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ACTIVE TRANSPORT OF L-PROLINE BY MEMBRANE VESICLES ISOLATED FROM RAT BRAIN

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Summary

(1) Active transport of L-proline has been demonstrated in membrane vesicles isolated from rat brain. The process is dependent on external Na^+ and is observed only under conditions when artificial Na^+ gradients ($C_{out} > C_{in}$) are imposed across the vesicle's membrane.

(2) The transport process, the K_m value of which has been determined to be 140 μM, is strongly inhibited (75—85%) by nigericin. The process is stimulated by valinomycin and, in the absence of a pH gradient, inhibited (about 50%) by the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone. These data are consistent with the concept that proline transport is electrogenic. It seems optimal in the presence of a membrane potential (interior negative). In contrast, transport was not affected by ouabain and only slightly inhibited by arsenate. Optimal transport is obtained in the presence of external Cl^-. This dependency is only partial, but even persists when K^+-loaded vesicles are used in the presence of valinomycin. In addition, the process is inhibited by alkaloids like veratridine and aconitine and this inhibition is prevented by tetrodotoxin. The results provide direct evidence for Na^+-coupled active transport by rat brain synaptic plasma membrane vesicles.

(3) The proline carrier has been solubilized from the membrane vesicles using cholate. The solubilized transporter was reconstituted in the presence of soybean phospholipids and potassium phosphate using the cholate dialysis technique. The reconstituted proteoliposomes catalyzed Na^+-dependent proline transport, which was 6—10-fold stimulated by valinomycin.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Mes, 4-morpholineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Introduction

Recently the transport of neurotransmitter amino acids has been studied using isolated membrane vesicles obtained after osmotic shock of synaptosomal fractions derived from rat brain [1,2]. These transport studies have provided direct evidence that the general concept that solute accumulation can be achieved by cotransport with ions [3—5] also applies to the brain. Thus, it appears that the electrochemical potential gradient Na^+ serves a direct driving force for the transport of γ-aminobutyric acid [1] and L-glutamic acid [2]. Surprisingly, these studies revealed that the neurotransmitter amino acid transport is absolutely dependent on additional ions, such as external Cl^- or small monovalent anions in the case of γ-aminobutyric acid [1] and internal K^+ in the case of L-glutamate [2]. The gradients of these ions may also serve as additional driving forces for the above transport systems [1,2].

In view of these remarkable observations, it seems of interest whether this requirement for ions other than Na^+ also applies to transport in brain of other amino acids. Studies with relatively intact preparations such as synaptosomes indicate that proline is suitable in this regard since it is one of the few amino acids of which the uptake is Na^+ dependent [6,7]. Therefore, we have chosen to determine the ionic requirements and the energetics of proline transport using isolated membrane vesicles derived from rat brain.

Materials and Methods

Materials

L-[G-^{3}H]Proline was obtained from New England Nuclear. Valinomycin gramicidin, CCCP, veratridine, acontine and tetrodotoxin were purchased from Sigma Chemical Co. Nigericin was a generous gift of Dr. R. Hosley from Eli Lilly. Cholic acid from Schwarz/Mann was recrystallized from 70% ethanol [8] and soybean phospholipids (asolectin, Associated Concentrates) were partly purified [8].

Methods

Preparation of membrane vesicles. Membrane vesicles were isolated after osmotic shock of synaptosomal fractions, as described previously [1,2]. Aliquots were stored in liquid air. Under these storage conditions the transport ability of the membrane vesicles was stable for at least 2 months. Variations in specific activity were observed, dependent on the preparation, but with a given preparation the specific activity for each aliquot was very similar.

Solubilization and reconstitution of the proline carrier. The stored membrane vesicles were solubilized essentially as described earlier [9], except that the membrane protein concentration ranged from 10—12 mg/ml and dithiothreitol was used at 2 mM. The solubilized proteins were reconstituted as described in Ref. 9.

Transport assays. Transport assays were performed essentially as described earlier [1,2]. Membrane vesicles were loaded [1,2] with 0.1 M potassium phosphate (pH 6.8) and 1 mM $MgSO_4$, unless stated otherwise. Of the latter suspension, 20 μl were added to 180 μl of external solution, consisting of 0.1 M NaCl

and 0.68 μM L-[G-^{3}H]proline (3.7 Ci/mmol), unless stated otherwise. Inhibitors and ionophores, when present, were usually added to the external solution prior to the addition of membranes. The transport reactions were terminated as described previously; the membrane filters were washed [1,2] and radioactivity was determined using liquid scintillation counting.

