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Stoichiometry of Sodium- and Chloride-Coupled γ-Aminobutyric Acid Transport by Synaptic Plasma Membrane Vesicles Isolated from Rat Brain†

Rodica Radian and Baruch I. Kanner*

ABSTRACT: Transport of γ-aminobutyric acid (GABA) into synaptic plasma membrane vesicles exhibits an absolute dependency on both sodium and chloride. The requirement for chloride is not due to its ability to serve as a permeant anion. Chloride ion does not merely fulfill a need for a permeant ion since GABA accumulation still requires external chloride when a K^+ diffusion potential (interior negative) is imposed across the vesicle membrane with valinomycin. K_m is lowered and V_{max} is raised by either sodium or chloride ions. A plot of the logarithm of the concentration ratio of GABA (internal/external) at steady state vs. the logarithm of the concentration ratio of sodium or chloride ions (both external/internal) yields straight lines with slopes of 1.50 ± 0.20 or 0.47 ± 0.02, respectively. Both GABA and tetraphenylphosphonium ion transport are affected to a similar extent by either valinomycin (enhanced) or carbonyl cyanide *m*-chlorophenylhydrazone (inhibited). In the presence of K^+/valinomycin a plot of the logarithm of the concentration ratio of GABA (internal/external) at steady state vs. the logarithm of the concentration ratio of potassium (internal/external) yields a straight line with a slope of 0.90 ± 0.08. The simplest stoichiometry for the translocation cycle catalyzed by the GABA transporter is the influx of two sodium ions and one chloride ion per GABA zwitterion.

Membrane vesicles isolated from various bacterial and mammalian cells have proved extremely useful for the study of active transport [cf. Kaback (1974), Aronson & Sactor (1974), Hopfer et al. (1973), Colombini & Johnstone (1974), Lever (1977), and Rudnick (1977)]. Some of their advantages include the possibility of using well-defined energy sources and the lack of metabolism and storage in subcellular organelles. Recently, the use of membrane vesicles has been extended to the study of the synaptic plasma membrane (Kanner, 1980) for the investigation of sodium-dependent neurotransmitter transport in rat brain (Kanner, 1978; Kanner & Sharon, 1978). These transport systems have been implicated in the termination of transmitter action on postsynaptic receptors (Iversen, 1971).

Using the synaptic plasma membrane vesicles, it has been shown that the general concept that solute accumulation can be achieved by cotransport with ions (Crane, 1965; Riggs et al., 1958; Mitchell, 1963) also applies to neurotransmitters in the brain. Thus, the electrochemical potential gradient of Na^+ serves as a direct driving force for the transport of GABA[1] (Kanner, 1978) and L-glutamic acid (Kanner & Sharon, 1978). Surprisingly, these studies revealed that neurotransmitter transport is absolutely dependent on additional ions, such as external Cl^- or small monovalent anions in the case of GABA (Kanner, 1978) and internal K^+ in the case of L-glutamate (Kanner & Sharon, 1978). Recent experiments have provided strong evidence that the GABA transporter catalyzes influx of GABA coupled with the influx of both sodium and chloride ions (Kanner & Kifer, 1981). Similarly, it appears that the L-glutamate transporter catalyzes influx of L-glutamate coupled with the influx of sodium ions and the efflux of potassium ions (Kanner & Marva, 1982). Other recent examples of participation of ions in addition to sodium in the translocation cycle of solute transport systems include the serotonin transporter from platelets (Nelson & Rudnick, 1979; Nelson & Rudnick, 1982) and the L-glutamate transporter from renal brush border vesicles (Burckhardt et al., 1980; Schneider & Sacktor, 1980; Sacktor et al., 1981).

The studies described in this paper focus on the stoichiometry of the GABA transporter. Because of the electrogenicity of the transporter (Kanner, 1978), it is predicted that the stoichiometry will be nNa$^+$:mCl$^-$:GABA with $n > m$. Studies on the sodium dependence of GABA transport in intact synaptosomes indicate a positive cooperativity for sodium ions with a Hill coefficient of slightly over (Martin & Smith, 1972) or under 2 (Blaustein & King, 1976). Recently, similar studies have indicated that the Hill coefficient for the chloride dependence is about 1 (Kuhar & Zarbin, 1978). These observations are also consistent with $n > m$.

This paper describes a thermodynamic approach to study the stoichiometry of the process in synaptic plasma membrane vesicles. It is concluded that the stoichiometry for the translocation cycle catalyzed by the GABA transporter is the

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[1] Abbreviations: GABA, γ-aminobutyric acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPP^+, tetraphenylphosphonium ion; $\Delta\tilde{\mu}_{Na^+}$, (RT/F) ln $([Na^+]_{out}/[Na^+]_{in}) - \Delta\psi$; $\Delta\psi$, membrane potential (outside is zero); $\Delta\tilde{\mu}_{Cl^-}$, (RT/F) ln $([Cl^-]_{out}/[Cl^-]_{in}) + \Delta\psi$.

