated membrane vesicles are an extremely useful tool for studying transport. Some of their advantages include the possibility of using a well-defined energy source and the lack of metabolism and storage in subcellular organelles. Thus, the transport process may be studied in isolation from other processes. This approach, pioneered by Kaback,[35] who developed procedures to isolate bacterial membrane vesicles, has been extended to mammalian systems.[36-40]

As outlined in Section III, the basic mechanism for sodium as well as for proton-coupled neurotransmitter transport is a chemiosmotic one; accumulation of the neurotransmitters occurs as a consequence of coupling the process to ions which move down their electrochemical gradients. Since the composition of both the external and internal medium of the membrane vesicles can be manipulated very easily, it is simple to define not only the dependence of the process on internal and external ions, but to test the chemiosmotic mechanism directly by driving the process by the appropriate transmembrane ion gradients. These types of studies have given rise to a greater understanding of those transport mechanisms and the conclusions have been quite interesting. These such studies are discussed in detail in Sections IV and V of this review.

In addition to the use of membrane vesicles, the pioneering studies on reconstitution by Racker and co-workers have also stimulated studies of neurotransmitter transport on reconstituted systems. Upon solubilization of the transporter with detergent, it is reinserted into liposomes and the resulting proteoliposomes are capable of displaying neurotransmitter transport with the same characteristics as that in the membrane vesicles.[41,42]

A similar development has occurred with the biogenic amine-containing storage organelles containing proton-coupled transporters. Upon lysis, transport-competent membrane vesicles have been isolated.[43,44] and the proton-coupled amine transporter has been reconstituted.[45,46] Recently, a representative of each class of systems has been identified and purified.[47-49]

## III. A CHEMIOSMOTIC MECHANISM OF TRANSPORT

### A. $Na^+$-Coupled Neurotransmitter Transport

High-affinity uptake of neurotransmitters in brain preparations such as synaptosomes or slices appears to be active. It is observed in the presence of metabolic energy provided by glucose and is inhibited by inhibitors interfering with ATP synthesis, such as cyanide or dinitrophenol. Ouabain, which interferes with the utilization of ATP to generate ion gradients,[50,51] has also been shown to inhibit the uptake of neurotransmitters. These observations and the fact that these systems are dependent on external sodium have suggested that the sodium ions and also possibly the potassium ion gradients provide the driving forces for active neurotransmitter uptake.[52,53] This is in accordance with the hypothesis that solute accumulation can be achieved by co-transport with ions, which move down their electrochemical potential gradient into the cell or cell-organelle.[52,54] In the case of the cellular plasma membranes, the coupling ions are sodium ions and the device which creates the gradient is the ($Na^+ + K^+$)-ATPase. From the above, it is clear that this is also the situation in neuronal membranes. Direct evidence for this concept has been obtained with membrane vesicles from rat brain for γ-aminobutyric acid[31] and L-glutamate[32] as well as for serotonin transport in platelet membrane vesicles,[40] a model system for neuronal serotonin transport. Active transport of these neurotransmitters into membrane vesicles can be driven by an

artificially imposed sodium ion gradient (out > in) and this process is not inhibited by ouabain,[31,32,40] Thus, the function of the $(Na^+ + K^+)$-ATPase is merely to convert the energy of cellular ATP into osmotic energy (e.g., the electrochemical sodium and potassium ion gradients). Subsequently, the electrochemical sodium ion gradient is utilized via co-transport catalyzed by tightly coupled sodium/neurotransmitter symporters to drive the accumulation of neurotransmitters (Figure 1). When artificial sodium gradients are applied, the $(Na^+ + K^+)$-ATPase is not needed and ouabain will not affect the process. Subsequently, similar evidence has been obtained for glycine.[55] However, the stoichiometry of many neurotransmitters is actually more complex than that outlined in the schematic Figure 1. As shall be discussed in Section IV, in many cases the flux of more than one sodium ion is coupled to the flux of one neurotransmitter molecule and, moreover, other ion gradients may serve as additional driving forces.

### B. $H^+$-Coupled Neurotransmitter Transport

Most of our knowledge about $H^+$-coupled biogenic amine transport into storage organelles stems from studies of the chromaffin granules.

The isolated granules from the adrenal medulla catalyze uptake of large amounts of adrenaline in an ATP-dependent process[56] which is inhibited by drugs such as reserpine and by proton ionophores.[56,57] The granules have been found to contain a membrane-bound $Mg^{2+}$-dependent ATPase which is stimulated by proton ionophores.[58-60] Evidence has accumulated indicating that the ATPase translocates protons and creates a proton electrochemical gradient across the chromaffin granule membrane. Thus, ATP-dependent proton translocation and ATP-dependent changes in the fluorescence of 1-anilino naftalene-8-sulfonate (ANS)[60] have been detected in intact granules and/or membrane vesicles derived from the granules by osmotic shock. Moreover, it has been demonstrated that an uncoupler sensitive pH gradient (interior acid) of 2 to 3 pH units is maintained across the chromaffin granule membrane.[62-64] However, the intact granules are not suitable to quantitate the role of pH and/or transmembrane electrical potential ($\Delta\Psi$) as a driving force for accumulation of biogenic amines. This is mainly due to the fact that the intact granules contain catecholamines at a concentration of 0.8 *M* together with high concentrations of ATP and bivalent cations. Thus, the amount of free or unbound components in the granules is uncertain. It is therefore difficult to evaluate whether the transport measured in these preparations is indeed against a concentration gradient. This difficulty can be circumvented by using chromaffin granule membrane vesicles derived from the intact granules by osmotic shock.[57,65] These vesicles still catalyze ATP-dependent accumulation of adrenaline and 5-hydroxytryptamine[57,65,66] as well as ATP-dependent proton translocation.[61] Accumulation of adrenaline by chromaffin granule membrane vesicles is dependent upon addition of ATP and inhibited by the appropriate ionophores. Direct evidence has been provided that $\Delta$pH plays a primary role in catecholamine and serotonin transport. It has been shown that a pH gradient generated artificially by a variety of methods can induce reserpine-sensitive biogenic amine transport against its concentration gradient in ghosts[66-68] as well as in the intact granules.[69] After correlations between $\Delta\Psi$ and uptake of catecholamines had been made in intact granules[70,71] or in ghosts[72-74] it was possible to show that induction of a membrane potential (interior positive), imposed by a potassium gradient ($[K^+]_{out} > [K^+]_{in}$) in the presence of valinomycin, can drive biogenic amine transport or enhance pH driven transport.[75-77,78]

In summary, all available experimental evidence clearly supports the concept that ATP-driven accumulation of biogenic amines is a result of two sequential processes: (1) generation of a proton electrochemical gradient by the membrane-bound ATPase; and (2) utilization of $\Delta\tilde{\mu}_{H^+}$ to drive the carrier-mediated accumulation. This is illustrated in Figure 1, step 4.

In Section V we will discuss some recent kinetic biochemical and pharmacological studies. We will also present data available on other storage organelles and on the consensus that seems to be evolving about the nature of the $H^+$-ATPase.

# IV. SODIUM-COUPLED NEUROTRANSMITTER TRANSPORT

Recent studies with isolated membrane vesicles obtained after lysis and resealing of synaptosomes have revealed that in several instances, in addition to the expected sodium dependence, the process is absolutely dependent on the simultaneous presence of additional ions such as chloride or potassium. Those transport systems have recently been characterized, and one of the interesting conclusions is that those additional ions appear to participate directly in the transport cycle. Below follows a review of these systems.



## A. The ($Na^+$ + $Cl^-$)-Coupled γ-Aminobutyric Acid Transporter From Rat Brain

In earlier studies, a high-affinity sodium-dependent γ-aminobutyric acid transport system ($K_m$ in the micromolar range) had been identified in relatively intact preparations from rat brain, such as slices and synaptosomes.[50,79,80] It was shown that in such preparations transport was inhibited by compounds such as dinitrophenol and ouabain[50,80] and was postulated that accumulation of γ-aminobutyric acid was driven by the sodium electrochemical gradient.[79,80] One of the complications of these preparations, however, is their high endogenous γ-aminobutyric acid content.[81,82] Indeed, the question as to whether most, if not all, of the uptake into synaptosomes reflects exchange rather than net flux, is a legitimate one. In earlier studies with synaptosomes, failures to demonstrate net uptake of γ-aminobutyric acid were reported.[83,84] However, this problem could be overcome by the use of membrane vesicles which are depleted of endogenous compounds as a consequence of the osmotic shock[30] and which are fully competent to perform γ-aminobutyric acid transport.[31] It has been demonstrated that the increase in the internal radioactive γ-aminobutyric acid was paralleled by an increase in the γ-aminobutyric acid concentration (by direct analysis of the γ-aminobutyric acid).[85] This was to be expected, since it was possible to obtain γ-aminobutyric acid accumulation into reconstituted proteoliposomes which of course are devoid of any endogenous substances.[41] Those vesicles also catalyze dilution induced efflux[86] (conditions where influx is negligible), and thus it is clear that the transporter can catalyze net influx and net efflux. In addition, it has also been possible to show that, even with synaptosomes, a substantial portion of the uptake is due to net influx.[14,87]

### *1. The Electrochemical Sodium Gradient as a Driving Force for γ-Aminobutyric Acid Accumulation*

One of the main virtues of membrane vesicles is that it has been possible to drive active γ-aminobutyric acid transport in such a preparation with artificially imposed ion gradients as the sole driving force.[31] γ-Aminobutyric acid accumulation could be driven by a sodium ion gradient (out > in) and, importantly, this process was not inhibited by ouabain and not influenced by ATP.[31] Cation-exchange ionophores like nigericin, which under these conditions are expected to collapse the sodium ion gradient across the vesicle, strongly inhibited the process when the vesicles were loaded with potassium. The initial rate as well as the extent of γ-aminobutyric acid transport were stimulated by the potassium-specific ionophore valinomycin, which is expected to create or enhance an interior negative membrane potential (with $[K^+]_{in} > [K^+]_{out}$). The proton ionophore, CCCP, which abolishes the membrane potential under these conditions ($pH_{in} = pH_{out}$), inhibits uptake.[31] However, the proton ionophore did not inhibit when a pH gradient (interior acid) was applied.[219] Under the latter condition, CCCP should create an interior negative membrane potential (proton diffusion potential) rather than collapse it. Thus, it appears that both components of the sodium electrochemical gradient, i.e., the concentration gradient and the membrane potential, act as a driving force for γ-aminobutyric acid accumulation. In addition, as discussed below, a chloride gradient (out > in) also serves as a driving force for this process.[31,86,88]

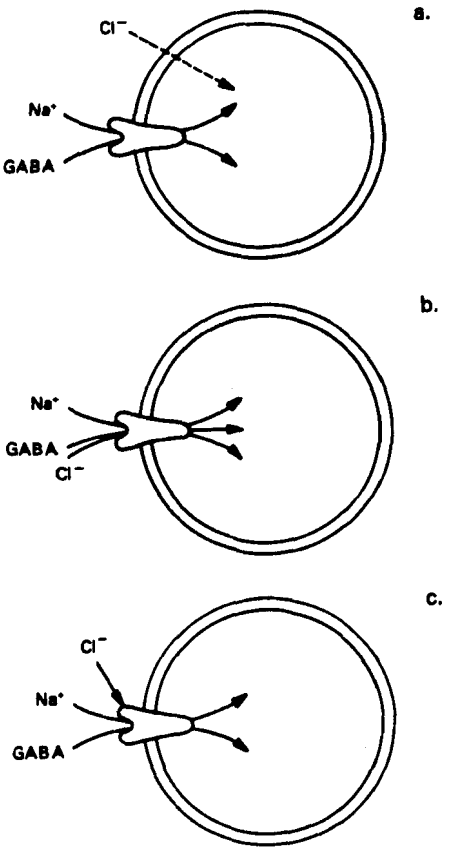


FIGURE 2. Possible explanation for the absolute chloride dependency of γ-aminobutyric acid (GABA) transport. As described in subsection VA-2, the following roles for chloride have been considered: (a) $Cl^-$ following sodium-coupled γ-aminobutyric acid influx to maintain electroneutrality; (b) ($Na^+$ + $Cl^-$)-coupled γ-aminobutyric acid transport; (c) $Cl^-$ activates the $Na^+$-coupled γ-aminobutyric acid transporter from the outside. As discussed, only model (b) is compatible with the data. (From Kanner, B. I., *Biochim. Biophys. Acta*, 726, 293, 1983. With permission.)