In the case of reconstituted vesicles transport was measured by adding 30—40 μl of proteoliposomes (1—1.5 mg protein/ml) to 0.36 ml incubation medium containing (unless indicated otherwise in the figure legends) 0.15 M NaCl, 1% (v/v) glycerol, 2 mM $MgSO_4$, 1.36 μM L-[G-^{3}H]proline (3.7 Ci/mmol) and the additions indicated in the figure legends. The reactions were carried out at room temperature (21—24° C). After termination, filtration and washing [10], the retained radioactivity was determined using liquid scintillation spectrometry. The counting efficiency was 25%.

Protein determinations. Protein was determined as described by Lowry et al. [11].

Results

The data presented in Fig. 1 illustrate the uptake of L-[G-^{3}H]proline into membrane vesicles isolated from rat brain. An artificial Na^+ gradient is created by diluting (at time zero) the membrane vesicles loaded with potassium phosphate 10-fold into an NaCl solution containing L-[G-^{3}H]proline. In this experiment approx. 30 pmol of proline/mg protein were taken up. The accumulated radioactivity in fact represents unmodified L-proline, since after release from the vesicles it was found to co-chromatograph with authentic L-proline using thin-layer chromatography (solvent system: 40% (v/v) chloroform/40% (v/v) methanol/20% (v/v) ammonia). The uptake process is temperature-dependent since no uptake at all was detected at 0° C (data not shown). When external Na^+ is replaced by Li^+, K^+ or NH_4^+, almost no proline uptake is observed (Fig. 1).

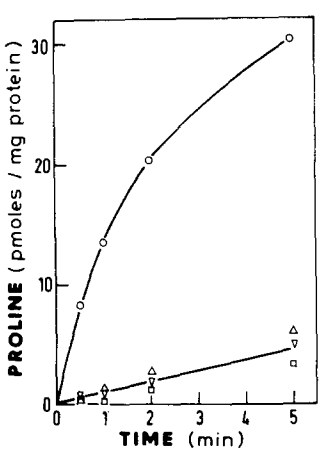


Fig. 1. The effect of external cations on proline uptake by rat brain membrane vesicles. Membrane vesicles were loaded and transport assays were made as described in Materials and Methods, using 182 μg protein per assay. The external medium contained: 0.1 M NaCl (○———○); 0.1 M LiCl (△———△); 0.1 M KCl (▽———▽); 0.1 M NH_4Cl (□———□).

Moreover, nigericin does not inhibit the latter residual uptake (data not shown) in contrast to uptake in the presence of Na^+ (Table I). This ionophore, which under the experimental conditions is expected to collapse the Na^+ gradient, strongly inhibits proline uptake. The effect of nigericin might, in principle, be attributed to collapse of the K^+ gradient ($C_{in} > C_{out}$). This is unlikely to be the cause of the strong inhibition of proline transport by the ionophore, since internal K^+ is not required for the process (Table I). The role of the Na^+ gradient ($C_{out} > C_{in}$) as driving force for proline transport is further substantiated by the low uptake observed in the absence of an inward-directed Na^+ gradient; i.e., when internal K^+ is replaced by internal Na^+ (Table I). This low uptake is not inhibited by nigericin, in contrast to the uptake under all other conditions indicated in the table (inhibition ranging from 75 to 85%). Thus, uptake in the presence of internal Na^+ is similar to that in the presence of any of the other internal ions in the presence of nigericin. Gramicidin, another ionophore capable of dissipating the Na^+ gradient is also inhibitory (Table I).

Valinomycin, which is expected to induce or to enhance the magnitude of the membrane potential (interior negative), stimulated proline transport about 1.7-fold (Table I). Although small, this stimulation is consistent and, depending on the batch of vesicles, sometimes reaches up to 2.5-fold.

The fact that significant transport is observed in the absence of valinomycin

TABLE I

THE EFFECT OF INTERNAL IONS, INHIBITORS AND IONOPHORES ON PROLINE TRANSPORT

Transport was measured as described in Materials and Methods. Membrane vesicles were loaded with the indicated loading solutions (all adjusted to pH 6.8 and all containing 1 mM $MgSO_4$). The external NaCl-containing solution contained the indicated final concentration (100 mM, unless stated otherwise) of compounds. In the case of *p*-hydroxymercuribenzoate, the membrane vesicles were also preincubated on ice with the same concentration of inhibitor used during the assay. For additions, concentrations in μM are given in parentheses.