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influx of two sodium ions and one chloride ion per GABA zwitterion.

Experimental Procedures

Materials

[2,3-$^3H_2$]GABA was obtained from New England Nuclear. [^{3}H]TPP$^+$ was obtained from the Nuclear Research Center, Negev, Israel. Valinomycin and CCCP were purchased from Sigma Chemical Co. All other materials were of the highest purity commercially available.

Methods

Preparation of Membrane Vesicles. Membrane vesicles from 14-day-old female rats were prepared and stored as described previously (Kanner, 1978). The vesicles were preloaded prior to the transport assays with 0.1 M KP_i plus 1 mM $MgSO_4$, pH 6.8, unless indicated otherwise in the legends to the figures. The molarity in all cases is with respect to the anion.

Transport Assay. Transport of GABA was measured as described previously (Kanner, 1978). TPP$^+$ uptake was measured essentially as described previously (Kanner, 1978), except that [^{3}H]GABA was replaced by [^{3}H]TPP$^+$ (888 Ci/mol). In this case influx media were supplemented with 1 μCi of [^{3}H]TPP$^+$/200 μL of assay medium. Furthermore, cellulose acetate filters (Schleicher and Schull, 25-mm diameter, 0.45-μm pore size) were used instead of cellulose nitrate filters. Stopping of the reactions, filtration, washing, and counting were done exactly as described for GABA transport (Kanner, 1978). Experimental values were obtained by subtracting zero-time values (stop solution added before the vesicles).

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

Calculation of Internal Concentrations. It was found that about 50% of the vesicles have a functional GABA transporter (Kanner, 1978). Thus the GABA concentration inside the vesicles was calculated from the amount of GABA taken up by the vesicles, using a value of 3.8 μL of internal volume accessible to GABA/mg of membrane protein (Kanner, 1978). For calculation of the internal TPP$^+$ concentration, the value of 7.4 μL of total volume/mg of protein (Kanner, 1978) was used. The membrane potential is calculated by using

$$\Delta\psi = -(RT/F) \ln ([TPP^+]_{in}/[TPP^+]_{out})$$

Results

Sodium and Chloride Ion Requirement for Influx. As reported previously, GABA accumulation into synaptic plasma membrane vesicles from rat brain is absolutely dependent on both external sodium and chloride ions (Kanner, 1978). Although this uptake appears to be electrogenic, evidence has been provided to exclude the possibility that the chloride requirement of the process is due to the ability of this ion to serve as a permeant anion. As will be shown below, direct measurements of the membrane potential with the lipophillic cation TPP$^+$ unambiguously exclude this explanation. The validity of this method for a wide range of systems is established [cf. Skulachev (1971) and Lichtstein et al. (1979)]. As shown in Figure 1, this method can also be applied to measurements of the membrane potential across the membrane of synaptic plasma membrane vesicles. The validity of the method is checked by diluting potassium-loaded vesicles (about 110 milliequiv of K$^+$ internal concentration) into an external medium containing 150 milliequiv of K$^+$ in the presence of the potassium ionophore valinomycin and [^{3}H]TPP$^+$. One can

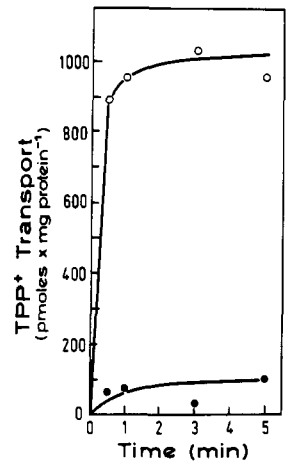

FIGURE 1: Effect of the external ion composition on TPP$^+$ uptake. Transport of TPP$^+$ was performed with vesicles (10 μL, 38 μg of protein) loaded with 40 mM KP_i, pH 6.8, + 50 mM KCl. The vesicles were diluted into 180 μL of either 0.15 M NaCl (○) or 0.15 M KCl (●) containing 5.63 μM [^{3}H]TPP$^+$ (888 Ci/mmol) and 2.5 μM valinomycin.