### 2. *Chloride as an Additional Coupling Ion*

The study of the dependence of the process on external and internal ions has revealed an absolute dependence of the influx process not only on external sodium but also on external chloride.[31] Since the process is electrogenic, the explanation for the chloride requirement could be a trivial one. Chloride is a rather permanent anion and therefore it could maintain electroneutrality of the sodium-γ-aminobutyric acid cotransport (Figure 2A) or create the internal negative membrane potential needed as driving force. This possibility appears unlikely, since more permeant anions like thiocyanate and nitrate are only poor substitutes for chloride.[31] Moreover, this chloride requirement persists in the presence of internal potassium, which is a permeant ion in nerves,[89] even in the presence of valinomycin.[31,88] Thus, potassium should be able to move out to charge compensate the positive charge moving inward via the transporter at least as efficiently as the inward movement of chloride. The explanation, i.e., the ability of chloride to create a membrane potential, is the reason for its being required and therefore can be discarded on the basis of the following experiment (Figure 3). Membrane

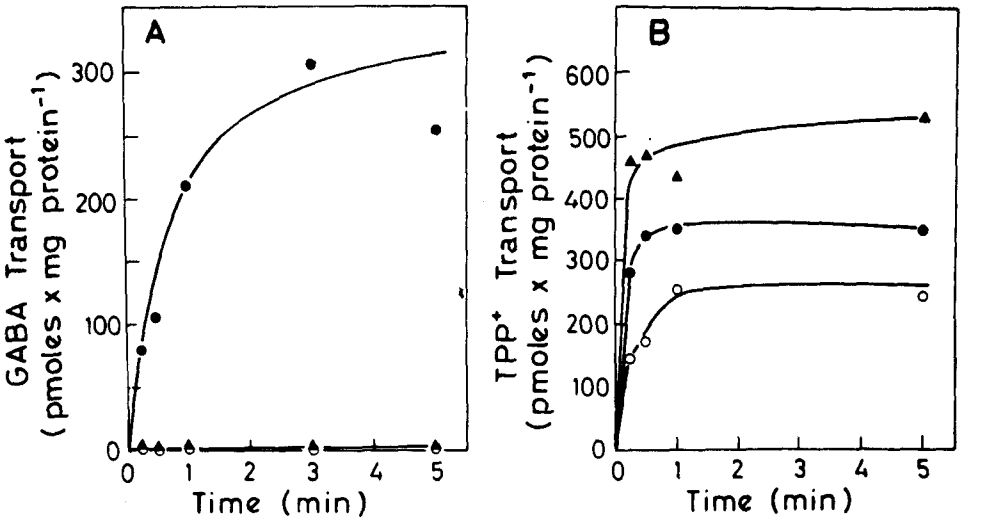


FIGURE 3. The effect of exterenal chloride on γ-aminobutyric acid (GABA) accumulation and on the membrane potential. Transport of γ-aminobutyric acid (A) or tetraphenylphosphonium cation ($TTP^+$) (B) was performed with $KP_i$ loaded vesicles. In addition to the isotope and 1 m*M* $MgSO_4$ the composition of the external media was (●—●) 0.1 *M* NaCl; (○—○) 0.1 *M* $NaP_i$; (▲—▲) 0.1 *M* $NaP_i$ + 2.5 μ*M* valinomycin. The external concentrations of the solutes were γ-aminobutyric acid 0.16 μ*M* (29.3 Ci/mmol); $TPP^+$ 5.4 μ*M* (888 Ci/mmol). (From Radian, R. and Kanner, B. I., *Biochemistry*, 22, 1236, 1983. With permission.)

vesicles are loaded with potassium phosphate and upon creation of an electrochemical sodium ion gradient (by dilution of the vesicles into sodium-containing media) those vesicles are tested for their ability to catalyze sodium-coupled γ-aminobutyric acid transport (Figure 3A) and to maintain a negative-interior membrane potential (Figure 3B), assayed by the accumulation of the tritiated lipophilic cation tetraphenylphosphonium ($TPP^+$). When the vesicles are diluted into sodium phosphate, an appreciable membrane potential (−51 mV) is generated which is not much less than when sodum chloride is employed as dilution medium (−59 mV) (Figure 3B). However, absolutely no γ-aminobutyric acid accumulation is observed in the chloride-free medium (Figure 3A). In the presence of valinomycin, dilution of the vesicles in the sodium phosphate-containing medium results actually in a more negative membrane potential (−70 mV) than dilution into the sodium chloride-containing medium (Figure 3B), but γ-aminobutyric acid transport occurs solely in the chloride-containing media (Figure 3A). Thus the role of chloride in the process must be due to a specific interaction of chloride with the transporter.

Two obvious possibilities come to mind regarding this interaction. First, the transporter may actually function by catalyzing cotransport of three species: sodium, chloride, and γ-aminobutyric acid (Figure 2B). The alternative to transport would be that chloride binds to the transporter and this causes a conformational change such that now the transporter can accept the sodium and γ-aminobutyric acid and subsequently transport those two species (Figure 2C). In order to distinguish further between these two possibilities, we have examined the effect of internal ions on the efflux of γ-aminobutyric acid. If cotransport with chloride occurs, then efflux of internal γ-aminobutyric acid by the transporter should require not only internal sodium but also internal chloride (Figure 2B), whereas for the option depicted in Figure 2C this is not expected to be the case. Indeed, it appears that both internal sodium and chloride are required for efflux of γ-aminobutyric acid,[86] and therefore it appears that the model of Figure 2B is probably the correct one. Consistent with this is that a chloride gradient (out > in) can serve as a driving force for γ-aminobutyric acid accumulation.[31] The present preparation of membrane vesicles is too leaky to sodium and chloride[88] to test

the ultimate prediction of the model, namely that one should be able, in a tight enough system, to observe sodium- and γ-aminobutyric acid-dependent chloride fluxes and also chloride- and γ-aminobutyric acid-dependent sodium fluxes. However, there are some indications of vesicle heterogeneity. Thus, the γ-aminobutyric acid gradient holds for a relatively long time in relation to flux of $Na^+$,[33] and therefore it seems that at least some of the γ-aminobutyric acid-transporting vesicles are tight. As a consequence, it might be possible to isolate the tight population and to perform the above experiments. The alternative approach is to isolate a highly purified γ-aminobutyric acid transporter preparation and to reconstitute this into single-walled liposomes and use this for the experiments. As described below, it has been possible to solubilize the transporter, to reconstitute,[41] and to purify it.[47,48]

Since it is believed that in nerve tissue chloride ion might be quite close to electrochemical equilibrium, one may wonder why it wold be advantageous to have cotransport also with chloride. It seems that it is still premature to answer this question, since it is not clear that across those very membranes where high-affinity uptake of γ-aminobutyric acid is taking place, the chloride ions are indeed at electrochemical equilibrium.

Finally, in view of the chloride requirement of the process the question may be asked whether γ-aminobutyric acid transport is indeed electrogenic or if the interior-negative membrane potential is stimulatory by slowing the dissipation of the chloride gradient. In the latter case, however, an increase of the stimulation with time is expected, while an electrogenic process should be stimulated immediately. The latter is indeed observed. Stimulation of the transporter by gating, i.e., the interior negative membrane potential promotes a more active conformation, is also unlikely since the effect of the membrane potential is not only on the initial rate but also the extent of transport is stimulated. Furthermore, exchange does not appear to be potential dependent.[86] Thus, it may be concluded that indeed charges are being translocated during net γ-aminobutyric acid flux.

*3. Stoichiometry*

The above mechanism for γ-aminobutyric acid transport (Figure 2B), and the fact that the process is electrogenic, impose restrictions on the possibilities for the stoichiometry of the process. Assuming that γ-aminobutyric acid is transported in its predominant form, the zwitterion (although there is no direct evidence for this), the stoichiometry obviously cannot be 1 $Na^+$:1 $Cl^-$:1 γ-aminobutyric acid. Thus, more than one sodium should be moving per chloride and γ-aminobutyric acid; in other words, the stoichiometry should be n $Na^+$:m $Cl^-$: γ-aminobutyric acid with $n > m$. However, the preparation is too leaky to sodium and chloride to obtain precise stoichiometric data. Still, it is possible to show that the stoichiometry for sodium is larger than 1. At the steady state the γ-aminobutyric acid gradient can be expressed as a function of the other ion gradients as follows:

$$\ln \frac{[\gamma\text{-aminobutyric acid}]_{in}}{[\gamma\text{-aminobutyric acid}]_{out}} = n \ln \frac{[Na^+]_{out}}{[Na^+]_{in}} + m \ln \frac{[Cl^-]_{out}}{[Cl^-]_{in}} - \frac{(n - m)F}{RT} \Delta\psi \quad (1)$$

Thus, when the chloride gradient and the membrane potential are kept constant, a plot of the log of the γ-aminobutyric acid gradient vs. the log of the sodium ion gradient should give a straight line with a slope equal to the stoichiometry. By the same approach, the stoichiometry for chloride and the number of charges can be determined. However, while it is trivial to determine the γ-aminobutyric acid gradient at the steady state, we do not know the sodium gradient across the very vesicles which transport γ-aminobutyric acid. The only time at which this parameter is known for the γ-aminobutyric acid-ergic vesicles is at time

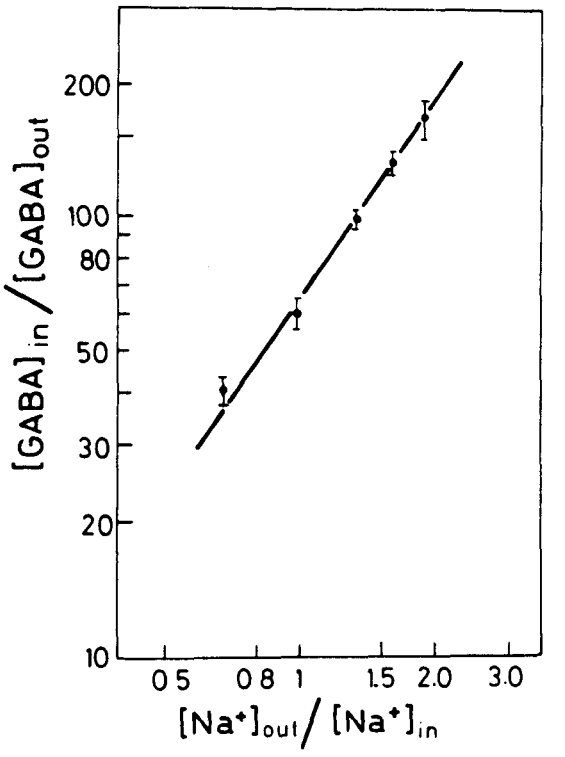


FIGURE 4. Relationship between sodium gradient and γ-aminobutyric acid (GABA) gradient. Membrane vesicles (15 μℓ, 76.5 μg of protein) loaded with 50 m*M* $NaP_i$ + 50 *M* $KP_i$ (pH 6.8); were diluted with 10 volumes of influx solutions containing mixtures (sum 150 m*M*) of NaCl and choline chloride + 0.153 μ*M* γ-amino[2,3-$^3$H]butyric acid (29.3 Ci/mmol). The final ratio of external to internal sodium was that shown on the abscissa. Reactions were stopped at 3 min and the γ-aminobutyric acid concentration ratio was determined as described under "Experimental Procedures". (From Radian, R. and Kanner, B., *Biochemistry*, 22, 1236, 1983. With permission.)