Loading solution	Addition	30 s transport (% of control)	2 min transport (% of control)
Potassium phosphate	—	100	100
Tris-phosphate	—	72	58
Ammonium phosphate	—	100	77
Sodium phosphate	—	30	26
K^+-Mes	—	102	96
50 mM K^+-Mes + 50 mM potassium arsenate	—	102	78
K^+-Tricine	—	133	128
50 mM K^+-Tricine + 50 mM potassium arsenate	—	134	104
Potassium phosphate	valinomycin (2.5)	174	128
	ouabain (100)	101	108
	p-hydroxy-mercurybenzoate (200)	14	34
	gramicidin D (50)	68	67
	nigericin (5)	18	24
	nigericin (5) + valinomycin (2.5)	16	15
	CCCP (5)	60	56

is consistent with the observations on intact nerve preparations. These suggest that the permeability of the neuronal membrane to K^+ is sufficiently large so that the K^+ gradient ($C_{in} > C_{out}$) may generate a membrane potential also in the absence of valinomycin [12]. This potential may thus contribute to L-proline accumulation. Consistent with this is the partial inhibition of accumulation observed with the proton ionophore, CCCP (Table I). Since the concentration of protons is equal on both sides of the membrane, this ionophore would be expected to abolish any existing membrane potential. This contention is further supported by the experiment depicted in Fig. 2. The membrane potential induced by CCCP will depend on the magnitude of the pH gradient present across the membrane. Thus, if the membrane vesicles loaded at pH 6.8 are diluted into a reaction medium of pH 7.5 in the presence of CCCP, the compound is no longer inhibitory and even slightly stimulatory (Fig. 2). If the same experiment is performed with the external medium buffered at pH 6.0 (tendency to set up a membrane potential of the opposite polarity), a strong inhibition of proline transport by CCCP is observed (Fig. 2). In view of these observations as well as those on the reconstituted system (Fig. 7) it appears that Na^+-dependent proline transport is electrogenic.

The notion that the potent inhibition of proline transport by nigericin is due to the collapse of the Na^+ gradient is further supported by the results depicted in Fig. 3. When nigericin is added to vesicles which have previously been allowed to accumulate proline, a rapid efflux ensues until a level of uptake is reached similar to that where nigericin was added from the beginning (Fig. 3).

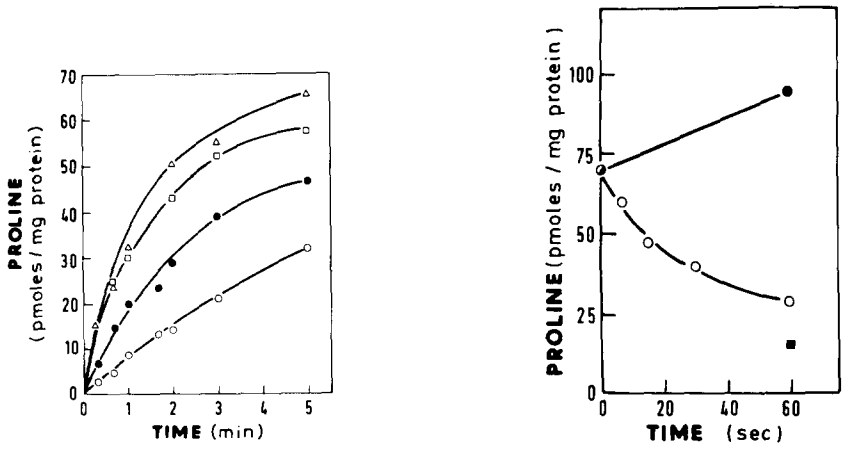


Fig. 2. The effect of the proton conductor, CCCP, on proline transport. Transport assays were performed as described in Materials and Methods, using 98 μg protein per assay. The external medium contained: 50 mM NaCl and 50 mM sodium phosphate, pH 6.0, without (●———●) or with 5 μM CCCP (○———○); 50 mM NaCl and 50 mM sodium phosphate, pH 7.5, without (□———□) or with 5 μM CCCP (△———△).