calculate using the value of 7.4 μL/mg of protein for the internal volume of the vesicles (Kanner, 1978) that under the conditions for this experiment (external TPP$^+$ concentration of 5.63 μM) only about 30 pmol of TPP$^+$/mg of protein should be accumulated. Instead, a value of 90–100 pmol/mg of protein is observed at steady state (Figure 1). Thus the difference of these two numbers—about 60 pmol/mg of protein—probably represents binding of TPP$^+$ to the outside of the vesicles or to the filters. However, when these potassium-loaded vesicles are diluted 19-fold under the same conditions but with NaCl replacing the KCl—a condition where a potassium diffusion potential (interior negative) is expected to be generated—the level of TPP$^+$ uptake is about 1000 pmol/mg of protein (Figure 1). Thus the binding (60 pmol/mg of protein) is only a small fraction of the potential-dependent uptake. This uptake corrected for binding is about 940 pmol/mg of protein, which, on the assumption that it reflects distribution in response to an interior negative membrane potential, reflects an internal concentration of about 127 μM. This represents a concentration gradient of about 22-fold. This is somewhat higher than the predicted value of 19-fold (19-fold dilution into valinomycin-containing K$^+$-free medium) or −74 mV, the calculated potassium diffusion potential. As discussed recently, such overestimates are to be expected as result of an increased binding to the membrane when the internal concentration is elevated due to an internal negative membrane potential (Zaritsky et al., 1981). It appears that the method can be used to obtain reasonably close estimates of the membrane potential in this system. It should be anticipated, however, that all calculated values of the membrane potential in this paper are somewhat overestimated. As shall become clear, this does not affect any of our conclusions.

In the experiment depicted in Figure 2, the question whether the dependence of GABA transport on chloride is due to the latter being a permeant anion is examined directly. When KP_i-loaded vesicles are diluted into a sodium phosphate containing medium, no GABA transport can be detected even in the presence of valinomycin (Figure 2A). This condition is expected to provide both the proper sodium gradient (out > in) as well as the appropriate membrane potential (interior negative). That the latter indeed is the case is seen when the lipophillic cation TPP$^+$ is used to estimate the membrane potential. From a parallel experiment to that described in

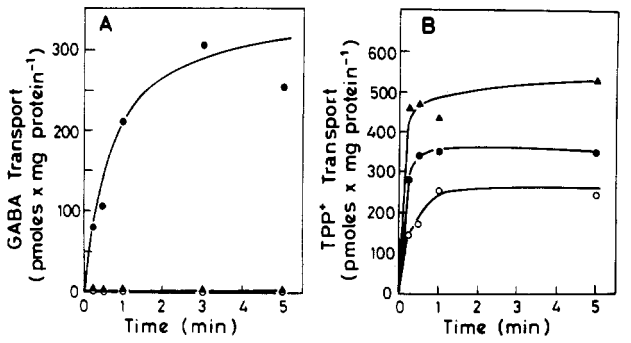

FIGURE 2: Effect of external chloride on GABA accumulation and on membrane potential. Transport of GABA (A) or TPP^+ (B) was performed with KP_i-loaded vesicles, 58 μg of protein/assay point, as described under Experimental Procedures. In addition to the isotope and 1 mM $MgSO_4$, the composition of the external media was as follows: (●) 0.1 M NaCl; (○) 0.1 M NaP_i; (▲) 0.1 M NaP_i + 2.5 μM valinomycin. The external concentrations of the solutes were 0.16 μM GABA (29.3 Ci/mmol) and 5.4 μM TPP^+ (888 Ci/mmol).

Table 1: Effect of Sodium and Chloride Concentrations on Apparent K_m and V_{max} for GABA[a]

	K_m (μM)[b]	V_{max} [pmol of GABA/(min·mg)][b]
(A) 25 mM Na^+	12.3 ± 6.6 (5)	580 ± 180 (5)
(B) 25 mM Cl^-	6.4 ± 1.8 (5)	800 ± 300 (5)
(C) 100 mM NaCl	4.2 ± 0.54 (9)	2093 ± 363 (9)

[a] Membrane vesicles (10 μL, 65–100 μg of protein) loaded with 0.1 M KP_i, pH 6.8, + 1 mM $MgSO_4$ were diluted into 190 μL of influx solutions containing (A) 25 mM NaCl + 75 mM LiCl + 1 mM $MgSO_4$, (B) 25 mM NaCl + 75 mM sodium glucuronate + 1 mM $MgSO_4$, and (C) 100 mM NaCl + 1 mM $MgSO_4$. The solutions also contained increasing concentrations (2–25 μM) of GABA and either 2.7 (A and B) or 0.9 (C) μCi of [2,3-$^3H_2$]GABA (25 or 29.3 Ci/mmol). In order to determine the initial velocity, it was necessary to measure transport at time zero and at 30 s, as described under Experimental Procedures. The values of K_m and V_{max} were calculated from Lineweaver-Burk plots. [b] Average ± SD calculated from the number of experiments given in parentheses.