zero (the time of the imposition of the gradient). It is clear that when that value is used in the log/log plot, an underestimate of the stoichiometry will be found (at the steady state the actual sodium gradient will be smaller than the plotted gradient). Yet, when the data are plotted in this fashion (Figure 4), a stoichiometry of 1.5 ± 0.2 (20 experiments) is found and thus one can conclude that more than one sodium is transported per translocation cycle.[88] The values for chloride and the number of charges are found to be 0.47 ± 0.02 and 0.9 ± 0.08, respectively.[88] Thus, the simplest stoichiometry consistent with these data is $2Na^+$:$Cl^-$:γ-aminobutyric acid, but more complex stoichiometries cannot be excluded. In a recent study, the stoichiometry has been determined on intact synaptosomes using a basically similar approach.[14] The sodium and potassium gradient present at the steady state of γ-aminobutyric acid transport was plotted, but the problem remains that this will be an average result from all the synaptosomes (not only the γ-aminobutyric acid-ergic ones). It is of interest to note that a stoichiometry of $2Na^+$ per γ-aminobutyric acid was determined.[14] The plot of the log of the γ-aminobutyric acid gradient vs. that of the potassium gradient (as an indicator of the membrane potential, a reasonable though not firmly established assumption) yielded a slope of close to 2, suggesting that two charges are moving per

translocation cycle.[14] It should be noted that in view of our own results, discussed above, these results are not incompatible with each other. On the other hand, in the study with synaptosomes, the authors found only a slight dependence of the γ-aminobutyric acid gradient on the external chloride concentration, and they concluded therefore that chloride is not translocated with γ-aminobutyric acid. However, endogenous γ-aminobutyric acid is present in the intact synaptosomes and in a recent study with membrane vesicles from our own laboratory (reviewed below), it has been found that exchange of γ-aminobutyric acid does not require external chloride.[90] Thus, it is likely that under conditions of low external chloride concentrations, where net flux in membrane vesicles is impaired yet exchange is operative, the uptake into the intact synaptosomes is due to exchange. An additional reason for the failure of Pastuszko et al.[14] to detect a steeper dependence of γ-aminobutyric acid transport on the chloride gradient may be that they have used relatively long intervals (15 min) so that it is possible that at this time the chloride gradient may have dissipated considerably. In fact, with the γ-aminobutyric acid transporting synaptic plasma membrane vesicles we have indirect evidence that within 5 min a considerable dissipation of the chloride gradients occurs.[86] Using proteoliposomes inlaid with the γ-aminobutyric acid transporter (Section IV.A.6) we have obtained direct evidence that chloride is translocated by the transporter together with sodium ad γ-aminobutyric acid. Thus it was possible to demonstrate γ-aminobutyric acid- and sodium-dependent chloride fluxes.[220] With regard to the stoichiometry of sodium, results from kinetic approaches are consistent with the idea that two to three sodium ions are involved.[80,91] Furthermore, with the reconstituted GABA transporter we have been able to demonstrate γ-aminobutyric acid and chloride-dependent sodium fluxes. Preliminary results indicate a stoichiometry of more than one for sodium/γ-aminobutyric acid.[220] It follows from Equation 1 that a complex stoichiometry will make it possible to achieve very high concentration ratios. Indeed, gradients of up to $10^4$ have been observed in synaptosomes, and taking into account the internal and external ion concentrations and the membrane potential, one can calculate that there is enough driving force to achieve these ratios.[14] This ability is of course very important in light of the function of the transporter, which is to remove γ-aminobutyric acid from the synaptic cleft.

*4. Binding Order of Substrates*

The ion dependencies of net flux and exchange of γ-aminobutyric acid in membrane vesicles have recently been compared. It has been observed that external γ-aminobutyric acid enhances efflux[86] and the usual interpretation for this is that a step distinct from γ-aminobutyric acid translocation (its binding from one side of the membrane, translocation and release from the other side) is rate-limiting for the efflux. Interestingly, although the stimulation by external γ-aminobutyric acid requires external sodium, it does not require external chloride.[90] the interpretation for this is that when labeled γ-aminobutyric acid is released on the outside, unlabeled γ-aminobutyric acid can rebind there before the chloride (translocated from the inside) is released. Thus unlabeled γ-aminobutyric acid is carried inward where it exchanges with labelled γ-aminobutyric acid and so forth. This is consistent with an ordered mechanism in which chloride binds first to the outside, followed by γ-aminobutyric acid and finally by sodium (Figure 5). Although kinetic experiments with intact synaptosomes appeared to fit best with another model[92] (which, however, allowed for binding of γ-aminobutyric acid to the transporter in the absence of sodium), a recent study with synaptosomes combining kinetic and exchange experiments indicates that γ-aminobutyric acid has to bind to the transporter prior to the sodium.[10] Studies with the membrane vesicles are consistent with the idea that on the inside the binding order to the transporter is different from that on the outside, so that it is possible that the transporter is asymmetric (Figure 5). Further evidence for this may be obtained when it becomes possible to prepare inside-out vesicles.

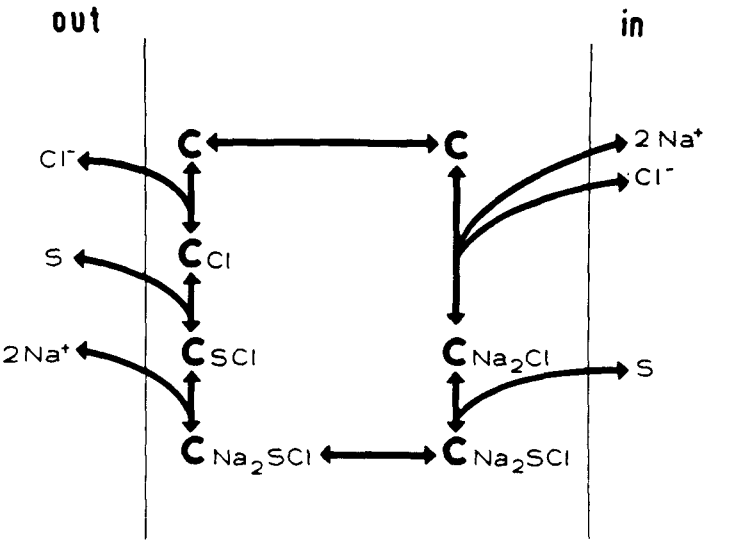


FIGURE 5. Proposed model for the translocation cycle catalysed by the γ-aminobutyrate transporter, S., γ-aminobutyrate. (From Kanner, B. I., Bendahan, A., and Radian, R., *Biochim. Biophys. Acta*, 731, 54, 1983. With permission.)

*5. Origin and Orientation of the Membrane Vesicles*

The membrane vesicles from rat brain cortex appear to be right-side-out (orientation of the intact cell) and of neuronal origin.[33] It appears from autoradiographic studies with slices that radioactive γ-aminobutyric acid is taken up by neuronal elements and about 30 to 50% of the terminals are involved.[93-95] Although the γ-aminobutyric acid system is not neuronal in all tissues, there is, at least in rat brain cortex, no reason to invoke the complicated glutamine cycle[96] as the mechanism for reuptake. This cycle envisions uptake of γ-aminobutyric acid by glial cells, conversion via glutamate (transamination with α-keto-glutarate) to glutamine, transfer of glutamine from the glial cells to the nerve terminal, and its metabolism to γ-aminobutyric acid. Experiments performed with synaptosomes[91] as well as with membrane vesicles[33] give further support for the neuronal origin of the γ-aminobutyric acid reuptake system from rat brain cortex. It has been shown that the same population of vesicles which transports γ-aminobutyric acid also has action-potential sodium channels.[33,91] Opening of the channels by veratridine should decrease the sodium electrochemical gradient and thus diminish the driving force for γ-aminobutyric acid transport, while tetrodotoxin (TTX), which is dominant over veratridine, should reverse the effect (Figure 6). This prediction has indeed been verified.[33,91] Although veratridine is hydrophobic and thus cannot be used as indicator for the sidedness of the membrane vesicles, tetrodotoxin is membrane impermeant and acts from the outside of the cell.[97] Since in rat brain vesicles all of the veratridine-stimulated sodium flux is inhibited by TTX,[33] it appears that all those vesicles are right-side-out. On the other hand, with a similar preparation from guinea pig brain, it appears that about 75% of the vesicles are inside-out as judged by the same criterion.[34] Although the reason for this serious difference is not yet known, comparison of the two methods may lead to development of both types of preparation from both sources. In terms of the mechanism of neurotransmitter transport, it is very important to be able to produce both types of vesicle from the same tissue so that it can be established directly whether the transporters are indeed asymmetric.

*6. Solubilization, Purification, and Reconstitution of the Transporter*

The γ-aminobutyric acid transporter has been solubilized and reconstituted into lipo-

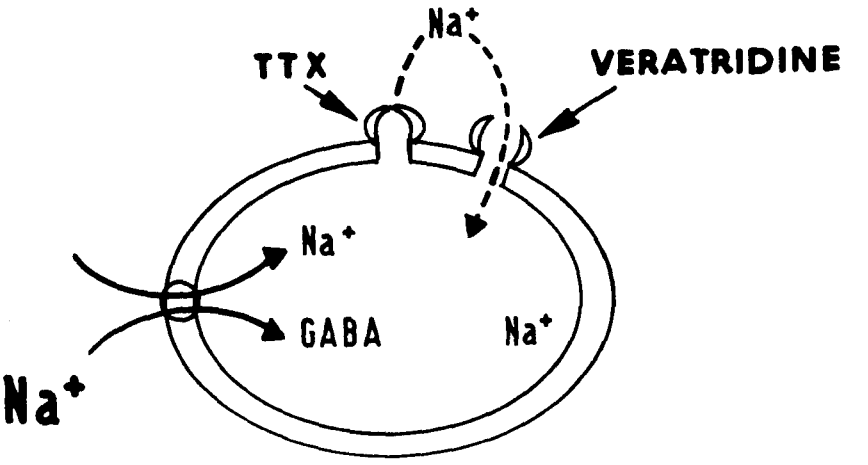


FIGURE 6. Effect of the state of $Na^+$-channel on γ-aminobutyric acid (GABA) transport. (From Kanner, B. I., *Biochim. Biophys. Acta*, 726, 293, 1983. With permission.)

somes.[41] The transport characteristics, such as sodium and chloride dependence, in the reconstituted system were the same as in the native membrane vesicles. The reconstitution assay enables one to assay the transporter functionally (ability to transport upon reconstiution).

Using this criterion, we have purified this transporter to an apparent homogeneity.[47,48] The development of a very fast reconstitution procedure using "minicolumns" has permitted the fast and simultaneous reconstitution of many samples, and this has enabled us to perform the purification of the reconstitutively active transporter. The option of affinity chromatography with substrate analogues was not feasible because of the extremely high specificity of the GABA transporter. The procedure is based on rapidity, using small fast-flowing columns and is completed within 8 hr. This rapidity is absolutely essential since, although the transporter is relatively stable, in the last stages the transporter is very diluted and undergoes partial inactivation. Briefly, the transporter is extracted with cholate in the presence of high salt (0.4 *M*). The soluble extract is fractionated with ammonium sulfate. Most proteins precipitate at 50% saturation, but the transporter remains in the supernatant.[47] The activity is precipitated at a saturation of 70% and is recovered as a floating pellet. Further purification requires the removal of both the ammonium sulfate and the cholate. This is achieved by gel filtration on Sephadex®-G-50, which is equilibrated with the detergents of choice for the next column, namely a mixture of Triton® X-100 and octyl-glucoside. The use of this mixture is critical. The void volume is collected and run on a DEAE column equilibriated with the above-mentioned detergents.

Recently, we have made further progress by observing that the transporter is a glycoprotein.[48] It can be retained by wheat germ agglutinin sepharose-6MB and eluted with *N*-acetyl-glucosamine containing detergent solution. When this step is done with the DEAE peak fraction a single polypeptide (Mr = 80 kdalton) is observed in the active fractions which tends to aggregate upon lyophillization. All the essential transport properties, such as sodium and chloride dependence, electrogenicity, and affinity, are retained by the preparation. An increase in specific GABA transport activity (upon reconstitution) of almost 400-fold with regard to the crude extract is observed. Since there is inactivation during the last stage when the protein is very dilute and actual purification is almost 1000-fold.[48] We think that it is extremely likely that the 80-kdalton polypeptide represents the ($Na^+ + Cl^-$)-coupled transporter.

We have raised antibodies against the 80-kdalton polypeptide and shown that these antibodies immunoprecipitate GABA transport activity.[48] Several up-scaled, highly purified

preparations of the transporter were dialyzed against a low ionic strength buffer, lyophilized, and purified by preparative SDS gel electrophoresis. This material was injected with adjuvant into rabbits, and followed by several booster injections. The serum was analyzed upon immunoblotting of partially purified transporter preparation (detection with $^{125}$I-Protein A). The antibody is specific for the 80-kdalton polypeptide and the small amount of labeling of the higher molecular weight band represents that of the dimerized transporter. Using protein-A coupled to sepharose beads it has been shown that the antibodies of the experimental serum were able to immunoprecipitate the transport activity of a crude solubilized GABA transporter preparation. This represents direct evidence that the 80-kdalton polypeptide indeed represents the sodium- and chloride-coupled GABA transporter.