Fig. 3. Influx and subsequent efflux of proline from rat brain membrane vesicles induced by nigericin. Transport assays were made as described in Materials and Methods, except that the external solution contained 2.7 μM L-[G-^{3}H]proline (3.7 Ci/mmol) and valinomycin (2.5 μM). Nigericin (5 μM) was added at time zero on the time scale of the figure, after 2 min of transport. At the indicated times after the additions, the amount of L-proline retained by the vesicles was determined. Additions: none (●———●); nigericin (○———○). In addition the level of transport after 3 min with nigericin present from the beginning was determined (■). 160 μg of protein were used in each assay.

Independent evidence on the importance of the electrochemical Na^+ gradient as a driving force for proline transport can be derived from the following considerations which are valid if proline transport is catalyzed by membrane vesicles originating from the synaptic plasma membrane. The latter should contain action potential Na^+ channels. These channels can be opened by alkaloids such as veratridine [13] or aconitine [14]. This opening should lead to a decrease in the electrochemical Na^+ gradient and thus result in an inhibition of proline transport. These effects should be reversed by tetrodotoxin. The latter poison closes the channel [15] and its effect is dominant over that of the alkaloids. These predictions have alrady been shown to be correct in the case of transport of γ-aminobutyric acid and l-glutamic acid [16]. As can be seen in Fig. 4, either veratridine or aconitine progressively inhibits the transport of proline. This indicates that the inhibitory effect is due to dissipation of the Na^+ concentration gradient, rather than to depolarization. In the latter case, inhibition would be instantaneous. Tetrodotoxin, which by itself has not much of an effect, completely reverses the effects of either alkaloids (Fig. 4).

The extent of proline uptake is sensitive to the medium osmolarity (data not shown), providing additional evidence that proline is in fact transported across the vesicle's membrane. Moreover, when the membranes are washed with water rather than with isotonic salt solution, most of the accumulated radioactivity is lost (data not shown). The extent of uptake is dependent on the batch of vesicles and ranges from 30 to 100 pmol/mg protein under standard conditions. Using the determined value of the total intravesicular space of 7.4 μl/mg [1], the internal proline concentration ranges from 4 to 13.5 μM. This is a minimal estimate, since possibly not all vesicles in the preparation have the ability to accumulate proline. Thus, a concentration gradient of at least 6—20-fold is reached.

In addition to ionophores capable of abolishing the artifically imposed Na^+ gradient and/or membrane potential, the sulfhydryl reagent, *p*-hydroxymercuribenzoate, also appears to be an inhibitor of the transport process (Table I), possibly inhibiting at the level of the transporter.

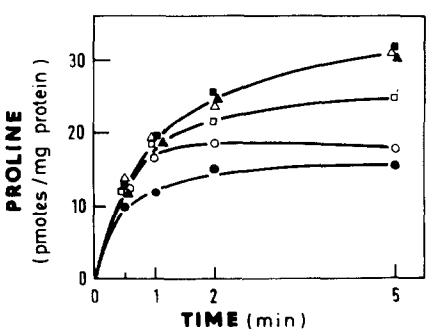
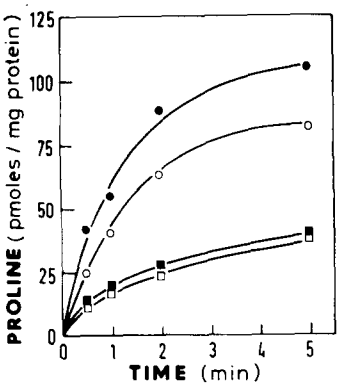


Fig. 4. Effects of veratridine, aconitine and tetrodotoxin on L-proline transport. Transport assays were performed as described in Materials and Methods using 206 μg protein per assay. The standard external medium contained the following additions: (□———□) none; (●———●) aconitine, 20 μM; (○———○) veratridine, 25 μM; (■———■) tetrodotoxin, 1 μM; (▲———▲) veratridine, 25 μM + tetrodotoxin, 1 μM; (△———△) aconitine, 20 μM + tetrodotoxin, 1 μM. Prior to the assays the membrane vesicles were preincubated for 15 min at 37°C with concentrations of the compounds identical to those present in the assay medium.



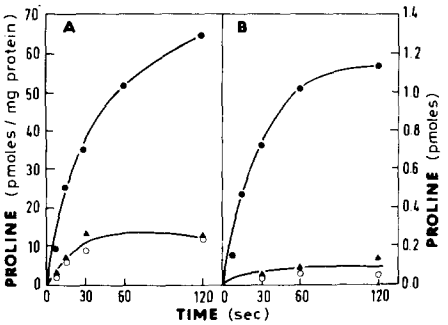


Fig. 5. Anion-dependence of proline transport. Membrane vesicles were loaded and transport assays were performed as described in Materials and Methods, using 56 μg of membrane protein. The external medium contained 100 mM NaCl with (●———●) or without (○———○) valinomycin or 50 mM Na_2SO_4 + 50 mM sucrose with (■———■) or without (□———□) valinomycin.

Fig. 6. The dependency of proline transport in reconstituted proteoliposomes on external Na^+, protein and phospholipids. (A) Cation-dependence: proteoliposomes were reconstituted and transport was measured in the presence of 2.5 μM valinomycin as described in Materials and Methods using 31.8 μg protein per transport assay. Composition of the external medium: standard NaCl medium (●———●), LiCl (▲———▲) or KCl (○———○) replacing NaCl. (B) Protein and phospholipid-dependence: vesicles were reconstituted containing protein without exogenous phospholipids (▲———▲); phospholipids alone (○———○), or both (●———●).

The insensitivity of the Na^+-dependent L-proline transport to ouabain (Table I) supports the view that the (Na^+ + K^+)-ATPase is not directly involved in the process. The resistance of the process to arsenate (Table I) also indicates that the possibility of indirect involvement of the (Na^+ + K^+)-ATPase to utilize the artificially created Na^+ and K^+ gradients (for energization) is unlikely.

The process exhibits saturation kinetics. From Lineweaver-Burk plots, a K_m value of 140 μM and a V value of 6.3 nmol/min per mg have been determined.

The external anion optimal for proline transport is Cl^- (Fig. 5). When external Cl^- is replaced by SO_4^{2-} (with sucrose to maintain identical osmolarity) both the initial rate and extent of transport are reduced by 60—70% (Fig. 5). This inhibition persists even in the presence of valinomycin (Fig. 5) and thus apparently is not due to a difference of the membrane permeabilities of these anions. Quantitatively similar results to those obtained with external SO_4^{2-} are obtained also in the presence of external phosphate and the sensitivity of the process to nigericin was similar to that observed in the Cl^--containing medium (data not shown). In contrast, optimal transport is obtained with any of the internal ions tested. (Table I).

A time-dependent L-proline uptake is observed when proteoliposomes formed in a potassium phosphate solution are diluted into 0.15 M NaCl in the presence of valinomycin (Fig. 6A). Under these conditions artificial gradients of ions such as Na^+ ($C_{out} > C_{in}$) and K^+ ($C_{in} > C_{out}$) are generated. The uptake of L-proline, both with regard to initial rate and extent is rather similar to that in the native membrane vesicles. The transport in the reconstituted proteoliposomes is also Na^+-dependent (Fig. 6A) and requires exogenously added phos-

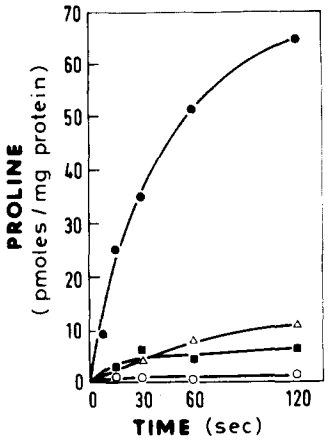


Fig. 7. The effect of ionophores on proline transport in reconstituted proteoliposomes. Proteoliposomes were reconstituted and transport was assayed as described in Materials and Methods. Additions: none (△———△); valinomycin, 2.5 μM (●———●); valinomycin, 2.5 μM + CCCP 5 μM (■———■); valinomycin, 2.5 μM + nigericin, 5 μM (○———○).

pholipids as well as the solubilized protein fraction (Fig. 6B).

When the cation gradients artificially imposed across the proteoliposomes are abolished using the ionophore nigericin, a complete inhibition of L-proline transport is observed (Fig. 7). Moreover, the addition of the ionophore, valinomycin, stimulates the initial rate of transport about 8-fold and this stimulation is completely abolished by the proton ionophore, CCCP (Fig. 7). Although the effects of nigericin, CCCP and valinomycin are similar to those observed in the native system (Table I), the stimulation by the last ionophore is considerably larger in the reconstituted system. This indicates that the passive permeability for K^+ is much lower in this system. It is likely that this is due to the excess of phospholipids added.