From preliminary experiments it appears that the same polypeptide is recognized by the antibody upon immunoblotting of membranes which have been resolved by electrophoresis. This renders unlikely the possibility that the 80-kdalton polypeptide is a proteolytic fragment of a larger unit occurring in the intact membrane.

### B. The ($Na^+$ + $K^+$)-Coupled L-Glutamic Acid Transporter From the Rat Brain

A high-affinity uptake system for L-glutamic acid has been identified in brain slices and synaptosomes.[98,99] This process is sodium dependent[99] and is inhibited by ouabain.[100] This suggests that, just like γ-aminobutyric acid transport, the process is sodium coupled. The idea that the process might be involved in termination of glutamate reuptake has been questioned.[81] However, subsequent studies with synaptosomes demonstrated their ability to catalyze net uptake of L-glutamate[101] as well as of aspartate,[102] which is transported by the same system. The ability to observe the process with right-side-out membrane vesicles of neuronal origin[32,33] has provided direct evidence for the idea that the process is sodium coupled. Such studies, together with the fact that the process can be observed in proteo-liposomes obtained after dialysis of cholate extracts in the presence of phospholipids, leave little doubt that the transporter is capable of catalyzing net influx. The membrane vesicles also catalyze dilution-induced efflux of L-glutamate[103,104] and thus it can be concluded that the transporter can catalyze both net influx and efflux. The interesting feature of the L-glutamate transporter is that, although no absolute requirement for chloride has been found (in contrast to the γ-aminobutyric acid transporter), the process appears to be absolutely dependent on internal potassium. Evidence that this requirement is due to countertransport of potassium by the transporter is presented below.

#### *1. The Sodium and Potassium Gradient as a Driving Force for* L-Glutamic Acid Accumulation

Membrane vesicles have been instrumental in showing that L-glutamic acid accumulation can be driven by an inward sodium gradient (out > in).[32] Transport driven by such a gradient is very sensitive to the ionophore nigericin,[32] which under such conditions is expected to collapse this gradient. Interestingly, not only is the process absolutely dependent on internal potassium,[32] but an outward-oriented potassium gradient (in > out) serves as an additional driving force for the process.[32]

As discussed below, it appears that transport of L-glutamic acid is electrogenic.[32] It is well known that in nerves potassium is a very permeable ion.[89] Therefore, the question may be asked whether the observed potassium requirement is due to the ability of the outward potassium gradient (in > out) to generate the interior negative membrane potential which serves as a driving force. This appears to be very unlikely, because in the absence of internal potassium a gradient of the highly permeant thiocyanate anion (out > in) (which also should generate an interior negative membrane potential, together with a sodium ion gradient (out > in) does not give rise to any glutamate transport.[32] Thus, there must be a specific interaction of potassium with the transporter. The fact that the potassium gradient (in > out) serves as

an additional driving force for transport, together with the observation that external potassium is required for efflux of glutamate,[103,104] strongly suggests that potassium is in fact translocated by the transporter. Thus, it appears that sodium and glutamate are being transported inward and that potassium exits via the transporter. It should be noted that the evidence on the role of potassium in glutamate transport is very much the same as that obtained on the role of chloride in γ-aminobutyric acid transport (Section IV.A.2). The potassium requirement also explains an observation made with intact synaptosomes. Thus, low concentrations of potassium or rubidium in the media were required for optimal transport, while thallium or ammonium ions, which were equally active as potassium and rubidium in their ability to stimulate the $(Na^+ + K^+)$-ATPase, could not replace the potassium.[105] In membrane vesicles it has been shown that the only cation which can take the place of potassium in glutamate transport is rubidium.[102]

*2. Electrogenicity of the Process*

When potassium-loaded vesicles are assayed for glutamate transport upon dilution into sodium chloride, addition of valinomycin (expected to create or enhance a negative interior membrane potential) results in a small but consistent stimulation.[32] Insensitivity of a process to such manipulation of the membrane potential is taken to be evidence for an electroneutral mechanism. However, a negative result by no means indicates that the process cannot be electrogenic. In such a case, valinomycin will potentially have a dual effect: (1) generation of an interior negative membrane potential and (2) partial dissipation of the outward potassium gradient. These two effects will have opposing effects on glutamate transport, and they may cancel out. The dissipation of the potassium gradient may take more time than the enhancement of the membrane potential. Since it is observed that the stimulation by valinomycin is more pronounced in the initial stages of the process, this may very well describe the situation in the case of glutamate transport. More clear effects of valinomycin can be observed by introducing thiocyanate, a permeant anion, internally. This will have a tendency to counteract the effect of potassium on the membrane potential and, indeed, a strong inhibition of glutamate transport is observed.[32] Addition of valinomycin now results in a large stimulation.[32] It is of interest to note that in a later study a consistent stimulation by valinomycin has also been observed.[102] The electrogenicity of the process is reinforced by studies with the transporter inserted into liposomes.[42] There is very clear stimulation of transport by valinomycin with sodium chloride as external medium.[42] This is probably due to the fact that the proteoliposomes have a relatively high lipid/protein ratio and therefore probably are tighter to potassium than native vesicles. With external sodium phosphate the transport is less than with external sodium chloride, but valinomycin stimulates to a larger extent. As a consequence, in the presence of valinomycin, the nature of the external anion is not important. Thus, at least in rat brain, the glutamate transporter appears to be electrogenic.

*3. Considerations on the Stoichiometry*

We do not know which form of glutamate is actually translocated by the transporter. If this is the negatively charged species, the predominant one at neutral pH, the above observations dictate that the stoichiometry **n** $Na^+$:**m** $K^+$:-glutamate is restricted and $\mathbf{n} > \mathbf{m} + 1$ should be fulfilled in order to explain the electrogenicity of the process. If the translocated species is neutral (zwitterionic), then according to the same considerations it follows that $\mathbf{n} > \mathbf{m}$. In the latter case **n** should be at least 2 and in the former it should be 3 or more. Consistent with these considerations is an **n**-value of 2, found by measuring directly the ratio of the initial fluxes of sodium and glutamate in a cerebellar cell line.[106] Kinetic studies are also consistent with $\mathbf{n} = 2$.[107] Information on the species of L-glutamate which is translocated is not available at present, but as discussed in Section IV.B.5, effects of pH gradients indicate that, in the case of the very similar transporter from kidney, a proton is

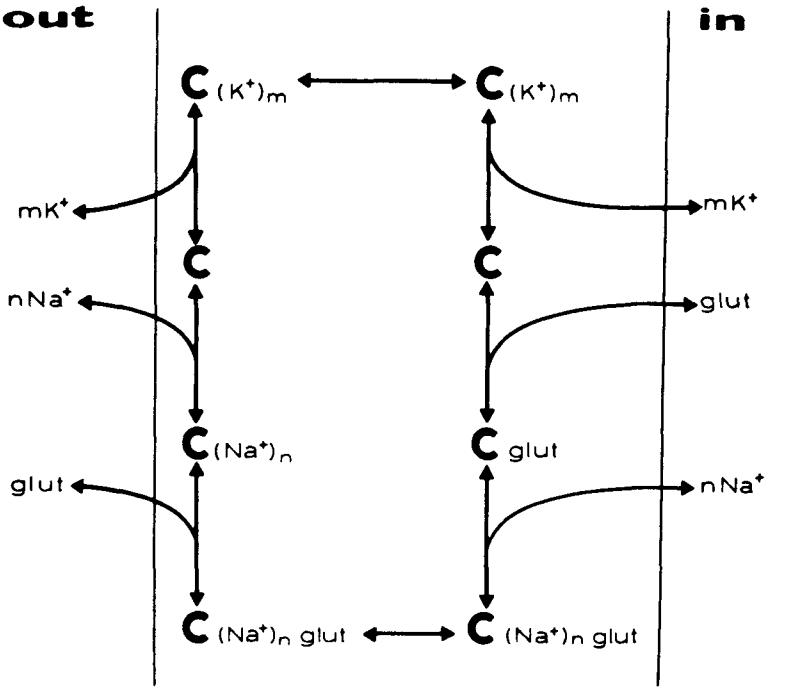


FIGURE 7. One of the possible models for the mechanism of L-glutamic acid (glut) translocation. The binding/debinding order of sodium and L-glutamic acid as indicated here for the inside and the outside may explain the data, but other possibilities exist. (From Kanner, B. I. and Bendahan, A., *Biochemistry*, 21, 6327, 1982. With permission.)

effectively moving inward. If both transporters have the same stoichiometry, one possibility is that two sodium ions are moving inward together with the neutral glutamate molecule (or the anion and a proton separately) while one potassium is moving in the other direction.

*4. Binding Order of Substrates*

As mentioned before, efflux of glutamate requires external potassium[103,104] and internal sodium.[103] However, there is also an additional mode of exit for glutamate. This is by exchange which occurs in the presence of external sodium and glutamate.[104] This suggests that potassium activates a step which is required for efflux but not for the translocation step[130] (Figure 7). The most obvious candidate for such a step is the return of the unloaded transporter. After sodium and glutamate have been released, potassium will bind to the transporter. This facilitates reorientation of the transporter so that upon translocation of potassium and its release, the binding sites for sodium and glutamate are available again at the original side of the membrane. Now a new translocation cycle can be initiated. This is true for influx (going counter-clockwise) as well as for efflux (going clockwise). Interestingly, it has been observed that the stimulation of efflux by external glutamate also occurs in the absence of sodium.[104] The following observations indicate that external glutamate is exerting its effects via the transporter. The stimulation of efflux by glutamate is concentration dependent; its half-maximal effect (about 1 $\mu M$) is very similar to the $K_m$ for L-glutamate influx.[104] Furthermore, the action of glutamic acid is stereospecific, since D-glutamic acid has a much smaller effect than L-glutamic acid. This is to be expected, since D-glutamic acid blocks influx of L-glutamic acid very poorly. Moreover, γ-aminobutyric acid, which is not a substrate for the L-glutamic acid transporter, does not induce much release of L-glutamic acid. On the other hand, L-aspartate acid, which effectively competes with

L-glutamic acid. On the other hand, L-aspartic acid, which effectively competes with L-glutamic acid for influx,[104] is able to release internal L-glutamic acid. Thus, the release of L-glutamic acid by external L-glutamic acid occurs via the transporter and is independent of external sodium. It is of interest to note also that L-aspartic acid-induced efflux of L-glutamic acid does not require external sodium. Thus, L-aspartate is also transported by the L-glutamate transporter via the same mechanism.

The easiest way to explain the independence of exchange on external sodium is that on the outside sodium binds first, followed by glutamate (Figure 7). Thus, during efflux, upon translocation, labeled glutamate is released on the outside and unlabeled glutamate binds before the sodium is released. Furthermore, it can be shown that exchange requires internal sodium.[104] Thus, the transporter seems to be asymmetric in its interaction with sodium. The observation can best be explained by postulating that on the inside, sodium is released prior to L-glutamate (Figure 7). Although the role of potassium was not taken into account, it is of interest to note that from kinetic studies the same binding order was deduced with regard to the binding of sodium and glutamate on the outside.[107] Furthermore, the sequence of steps involving sodium and glutamate is reminiscent of the glidesymmetry of the sodium-coupled glucose transporter from intestine,[108] where also first on-first off is proposed.