Discussion

Important differences exist between the transport of the neurotransmitters, γ-aminobutyric acid and L-glutamic acid, and that of proline. Transport of the two neurotransmitters was found to be absolutely dependent on the additional presence of ions other than Na^+, such as external Cl^- or small monovalent anions in the case of γ-aminobutyric acid [1] and internal K^+ in the case of L-glutamic acid [2]. No absolute dependency of proline transport on ions other than Na^+ was detected. Nevertheless, the process is strongly stimulated by the presence of Cl^- (Fig. 5). In the case of neurotransmitter transport, the gradients of these additional ions, Cl^- ($C_{out} > C_{in}$) for γ-aminobutyric acid and K^+ ($C_{in} > C_{out}$) for L-glutamic acid represent additional driving forces for accumulation of these neurotransmitters [1,2]. On the other hand, no evidence for such driving forces in addition to the electrochemical gradient for Na^+ has been detected for proline transport. Another important difference is the affinity for the respective solutes, the K_m value for proline (140 μM) being about 50-fold larger than that reported using isolated membrane vesicles for γ-aminobutyric

acid [1] or L-glutamic acid [2]. This is reasonable since the latter two amino acids are thought to serve as neurotransmitters. The main function of their high-affinity transport systems is apparently the termination of synaptic transmission by removal of the transmitters from the synaptic cleft [17]. Thus, the high affinity for the transmitters is of paramount importance to maintain low concentrations in the synaptic cleft. There is no strong evidence for proline being a neurotransmitter, although the Na^+-dependence of the process has been considered as an indication for it [7].

Proline transport in membrane vesicles has also many similar features to transport of γ-aminobutyric acid [1] and L-glutamic acid [2], the only two amino acids studied in any detail using this preparation. These features include Na^+-dependence and sensitivity to sulfhydryl reagents. This Na^+-dependence has also been observed using synaptosomal preparations [6,7]. This dependence is not merely for external Na^+, since the transmembraneous Na^+ gradient has been established to be a major driving force for the process (Table I and Figs. 1, 3, 6 and 7). Independent evidence for this contention comes from the experiment depicted in Fig. 4 in which it is shown that the opening and closing of the action potential Na^+ channel influence the transport of proline as predicted. This experiment also indicates that the membrane vesicles catalyzing proline transport are derived from the synaptic plasma membrane. The transport of neurotransmitters and proline in the membrane vesicles is not inhibited by ouabain (Refs. 1 and 2 and Table I), in contrast to that in the more intact preparations [6,18,19]. In the latter, energy is supplied in the form of glucose and the ATP derived from it by catabolism is used by the $(Na^+ + K^+)$-ATPase to create the ion gradients which appear to be the immediate driving forces for active Na^+-dependent amino acid transport.

Although there is no absolute dependence of proline transport on any ion other than Na^+, it is of interest to note that optimal transport is observed in the presence of external Cl^- (Fig. 5). Recently and independent of our observations of the absolute Cl^--dependency of the γ-aminobutyric acid process [1] and the partial Cl^--dependence of the proline transport, it has been observed that the transport of many solutes, including proline, in the relatively intact synaptosomal preparations is optimal in the presence of Cl^- [20]. Moreover, in membrane vesicles isolated from platelets, an absolute dependency of 5-hydroxytryptamine transport on external Cl^- has been observed [21]. It is unlikely that the partial dependence of proline transport on external Cl^- with the isolated membrane vesicles from brain can be explained by the imposition of a membrane potential or by charge compensation by this anion, as has been suggested [20], since the dependency persists in the presence of valinomycin using K^+-loaded vesicles (Fig. 5); a possible explanation for its partial requirements is that binding of Cl^- to the carrier may result in a conformation which is optimal for transport.

Accumulation of proline under standard conditions is 6—20-fold. Although this is a minimal estimate, because of the heterogenity of the vesicle population, the gradients reached with γ-aminobutyric acid [1] and L-glutamic acid [2] are larger by an order of magnitude, with similar ion gradients imposed. One possibility is that the proportion of proline-transporting vesicles is at least an order of magnitude smaller than that for the neurotransmitters. Alterna-

tively, the different levels of accumulation may be due to different translocation stoichiometries of the solutes with the coupling ions. If in the case of the neurotransmitters the additional ions indeed play a role in the translocation cycle, more than one coupoing ion is translocated per molecule of solute [1,2].

In order to further our knowledge on the mechanism of solute translocation, it is of paramount importance to purify the carriers and characterize them. For this purpose, a reconstitution assay has to be set up. Since we have been able to solubilize and reconstitute the proline transporter (Figs. 6 and 7), a start towards this important goal has now been made.

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