*5. Similar Glutamate Transporters from Kidney and Liver*

Interestingly, the glutamate transporter from other sources such as kidney [109-111] and liver [112] has many strikingly similar features to that of brain. Not only is the glutamate transport driven by an inward directed sodium gradient (out>in), there also is a requirement for internal potassium.[109,111,112] At least in the kidney, the potassium gradient (in > out) represents an additional driving force for glutamate transport,[109,111] and the concentration dependence of the effect of potassium is similar.[104,109,111] An important difference is that while in brain the requirement for potassium is absolute,[32] this is not the case in kidney and liver vesicles.[109,111,112] There, the requirement is only partial. Probably the transporters are slightly different. Another difference is the affinity of the various transporters for glutamate: the brain transporter has the highest affinity.[32] In the brush-border vesicles from kidney the potassium-independent process appears to be electroneutral.[110,111] However, in the case of the potassium dependent process there are differing views. While one group views the process as electroneutral,[109] another group proposes an electrogenic process.[111] It is of interest to note that stimulation of glutamate transport observed in response to conditions under which the magnitude of the membrane potential is expected to be enhanced is only apparent at early times.[111] As discussed in Section IV.B.2, this may be expected. One way to resolve the question is to postulate species differences (rabbit[109] vs. rat[111]) as the explanation, but this does not seem to be very appealing. Interestingly, the controversy on the electrogenicity of the transporter extends also to more physiological preparations.[113,114] In the case of the transporter from liver, the question is also still open.[112] Subsequently, it has been found that a pH gradient (alkaline interior) is capable of serving as a driving force for glutamate transport in kidney vesicles.[115] This suggests that glutamate is translocated as the zwitterion, or that the anion is translocated together with a proton (not bound to the anion). There is also evidence for the inhibition of the potassium stimulation by protons.[109,115] Thus, it is possible that in kidney (and maybe also in liver) the potassium-independent transport is proceeding with a proton taking the place of potassium. The inhibitory effect of internal protons further complicates the issue of the electrogenicity of the process in kidney. Stimuation by conditions which favor an interior negative membrane potential — set up by creating as pH gradient (acidic interior) in the presence of CCCP — may be explained by the glutamate translocation being electrogenic.[111] Alternatively, the stimulation by CCCP might be due to facilitation of outward movement of protons, relieving thereby the inhibition by internal pH.[116] Since the stimulation by CCCP is very rapid,[111] it remains an open question whether an increase of internal pH is fast enough to account for this. Obviously, further studies are required to decide the issue.

### C. Sodium-Coupled Biogenic Amine Transport: the ($Na^+$ + $K^+$ + $Cl^-$)-Coupled Serotonin Transporter from Platelets

It seems that the major transmitters, norepinephrine, serotonin, and dopamine, are transported by separate sodium-dependent neuronal uptake systems. Although there are many similarities between transport of serotonin (5-hydroxy-tryptamine) and norepinephrine, there are some differences. Thus, desimipramine is a much weaker inhibitor of serotonin transport than of norepinephrine.[117] Dopamine transport appears to be relatively insensitive both to imipramine as well as to desimipramine.[117] The serotonin transporter from the platelet plasma membrane is the only sodium-coupled biogenic amine transporter whose mechanism has been studied in great detail, including studies with membrane vesicles.[40,118] The similarities between serotonin transport into platelets on the one hand and synaptosomes and brain slices on the other have led to the proposal that the same transporter is present in both tissues.[119] Thus, the platelet system may serve as a model for neuronal serotonin transport. Below follows a discussion of the studies on the platelet system together with some recent observations on the possible analogous system from membrane vesicles from rat basophilic leukemia cells. The serotonin transporter appears to be even more complex than the γ-aminobutyric acid and L-glutamate transporters from rat brain, since it requires both external sodium and chloride as well as internal potassium. Furthermore, it appears that all those ions are participating directly in the translocation cycle.

#### *1. The Sodium and Chloride Gradients as Driving Forces for Serotonin Accumulation*

The influx of serotonin is a saturable energy-dependent process which displays an absolute requirement for $Na^+$ and $Cl^-$ in the external medium.[40,120,121] By using plasma membrane vesicles from platelets it has been observed that an inward sodium gradient (out < in) serves as a driving force for serotonin accumulation.[40] Consistent with this is the strong inhibition of this process by ionophores such as nigericin, monensin, and gramicidin, which collapse this gradient.[40] The requirement for external chloride is also absolute[40,121,122] and only small monovalent anions can substitute for chloride to some extent.[122] This is similar to that observed with the γ-aminobutyric acid transporter.[31,41] As is also the case with the γ-aminobutyric acid transporter,[31,86,88] the effect of chloride on the serotonin transporter is due to a specific interaction between the two. Thus serotinin accumulation even requires external chloride even when a $K^+$ diffusion potential (interior negative) is imposed across the vesicle membrane with valinomycin.[122] Furthermore, exchange which is electroneutral also requires external chloride.[122] Internal chloride inhibits the steady-state level of serotonin accumulation, i.e., the chloride gradient is a driving force for the process.[122] While influx requires external chloride, efflux requires internal chloride.[122] These observations taken together strongly suggest that chloride is cotransported by the transporter together with sodium and serotonin.[122] This is analogous to sodium- and chloride-coupled γ-aminobutyric acid transport discussed in Section IV.A.1.

#### *2. The Role of Internal Potassium in Serotonin Transport*

Influx of serotonin is stimulated by, but not absolutely dependent on, internal potassium ions.[40,123] External potassium inhibits influx, but it also enhances efflux of serotonin.[123] Since this stimulation is seen in the absence of valinomycin, an ionophore necessary for the imposition of potassium ion diffusion potentials,[123,124] it seems that potassium is exerting its effect directly on the transport and not by acting as a permeant ion. Stimulation of serotonin efflux can be observed under two sets of conditions: (1) in the presence of external serotonin and NaCl (exchange) or (2) by external potassium. The stimulation of efflux by converting it into exchange suggests that a step distinct from the serotonin translocation step, such as the return of the unloaded transporter (a step by which efflux and exchange differ), is the rate-limiting step in efflux. The stimulation of efflux by external potassium

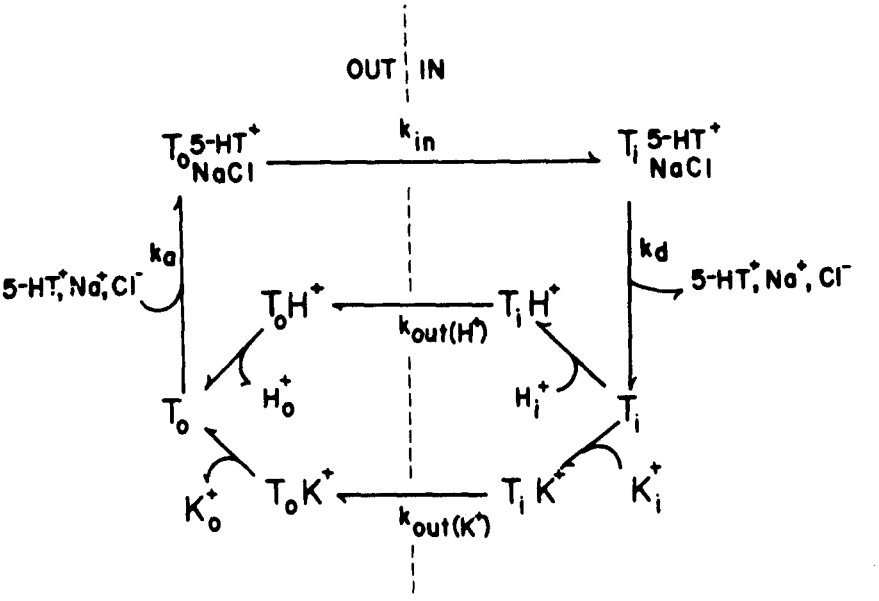


FIGURE 8. Proposed mechanism for stimulation of serotonin transport by $K^+$ or $H^+$. $T_o$ and $T_i$ represent two forms of the transporter with the serotonin binding site exposed on the exterior and interior surface of the membrane, respectively. 5-HT, serotonin. (From Keyes, S. R. and Rudnick, G., *J. Biol. Chem.*, 257, 1172, 1982. With permission.)

then may be explained by postulating that it binds to the unloaded transporter and that this form can translocate more readily. As a consequence, upon release of potassium, the sodium, chloride, and serotonin can bind again and initiate a new cycle (Figure 8). The question then is, what happens in the absence of potassium? Can the unloaded transporter reorient with a finite rate so as to enable net flux in the absence of potassium, or has some other ion (e.g., lithium, proton) bind to it to enable return of the transporter? Recent evidence[118] suggests that the latter possibility is the correct one and that protons may take the place of potassium. The experimental evidence for this is quite strong. First, it can be shown that an artificially imposed pH gradient (acidic interior) in the presence of sodium and chloride both at equal concentrations on both sides of the membrane can drive serotonin accumulation. This is mediated by the transporter because it is sensitive to the specific inhibitor, imipramine.[118] Furthermore, there appears to be a competition between internal protons and internal potassium. Thus, the stimulation of influx by internal potassium is much smaller at lower than at higher pH values and, vice versa, the stimulation by internal portons is diminished in the presence of internal potassium. Thus, it appears that protons (which are of course always present) can, to some extent, take over the role of potassium, which is to facilitate the return of the unloaded transporter[118] (Figure 8). An alternative explanation for the stimulation of influx by internal protons is the translocation of the unprotonated form of serotonin. Serotonin would accumulate on the inside in response to the pH gradient. However, this idea is less likely because it does not account for the competition between protons and potassium.

### *3. An Electroneutral or Electrogenic Mechanism?*

There appears to be some lack of clarity as to the question whether the process is electroneutral or electrogenic. As indicated above, mere charge compensation fails to account for the role of chloride and that of potassium. Although the process was initially thought to be electrogenic.[40] in his subsequent studies Rudnick[123,124] concludes that the process is

electroneutral, at least in the presence of potassium. This is based on the lack of correlation between the membrane potential and the level of serotonin accumulation. In the absence of valinomycin there is no appreciable membrane potential as judged by the tetraphenylphosphonium ion distribution, but high rates and extents of serotonin transport are observed.[123,124] Addition of valinomycin results in formation of an interior-negative membrane potential with no[123] or little[123] stimulation of serotonin transport. Only the simultaneous presence of valinomycin and the proton ionophore dinitrophenol results in inhibition of the extent, but, importantly, not of the initial rate of the process.[124] This suggests that the inhibition by dinitrophenol under these conditions is due to dissipation of the potassium concentration gradient (via exchange with protons). However, even in the absence of dinitrophenol, the potassium ion gradient may have decayed somewhat faster in the presence of valinomycin. Exactly as discussed for glutamate transport in Section IV.B.2, this would act to obscure a possible stimulation due to the imposition of an interior negative membrane potential. The basic problem is that electroneutrality of a process is inferred from experiments leading to negative conclusions, i.e., inability to see effects on transport under conditions which result in a change in the membrane potential. In this context, it is of interest to mention some of our own experiments with membrane vesicles derived from tumor cells, rat basophilic leukemia cells.[125] From these cells it is possible to isolate membrane vesicles which catalyze serotonin transport which is dependent on external sodium and chloride as well as on internal potassium. This process is sensitive to imipramine.[125] Not only the mere presence but the gradients of these ions are required for accumulation of serotonin. Under certain conditions the initial rate as well as the extent of the process appears to be stimulated by valinomycin in the presence of internal potassium.[125] Thus, this process is apparently electrogenic. Although the possibility cannot be excluded that the transporters from platelets and rat basophilic leukemia cells are different, the other similarities render this possibility unlikely. Obviously, the electrogenicity issue has implications on the expected stoichiometry of the process. For the sake of simplicity we shall assume for the moment that serotonin is transported in its positively charged form and that one potassium and one chloride are involved. If the cycle is electroneutral, the stoichiometry for sodium would also be 1, whereas this would be at least 2 for an electrogenic mechanism. If serotonin is transported in its neutral form or more chloride or potassium ions are translocated, the number of sodium ions must go up correspondingly. No positive cooperativity is observed when the initial rate of serotonin transport is plotted vs. the sodium ion concentration,[126] suggesting, but certainly not proving, that only one sodium is translocated. Determination of the stoichiometry by a thermodynamic approach as described for γ-aminobutyric acid in Section IV.A.3 yields a slope of 0.9,[126] but as mentioned this may lead to underestimates of the values. If the stoichiometry for sodium is indeed 1, the mechanism should be electroneutral and if this were the case, some other explanation for the stimulation by valinomycin would have to be sought. But the stoichiometry may very well be larger than 1 and then the valinomycin data indeed will point to an electrogenic mechanism.

*4. Imipramine Binding*

The tricyclic antidepressant, imipramine, is a potent inhibitor of serotonin transport[40] and displays sodium-dependent specific binding to platelet membranes.[127] This binding is inhibited by serotonin in a competive fashion and therefore it has been used to probe the mechanism of serotonin transport.[127] Imipramine inhibits all serotonin fluxes, i.e., influx, efflux, and exchange; this indicates that the compound is not transported.[127] This is also clear from the observations that preventing transport using nigericin, digitonin, or by omitting chloride does not inhibit imipramine binding.[126-128] A turnover number of 500 molecules of serotonin translocated per min at $V_{max}$ has been derived from the $V_{max}$ and the number of imipramine-binding sites, assuming that the number of imipramine-binding sites is equivalent to the number of serotonin transporters.

Imipramine has been used to probe the functionality of the serotonin transporter. Although the transporter can be reconstituted upon rupture of the membrane by cholate, the activity was not solubilized.[129] However, using the sodium dependence of imipramine binding as an assay, it appears that it is possible to solubilize the transporter in a potentially functional form with digitonin.[128]

Although imipramine binding is sodium dependent, it is of interest to note that, although it is stimulated by chloride, the activity is not absolutely dependent on this anion.[128] This is, of course, in contrast with accumulation of serotonin, which is absolutely dependent on external chloride.[40] One possible interpretation is that sodium is sufficient for imipramine and serotonin binding and binding of chloride occurs after this step and before the translocation step.

It has been argued that antidepressant activity cannot be fully explained by its ability to block biogenic amine transport,[130] although it seems that the serotonin transporter is the site of imipramine binding. Indeed, it has been shown that the two activities differ in their sodium requirements,[125] and thus clarification of the mechanism of action of imipramine on serotonin transport is needed before this tool can be used to draw further conclusions on the mechanism of serotonin transport. It is of interest that a similar situation may exist with regard to the inhibition of norepinephrine uptake by desimipramine. Desimipramine binding has been correlated with neuronal norepinephrine uptake (see References 131, 132), but this relationship has also been challenged.[133] Yet, desimipramine binding also requires sodium.[132]

### D. A Multitude of Sodium-Coupled Neurotransmitter Transport Systems Requiring Additional Ions

In addition to the transporters discussed in Sections IV.A to IV.C, there are other neurotransmitter transport systems which require additional ions. With these other systems it is not yet clear whether the additional ions are cotransported, but this is likely to be the case. Below follows a brief discussion on these, as well as a summary of the evidence on the various systems (Table 1).

In synaptosomes, transport of many neurotransmitters displays a chloride dependence.[134] This dependence is not always absolute and accurate. For instance, in synaptosomes the chloride dependence of γ-aminobutyric acid transport is not very pronounced,[134] whereas in membrane vesicles it is absolute.[31,86,88] As discussed in Section IV.A.4, studies with membrane vesicles have established that for γ-aminobutyric acid exchange, external chloride is not required.[90] Therefore, this, together with the well known fact that synaptosomes contain high endogenous γ-aminobutyric acid levels,[81,82] strongly suggests that the partial dependence of transport on chloride[134] of a variety of neurotransmitters — choline, glycine, dopamine, norepinephrine, and serotonin — may be an absolute one in several cases. On the other hand, there is the partial chloride dependence of proline transport in synaptosomes.[134] In membrane vesicles this dependence is also not absolute.[135] Thus, some unclarity exists in any interpretation of results obtained with synaptosomes on this issue. The authors[134] considered the effects of chloride, which they obtained in synaptosomes, most likely due to charge compensation (Figure 2A). This is unlikely, since ions more permeant than chloride (such as nitrate) are much less effective than chloride in supporting the process.[134] Indeed, recently it has been shown that the glycine transporter can be studied in membrane vesicles, that this electrogenic process is dependent on external sodium and chloride, and that their gradients (out > in) serve as driving forces.[55] These results, together with the observation that in such membrane vesicles there is a considerable (interior negative) membrane potential in the absence of chloride,[88] imply that the effect of chloride is not due to charge compensation (Figure 2A) but must be due to a specific effect on the transporter (Table 1). Recent studies indicate that the chloride requirement is sided, i.e., external chloride being required for influx and internal chloride for efflux.[136] Possibly the process may be driven with two sodium

## Table 1
## SODIUM-COUPLED NEUROTRANSMITTER[a] TRANSPORT SYSTEMS REQUIRING ADDITIONAL IONS

| System | Additional ions[b] | | Specific requirements | Sided effect on efflux and influx | Ref. |
|---|---|---|---|---|---|
| | $Cl^-$ | $K^+$ | | | |
| γ-Aminobutyric acid | + | − | + | + | 31, 41, 86, 88, 90 |
| Glutamate/aspartate brain | − | + | + | + | 32, 42, 103, 104 |
| Serotonin platelet | + | + | + | + | 40, 118—124, 126—129 |
| Serotonin rat basophilic leukemia cells | + | + | + | ND | 125 |
| Serotonin brain | + | ND | ND | ND | 134 |
| Glutamate kidney | − | + | + | + | 109—111 |
| Glutamate liver | − | + | ND | ND | 112 |
| Glutamate crab nerve | − | ? | ND | ND | 139 |
| γ-Aminobutyric acid insect nervous system | + | − | + | ND | 140 |
| Glycine brain | + | − | + | + | 55, 134, 136 |
| Glycine erythrocytes | + | − | + | ND | 137, 138 |
| Choline brain | + | ND | +? | ND | 134, 141 |
| Norepinephrine brain rat heart nerve | + | ND | +? | ND | 134, 142 |
| Norepinephrine PC 12 cells | + | + | ND | ND | 143, 144 |
| Dopamine brain | + | ND | +? | ND | 134 |
| Amino acids fish intestine | + | − | +? | ND | 145 |

Note: ND = Not determined.

[a] Including some possibly analogous systems.
[b] Protons are not included, but see Sections IV.B.5 and IV.C.2.

ions and one chloride ion. In this context it is of interest to recall some of the classical studies by Vidaver on the glycine transporter from pigeon erythrocytes. This system, which transports two sodium ions per glycine, also displays an absolute requirement for chloride ions[137] and the chloride requirement is probably not due to its ability to serve as a permeant anion.[138] In terms of Table 1 this is indicated as displaying a specific requirement. Thus, it is very likely that the transporter from these two sources is very similar if not identical. As noted previously, several neurotransmitter transporters can be identified in other tissues, and they appear to be very similar to each other. This includes the transporter of glutamate/aspartate (Section IV.B, possibly also including the system for crab nerve[139]) and the transporter of serotonin (Section IV.C). It also appears that the γ-aminobutyric acid transporter from insect nervous tissue[140] is very similar to that of its mammalian counterpart.[31,86,88] The requirement for chloride by the choline transporter also seems to be genuine, since it persists in reconstituted proteoliposomes.[141] The norepinephrine transporter also appears to be such a case. The process in rat heart adrenergic nerves has been shown to be absolutely dependent on external chloride.[142] Furthermore, recently transport competent membrane vesicles from the PC-12 cell line (a clonal line of rat phaeochromocytoma cells) have been isolated.[143] The influx of norepinephrine in these vesicles is dependent on both external sodium and chloride and on internal potassium and appears to be electrogenic.[144] Although in brain tissue serotonin transport has been measured only in intact synaptosomes (just as is the case for the choline system, the activity is lost upon osmotic shock,[141,219] the analogous situation in platelets[40,118,120] and RBL cells[125] makes it likely that the chloride requirement observed[134] is probably due to its involvement as a coupling ion. The involvement of additional ions

will probably turn out to be very widespread. An example of a nonneurotransmitter system where this occurs may be amino acid transport in the intestine of a herbivorous Mediterranean fish.[145]

## V. $H^+$-COUPLED NEUROTRANSMITTER TRANSPORT

As previously stated, most of our knowledge about $H^+$-coupled biogenic amine transport stems from studies of the chromaffin granule. The process of biogenic amine accumulation into these granules is a result of two sequential processes: (1) ATP-dependent generation of a proton electrochemical gradient and (2) utilization of the $\Delta\mu_{H+}$ by a reserpine and tetrabenazine sensitive transport.

### A. The Hydrogen Ion Pumping ATPase

It is well established that the acidification of the intravesicular space of chromaffin granules and the establishment of a membrane potential, positive inside, is due to the operation of a $H^+$-ATPase. Some confusion existed for several years as for the nature of the enzyme. This was mainly due to the fact that most preparations of chromaffin granules contain mitochondrial contaminants. Early findings led researchers to propose that the ATPase of chromaffin granule membranes closely resembled that of mitochondria.[146] However, Cidon and Nelson demonstrated that the presence of this enzyme was most likely due to contamination.[147] Thus, upon treatment of highly purified granule membranes with NaBr all the $F_1$-subunit, as measured with antibodies, dissociates, while up to 80% of the ATPase activity remains associated with the granule.

In an elegant series of experiments Rudnick and collaborators[148] characterized the inhibitor profile of the enzyme from chromaffin granules and from platelet dense granules: to avoid complications by other ATPases which might contaminate the preparation, they used an assay that measures only those ATPase molecules which are functionally inserted in the granule membrane and which, therefore, drive accumulation of the biogenic amine. As a control, the ability of an artificially imposed pH gradient to drive transport in the absence of ATP was measured. ATPase inhibitors should not block transport driven by imposed pH gradients, which depends only on the activity of the amine transporter and the relative impermeability of the membrane to $H^+$. In both preparations the pump activity displays an inhibitor sensitivity distinct from that of mitochondrial $F_1F_o$ ATPase or $Na^+$, $K^+$-ATPase: thus the enzyme is insensitive to azide, vanadate, oligomycin, ouabain, and efrapeptin. In addition, both NEM (*N*-ethylmaleimide) and Nbd-Cl (7-chloro-4-nitrobenz-2-oxa-1,3-diazole) preferentially inhibit the granule enzyme. These results suggest that the granule enzyme represents a new class of ATP-driven ion pump very similar to that described in endosomes, lysosomes, coated vesicles, and plant vacuoles.

The chromaffin granule ATPase has been solubilized, reconstituted, and partially purified.[149,150] Four polypeptides seem to be associated with the activity: 115, 72, 57, and 39 kdalton; the 115- and 57-kdalton peptides are labeled with NEM. Based on the hydrodynamic properties of a detergent solubilized preparation of chromaffin granules, the mass of the active protein has been estimated to be 134 kdalton.[151]

A second ATPase associated with granule fractions has been detected.[152] This activity displays a very distinct inhibitor profile: it is insensitive to DCCD or alkylating agents but is strongly inhibited by vanadate. Its structure and function is still obscure.

Many early attempts to purify and reconstitute the granule ATPase have been reported.[153,154] Also, the reversibility of the enzyme has been demonstrated by net ATP synthesis[155] and by ATP-$^{32}P_i$ isotope exchange.[156] An apparent stoichiometry of $2H^+$/ATP has been determined in two laboratories.[157,158] It is not clear as to whether enough care has been taken in all the studies to determine which enzyme is being studied. The pharmacology available at present makes this task possible in the foreseeable future.

For more details with respect to the $H^+$-ATPase of the chromaffin granule the reader is referred to several recent reviews.[159-161]

## B. Mechanism of Biogenic Amine Translocation

### *1. Molecular Species of Amine Transported*

There now seems to be general agreement that the process of catecholamine uptake is an electrogenic one. This is based on two lines of reasoning: (1) the contribution of components of $\Delta\mu_{H+}$ to steady-state amine uptake was measured qualitatively and quantitatively;[70-73] (2) amine uptake was tested upon imposition of artificially generated pH gradient[66-69] and membrane potential.[75-78] In summary, the findings demonstrate that amine uptake is driven by a membrane potential as well as by a pH gradient; the quantitative dependence of the gradient on each of the components is different[73] and fits best to the following equation:

$$\frac{RT}{F} \ln \frac{[\text{amine}]_{in}}{[\text{amine}]_{out}} = 2\left(\frac{RT}{F} \ln \frac{[H^+]_{in}}{[H^+]_{out}}\right) + \Delta\psi$$

This relation can be accounted by either of the following: (1) the exchange of an uncharged amine molecule with one proton and a further protonation of the amine in the intravesicular space and (2) exchange of the protonated amine with two protons. Several approaches have been employed to try to distinguish between the two possible mechanisms and thus far there is no clear-cut answer. One approach, utilized thus far in several laboratories, has been to test the dependence of the apparent Km on the medium pH; most of the published findings suggest that there is a steep decrease of the apparent Km with the increase of the pH (References 162, 163 but also see Reference 164). When the concentration of the uncharged amine is calculated at each pH, the apparent Km towards this form does not change, suggesting that the pH dependence observed reflects the change in the concentration of the uncharged species. Similar findings have been reported when binding of Dihydrotetrabenzine (TBZOH) (See Section V.C.1) was tested as a function of pH.[165] Further support to the proposal that the uncharged species is the substrate is provided by the finding that a charged analogue of epinephrine: dimethylepinephrine is not a substrate of the transporter.[166] However, in the latter case a methyl group is juxtaposed to the nitrogen of the amine group and this could, by itself, alter the interaction with the protein. Moreover, a charged substrate of the system has recently been reported: meta-iodobezylguanidine (apparent $K_m$: 2 $\mu M$).[167]

### *2. Asymmetry of the Transporter*

Studies performed in the intact organelle indicate a substantial but very slow spontaneous efflux of the endogenous amines. This is true even in the absence of exogenous ATP and when the gradients of the amine can be made as large as desired (and favoring efflux) by manipulation of the degree of dilution of the medium.[168-171] Under such conditions (in the absence of ATP) the electrochemical gradient of protons in the intact granule is very low, consisting of a pH gradient (interior acid) and a membrane potential of opposite magnitude (negative inside). Since, on the other hand, the amine gradients are very large it is clear that, under these conditions, the amine is not in thermodynamic equilibrium with the electrochemical gradient of protons.

The slow rate of efflux has been tentatively explained by complexation of the amine by the vesicle core which brings about a decrease in the concentration of the free species.[169] A further analysis of this phenomenon is difficult in the intact granule because of possible binding of the amine to the intravesicular proteins, nucleotides, and ions and also because even the slow leakage of the granule content generates considerable concentrations in the medium.

The reasons for the slow efflux rates have been studied in detail in membrane vesicles

depleted from the granule core and from most of the endogenous catecholamine content.[65,66] 5-hydroxytrypytamine (5-HT)[1], noradrenaline, and other biogenic amines are accumulated by a preparation of chromaffin granules membrane vesicles against concentration gradients of up to 4000. This uptake is dependent on the presence of ATP and the activity of a $H^+$-ATPase which generates a proton electrochemical gradient. However, once achieved the steady-state levels can be maintained for at least 1 hr even in the absence of ATP. Even when the external 5-HT is decreased up to 200-fold by dilution, after a steady state is reached, previously accumulated substrate does not efflux to a new steady state as dictated by simple energetic considerations.[173]

These findings would suggest that no input of energy is required to maintain the steady state once this is reached. This would be possible only if there are no considerable leaks under these conditions, i.e., the electrochemical gradient of protons does not decay and the transporter must be catalyzing a 1:1 exchange but no net efflux. In accordance with previous findings, addition of the ionophore nigericin at any time after the steady state has been reached does induce net efflux. It is concluded that the lack of net efflux must be due to a kinetic barrier induced by the acid intravesicular pH; when the intravesicular milieu is acidified below a certain value, the transporter can catalyze exchange of intravesicular amines with extravesicular substrate, but it cannot catalyze net efflux of the internal amine. Net efflux, however, can be induced by agents that bring upon an alkalinization of the internal pH, such as nigericin and ammonia salts.

There are several simple models that can explain the translocation cycle of amines in exchange for an hydrogen ion: two of them are schematically depicted in Figure 9. In the top part of the figure a sequential model is presented which is similar to others previously proposed:[69-73] a site on the transporter facing the cytoplasmic side binds substrate with a certain affinity and translocates it to the internal face of the membrane. At this side, the substrate is released and the transporter now becomes protonated. This protonation, which clears the free transporter sites from the intravesicular face of the membrane, is obviously strongly dependent on the pH and is probably a very fast reaction. We assume that, for net flux: (1) the equilibrium of the protonated form between the inside and the outside face and (2) the release of protons in the outside face are not rate-limiting steps since the rate of exchange is high, even when the inside pH is low. Under exchange conditions, the availability of the transporter-binding site inside is made higher because of the continuous flow of solute to the inside. The exchange process will use only the lower part of the cycle described in Figure 1 and is almost independent of pH.

The lower part of Figure 9 describes a simultaneous mechanism in which both the hydrogen ion and the substrate have to be bound to the transporter before translocation occurs. Also in this model binding of protons to the transporter from within will prevent *cis*-binding of the amine and therefore efflux. Exchange will occur when external amine binds to the protonated transporter and therefore will not be inhibited by the intravesicular low pH. In fact, if the order of the reaction is as described in the model, binding of substrates to the external side of the membrane should depend on trans-protonation of the transporter.

Some recent findings seem to support an effect of intravesicular pH on several parameters of the translocation cycle: (1) an increase in the intravesicular pH (at a constant given external pH) brings about an increase in the apparent $K_m$ for amine uptake, (2) reserpine binding is stimulated by an electrochemical gradient of protons,[174,175] and (3) the rate of labeling of the transporter with the specific probe ANPA-5HT (see below) increases with a decrease in the intravesicular pH.[221] Even though these data can be interpreted in a number of ways, they are consistent with both models described in Figure 9.

Regardless of the mechanism, the phenomenon described can, in fact, be considered as a gating device regulated by the intravesicular pH. Part of the mechanism of translocation, the protonation of the transporter in the interior phase of the membrane, is also responsible

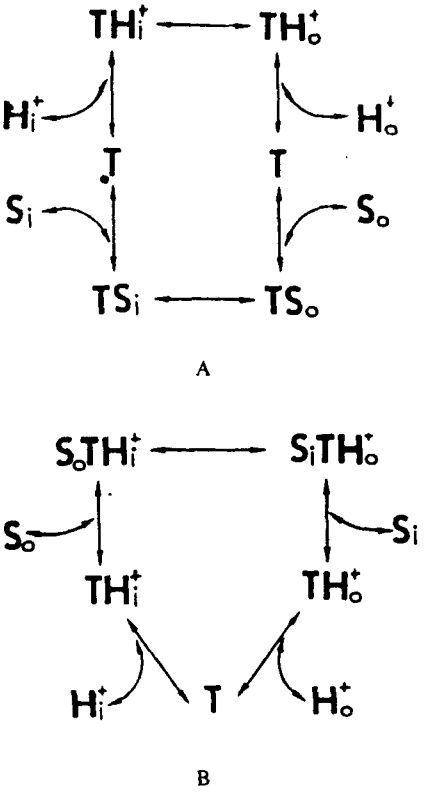


FIGURE 9. Schematic description of a translocation cycle. A sequential model (A); a simultaneous model (B). T, transporter; S, substrate. (From Schuldiner, S., Gabizon, R., Maron, R., Suchi, R., and Stern, Y., *Ann. N.Y. Acad. Sci.*, 456, 268, 1985. With permission.)

for the regulation of the process. The pH at which the gate will shut off depends on the pK of one or more residues on the protein. This type of gating mechanism has since been described in at least two other transport systems: the $Na^+/H^+$ antiporter from eukaryotes and prokaryotes.

The possibility that the unprotonated species of 5-HT is highly permeant through the membrane has been considered. Indeed, there have been reports that liposomes prepared with purified soybean lipids display a high permeability to the unprotonated form of various biogenic amines.[176,177] However, an interesting corollary of the findings is that this does not seem to be the case with the lipids from the chromaffin granules since dilution of the extravesicular medium does not induce efflux even when the intravesicular concentrations are up to 5 m*M* 5-HT.[173] Exchange, on the other hand, is quite rapid under the conditions where there is no net efflux. Also, photoinactivation of the transporter with ANPA-5-HT significantly slows down the rate of nigericin-induced efflux.[173] Furthermore, if the protonated amine were permeable through the membrane, it would be in equilibrium with the pH gradient. It has already been shown that this is not the case.[178] The low permeability of 5-HT is also shared by other membranes such as the cytoplasmic one in platelets, in which downhill movement of the amine requires $Na^+$ and $Cl^-$, the cotransported ions in this system, and is inhibited by imipramine, an inhibitor of the sodium-coupled transporter.[122]

The finding that there is a rapid exchange under conditions which are apparently in a steady state is in agreement with the possibility that the various transmitters, dopamine,

norepinephrine, and epinephrine, are synthesized on opposite sides of the membrane.[178] Recently, it has even been suggested that ATP may have a regulatory role on the exchange process.[171]

The significance of the findings described for the secretory cell is self-evident: the energetic burden of maintaining concentrations of amines of up to 0.6 to 0.7 *M* at 18 μ*M* cytoplasmic levels[178] could be prohibitive. Moreover, leakage of biologically active amines into the cytoplasm could have serious effects on the metabolism of the cell. In a steady-state system with no "gating" device, this would occur every time the levels of ATP in the cytoplasm decrease. A loss of intravesicular material would occur also upon decrease of the cytoplasmic levels of the amine.

Regardless of the mechanism, one of the most straightforward predictions of these findings is that in vivo inhibitors of the synthesis of the amines should not cause depletion of the stores. Indeed, even inhibition of synthesis by α-methyltyrosine, at the normally rate-limiting step, that of tyrosine hydroxylation, does not alter the catecholamine content of the adrenal medulla at rest.[179] The half-life of adrenaline and noradrenaline in different tissues ranges from 4 hr for brain to 300 hr for the adrenal medulla.[180] It has been shown that reserpine is a competitive and reversible inhibitor of amine influx.[76] Early experiments indicate enhancement of efflux by reserpine. However, these experiments are complicated by a nonspecific detergent-like effect at the ratio of reserpine-protein used.[181] Appreciation of this difficulty, together with our better understanding of the efflux process obtained in this study, should be helpful in the clarification of the mechanism of action of reserpine.

Reserpic acid, an impermeant derivative of reserpine, inhibits uptake from the outside but not from the inside.[182] This finding has been interpreted as evidence that the transporter is also structurally asymmetrical.

### C. Molecular Pharmacology of the Transporter

Impressive progress towards an understanding of the pharmacology of the amine transporter at a molecular level has been made during the last 5 years. Thus, several probes have been synthesized and their binding has been studied to learn about the properties of the binding site(s) and to identify the putative subunits of the transporter.

#### *1. Tetrabenazine Binding*

[$^3$H] TBZOH, a derivative of the drug tetrabenazine, was synthesized.[183] TBZOH binds to a single site on the chromaffin granule membrane with an apparent binding constant of about 3 n*M*. The density of the TBZOH binding site is about 60 pmol/mg protein. The $K_d$ is very similar to the apparent $K_i$ of the amine transport. Also, binding was correlated with inhibition of norepinephrine transport. However, displacement of TBZOH from its binding site by transport substrates such as 5-HT or NE is achieved only at concentrations about 100-fold higher than the apparent $K_m$ of the respective compounds.[175,183] Reserpine does not inhibit binding at concentrations which completely inhibit transport. At higher concentrations ($K_i$ = 20 n*M*), however, it does inhibit binding as well.

The component responsible for the binding has been solubilized by treatment with detergents: sodium cholate solubilized more than 70% of the binding sites.[184] High detergent concentrations had a reversible inhibitory effect. The binding characteristics ($K_d$ = 23 nM, $B_{max}$ = 90 pmol/mg protein) and the pharmacological properties of the binding sites were also similar to those of the membranes.

Radiation inactivation experiments have shown that the apparent $M_r$ of the TBZ binding protein is 65 kdalton.[185]

#### *2. Reserpine Binding*

[$^3$H] Reserpine was used to measure binding of this drug to granule membranes. Weaver and Dupree[174] and Scherman and Henry[175] found that reserpine binds to membranes energized

by the addition of ATP and is inhibited by protonophores. Scherman and Henry showed that binding also occurs in the absence of ATP and that ATP accelerates the rate of binding without affecting plateau values and equilibrium constants. They found that binding occurs on two classes of sites: $R_1$, $B_{max}$ = 7 pmol/mg protein and $K_d$ = 0.7 n*M*, and $R_2$, $B_{max}$ = 60 pmol/mg protein and $K_d$ = 25 n*M*. Deupree and Weaver[186] found only one class of sites with intermediate affinity and low density ($B_{max}$ = 7.8 pmol/mg protein and $K_d$ = 9 n*M*). Scherman and Henry[175] propose that sites $R_2$ are equivalent to TBZOH binding sites, as the densities are similar and because TBZ displaces reserpine from $R_2$ sites. Sites $R_1$, on the other hand are TBZ resistant and they seem to be involved in amine uptake since the $K_d$ values are similar to the $K_i$ values of reserpine for NE uptake. Moreover, transport substrates displace reserpine from $R_1$ sites at concentrations similar to their apparent $K_m$ values.

Radiation inactivation experiments show that the apparent $M_r$ of the reserpine binding component is 40 kdalton.[185]

*3. Photoaffinity Labels: Azido Derivatives of 5HT*

Three photoactive derivatives have been used to identify the transporter of bovine chromaffin granules:[187] pig platelet storage organelles, rat brain synaptic vesicles, and 5-HT-containing organelles in rat basophilic leukemia cells.[207]

The specificity of the first one, 4-azido, 3-nitrophenyl-azo-[5-HT] (ANPA-5HT), is supported by the following criteria. (1) ANPA-5HT is a competitive inhibitor of amine transport in the dark. The apparent $K_i$ is identical to the apparent $K_m$ of transport of 5-HT, its parent compound.[187] The latter indicates that the transporter recognizes the modified substrate. (2) Upon illumination, ANPA-5HT photoinactivates the amine transport. Transport of unrelated neurotransmitters or the generation of a pH gradient across the membrane are not inhibited.[187] (3) The rate of photoinactivation is lower in the presence of other substrates of the transporter. The concentrations required to "protect" the transporter correlate well with the known affinities of the various substrates. From the dependence of the rate of photoinactivation on the concentration of ANPA-5HT an apparent kinetic constant can be calculated. This constant is similar to the apparent $K_i$. The agreement suggests that the inactivation of transport is a result of a sequence of events which starts when ANPA-5HT binds to the transporter and is thereafter activated by light. (4) The rate of labeling of a membrane suspension with [$^3$H] ANPA-5HT in the light is lower in the presence of other substrates of the transporter.

Recently, ANPA-5HT has been iodinated with $^{125}$I.[222] The resulting compound displays properties almost identical to those of ANPA-5HT and dramatically shortens the time required for visualization of the label.

The pattern of labeling of the membrane polypeptides has been analyzed by separating them by SDS-gel electrophoresis and subjecting the gel to fluorography or autoradiography. More than 80% of the label in the protein fraction is associated with a 48-kdalton polypeptide and the labeling is inhibited by reserpine and by other substrates of the transporter. The concentration of reserpine required to inhibit labeling by 50% is similar to its apparent $K_i$ of transport.

The characteristics of the specific labeling of the amine transporter have been studied by assessment of the amount of radioactivity associated with the membrane proteins under several conditions. The criterion of the specificity of the labeling is that when reserpine or any of the other four transport substrates tested thus far, 5-hydroxytryptamine, epinephrine, norepinephrine, and dopamine are present during illumination at concentrations above their $K_m$, the amount of labeling is dramatically decreased.[187,188]

Since transmembrane gradients are not needed for labeling of the transporter with ANPA-5HT[188] we tested whether labeling requires at all the presence of an intact membrane. Extraction of chromaffin granule membranes with 1% cholate renders a soluble fraction that can be reconstituted into liposomes.[50] The proteoliposomes thus obtained display the trans-

porter activity. We have now found that even in the presence of the detergent, a protein is still specifically labeled by ANPA-5HT; however, labeling of the 48-kdalton polypeptide is inhibited at concentrations above 0.5%. A second polypeptide (apparent $M_r$ 56 kdalton) is labeled under these conditions. The concentrations of the substrates required to prevent labeling are higher than those required in the intact membrane. It is worth mentioning that in at least two other detergents, octylglucoside and Triton® X-100, labeling of the 48-kdaltor peptide is not inhibited even at concentrations that achieve full solubilization.[223]

*4. Azido Derivative of Tetrabenazine*

An azido derivative of tetrabenazine, a specific inhibitor of transport (see also Section V.C.1), has been synthesized.[189] The compound, [$^3$H]TBA, binds reversibly to the granule membranes in the dark with a $K_d$ of about 50 n*M* and a density of sites of 40 to 50 pmol/mg protein, consistent with reported densities of reserpine and dihydrotetrabenazine binding sites. Upon irradiation, TBA bound irreversibly to a polypeptide with an apparent $M_r$ of 70,000. Since TBA and TBZ compete for the same binding site ($R_2$ type), the authors suggest that the 70-kdalton polypeptide is the $R_2$ binding site.[189] This suggestion is supported by radiation inactivation studies in which it was found that the apparent $M_r$ of the TBZ binding site is 65 kdalton.[185]

## D. Chemical Modifications

Despite the profusion of noncovalent inhibitors and ligands of the catecholamine transporter very little information as been gathered about covalent modifiers of the protein. The native protein does not seem to have essential sulfhydryl groups accesible to NEM or pCMB. DCCD inhibits transport[66,190] and this inhibition can be prevented by TBZOH.[190] Diethylpyrocarbonate, which under proper conditions reacts specifically with histidine moieties, inhibits the transporter by lowering its $V_m$, without alteration of the $K_m$.[191] Interestingly, binding of TBZOH is not hampered.

## E. Reconstitution of the Amine Transporter

The amine transporter from bovine chromaffin granules has been solubilized with sodium cholate in the presence of soybean phospholipids.[177,192] The solubilized protein has been incorporated into liposomes after removal of the detergent either by gel filtration or by dialysis. Reserpine- and tetrabenazine-sensitive accumulation against concentration gradients has been achieved by imposition of artificially generated pH gradients and membrane potential. The apparent $K_m$ of the reconstituted transporter is similar or even lower than that of the native one. Two major problems have been encountered in the reconstitution of transport activity: (1) it is necessary to add asolectin prior to the detergent to maintain a fully functional transporter. Interestingly, this requirement is superseded when crude bovine brain lipids are added at reconstitution; (2) a large reserpine-insensitive accumulation is detected either in bare liposomes or in reconstituted proteoliposomes.[177] This is due to the relatively high passive permeability of the various amines across most membranes and subsequent equilibration with the imposed pH gradients. A further complication arises from the fact that reserpine is a very hydrophobic compound with detergent-like properties. Thus, at high concentrations it will induce leakiness even in liposomes and inhibit even unmediated transport.[181] Therefore, very rigorous criteria should be applied in every single reconstitution experiment to distinguish between mediated and unmediated transport.

## F. Purification of the Transporter

The putative amine transporter from bovine adrenal chromaffin granules has been partially purified in a single step utilizing affinity chromatography.[49] A 5-hydroxytryptamine moiety has been coupled to a sepharose 4B matrix in a position ortho to the hydroxyl group. When

membranes solubilized with sodium cholate are chromatographed on the above matrix a polypeptide is highly enriched. The enrichment is dependent on the presence of the proper ligand on the matrix and is inhibited if the column is previously equilibrated with a soluble ligand. Enrichment of the above polypeptide is accompanied by an increase in the specific activity of the transporter as measured by its labeling by 4-azido, 3-nitrophenyl-azo-(5-hydroxytryptamine). The ability of reserpine, a competitive inhibitor of binding and transport, to inhibit labeling of the purified transporter correlates well with its known kinetic constants in the native membranes.

The polypeptide purified was first thought to be identical to the one previously identified as the putative transporter based on specific labeling by a photoaffinity label.[49] However, a more detailed analysis of the electrophoretic pattern along with the realization that in the presence of 1% sodium cholate, a 56-kdalton peptide, but not a 48-kdalton peptide is labeled with $^{125}$I-ANPA-5HT and an antibody which is raised against the purified polypeptide cross-reacts both in the membrane and in the cholate extracts with a protein that has the mobility of a 56-kdalton and not a 48-kdalton polypeptide (unpublished results) indicates that the polypeptide enriched is not identical to the one labeled in intact membranes. It is suggested that the 56-kdalton polypeptide may be a subunit of the transporter.

### G. Amine Storage in Other Organelles

In addition to the catecholamine-rich chromaffin granules of adrenal medulla, other intracellular organelles from a wide variety of secretory cells accumulate biogenic amines. These include the adrenergic synaptic vesicles,[177,193,195] 5 hydroxytryptamine-containing secretory granules in platelets, enterochromaffin cells, and serotonergic neurons;[196-198] dopamine-rich vesicles in dopaminergic neurons;[199] and histamine-containing granules in mast cells and basophils.[200] Where it has been studied, the mechanism of amine accumulation into isolated storage organelles or membrane vesicles derived therefrom always involves exchange of extravesicular amine with intravesicular $H^+$, catalyzed by a reserpine-sensitive transporter.[124,177,195,201-203] A $H^+$-pumping ATPase in the organelle membrane generates an electrochemical gradient of $H^+$ (acid and positive inside) which provides the driving force for amine-$H^+$ exchange. All the organelles studied display the same inhibitor sensitivity, irrespective of the amine normally stored within.[204] These findings have led to the proposal that a closely similar or identical protein catalyzes amine-$H^+$ exchange in all biogenic amine storage organelles.[201]

Binding of TBZOH and the apparent $K_m$ for 5HT transport were measured in mice brain synaptic vesicles.[205] The values obtained (3 n*M* and 0.8 $\mu M$) are very similar to the values measured in adrenal chromaffin granules.

[$^3$H] Reserpine binding was measured in a synaptic vesicle preparation from bovine caudate nucleus.[206] Binding is of a high affinity type ($K_d$ = 1.2 n*M*; $B_{max}$ = 3.3 pmol/mg protein) and is dependent on ATP and inhibited by protonophores. Substrates displace reserpine at concentrations similar to those required for inhibition of dopamine transport.

ANPA-5HT was used in an attempt to identify the transporter in various organelles;[207] it inhibits ATP-driven reserpine-sensitive 5-HT transport into membrane vesicles prepared from porcine platelet dense (PL) granules, rat brain synaptic vesicles (SV), and histamine-containing granules of rat basophilic leukemia (RBL) cells. In addition, it specifically labels a polypeptide in each of the above-mentioned preparations. The apparent molecular weight of the labeled protein band varies from approximately 52 to 34 kdalton depending on the source of vesicles. The largest variation occurs between SV and RBL vesicles, while in both PL and chromaffin granule (CG) vesicles a 48-kdalton polypeptide is labeled. This result indicates that the molecular weight variation is not due to species difference, since RBL and SV membranes are both derived from rat, while CG and PL vesicles are from cow and pig, respectively. A more likely explanation is that the differences result from either

proteolysis or processing or both, since the 34-kdalton band is occasionally observed in overexposed samples of labeled CG vesicles. The 52-kdalton may be similar to the 56-kdalton band labeled in the presence of cholate in chromaffin granules. Still, the possibility that the differences reflect functionally similar but structurally different amine transporters cannot be ruled out at present. Analysis of the labeled polypeptides by peptide mapping is likely to help in resolving this issue.



### H. Storage of Neurotransmitters Other Than Biogenic Amines

The studies of the mechanism of storage of other neurotransmitters have considerably lagged behind the studies of amine storage. This is most likely due to the lack of a source of storage granules as rich and homogeneous as that found in the adrenal medulla, and until recently, to the lack of any pharmacology.

#### *1. Acetylcholine*

Cholinergic vesicles isolated from the electric organ of *Torpedo* display a complex pattern of AcCho transport activity. One component of the activity is passive, electrogenic and nonspecific;[208,209,212] a second component is ATP dependent and can generate relatively small gradients (about 5- to 12-fold).[12,210-212] This uptake is inhibited by ionophores of various kinds; it requires bicarbonate ions, but neither sodium nor potassium or chloride.[210] Uptake is sensitive to NBD and DCCD and is stimulated by DTT.

Isolated vesicles contain an ATPase.[213-215] It has been suggested that the enzyme is a proton pump,[216] and it has been solubilized in an active form.[217] Both the membrane-bound and the soluble enzyme are stimulated by bicarbonate ions[217] at the same concentration range that stimulates concentrative AcCho transport. The findings as a whole suggest that the overall mechanism of transport may have common attributes with the amine transport system. However, it is still early to propose detailed models.

Recently a potent inhibitor of AcCho transport has been described: 2-(4-phenylpiperislins) cydishexanol (AH5183).[218] The apparent $K_i$ of this compound is around 40 n*M*.

#### *2. Glutamate*

Synaptic vesicles from bovine brain were purified by immunoprecipitation with an antibody against Protein I, a neuron-specific synaptic phosphoprotein.[13] These vesicles were able to accumulate L-glutamate specifically in an ATP-dependent, temperature-dependent, but $Na^+$-independent manner.[13] Other neurotransmitters including aspartate, which shares with glutamate the sodium dependent plasma membrane transporter, were not accumulated. Non-hydrolyzable ATP analogues did not support the process. It does seem likely that also in the case of L-glutamate an ATP driven proton pump creates a membrane potential and/or pH gradient, which serve as driving forces for L-glutamate uptake in synaptic vesicles.

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