Figure 2A the membrane potential obtained is calculated to be −59 mV (Figure 2B). The membrane potential upon dilution of these vesicles into NaP_i (instead of into NaCl) with or without valinomycin is −70 and −51 mV, respectively (Figure 2B). Thus the absolute requirement of GABA transport for chloride is due to a specific requirement for this anion by the transporter. Observations on intact nerve preparations (Baker et al., 1962) suggest that the permeability of the membrane to potassium ions is sufficient so that the potassium gradient (in > out) may generate a membrane potential also in the absence of valinomycin. The data illustrated in Figure 2B provide direct evidence for this idea. As found previously, GABA transport is not dependent on internal potassium ions (Kanner, 1978). It is observed that with internal potassium higher levels of GABA accumulation are obtained than with internal lithium or other internal ions (Kanner, 1978). This discrepancy can be explained since the uptake of GABA is very well correlated with the levels of TPP^+ uptake, i.e., the membrane potential (data not shown). This result reinforces the conclusion that internal potassium per se is not required for GABA accumulation.

The direct interaction of sodium and chloride ions with the GABA transporter is further supported by kinetic experiments. The results presented in Table I show that in both the sodium and the chloride concentration influence K_m as well as V_{max} for GABA influx. Lowering of either ion concentration from 100 to 25 milliequiv/L results in a drop in V_{max} and an increase in K_m.

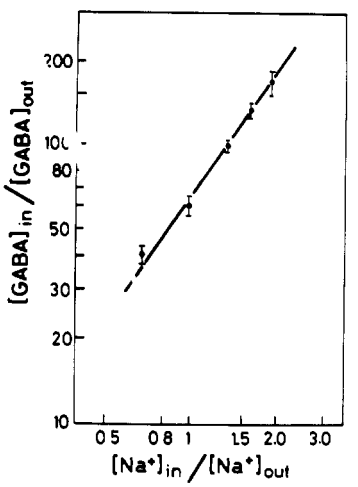

FIGURE 3: Relationship between sodium gradient and GABA gradient. Membrane vesicles (15 μL, 76.5 μg of protein) loaded with 50 mM NaP_i + 50 mM KP_i, pH 6.8, were diluted with 10 volumes of influx solution containing mixtures (sum 150 mM) of NaCl and choline chloride plus 0.153 μM [2,3-$^3H_2$]GABA (29.3 Ci/mmol). The final ratio of external to internal sodium was that shown on the abscissa. Reactions were stopped at 3 min, and the GABA concentration ratio was determined as described under Experimental Procedures.

Ion/GABA Stoichiometry: Rationale. It is customary to obtain ion/solute stoichiometries by either of the two following methods. One is a direct kinetic approach, which involves direct measurement of initial rates of fluxes upon imposition of a solute gradient (Zilberstein et al., 1979). Using $^{22}Na^+$, we find that the leak rate of sodium ions in the system is about 150 nmol/(min·mg) in 100 mM NaCl (data not shown). This is almost 2 orders of magnitude larger than the V_{max} for GABA. Thus, this method cannot be used here. The other approach is a thermodynamic one. It is assumed that GABA is translocated in its predominant form—the zwitterion—and the stoichiometry is $n Na^+$:$m Cl^-$:GABA. At equilibrium, the following condition should be fulfilled:

$$n\Delta\tilde{\mu}_{Na^+} + m\Delta\tilde{\mu}_{Cl^-} + \Delta\tilde{\mu}_{GABA} = 0 \quad (1)$$

Upon rearrangement

$$\ln([GABA]_{in}/[GABA]_{out}) = n \ln([Na^+]_{out}/[Na^+]_{in}) + m \ln([Cl^-]_{out}/[Cl^-]_{in}) - [(n-m)F/(RT)]\Delta\psi \quad (2)$$

Thus when both the chloride ion gradient and the membrane potential are kept constant and the sodium ion gradient is varied, the plot of the logarithm of the GABA concentration gradient (internal/external) vs. the logarithm of the sodium ion gradient (external/internal) should give a straight line, and the value of the slope should give the stoichiometry of n. Similarly, it is possible to obtain the value of m.

Sodium Ion/GABA Stoichiometry. In the experiment depicted in Figure 3, the vesicles are preloaded with a mixture of 50 mM potassium phosphate, pH 6.8, plus 50 mM sodium phosphate, pH 6.8. Then they are diluted (t = 0) into influx media containing variable concentrations (50–150 mM) of sodium chloride supplemented with choline chloride (so that the chloride concentration is constant; 150 milliequiv/L) and also [^{3}H]GABA. Under these conditions the chloride ion concentration gradient and membrane potential are expected to be constant. Indeed, it is found that the TPP^+ concentration gradient remains constant (data not shown). The plot of the logarithm of the GABA concentration gradient (internal/external) vs. that of the initial sodium ion gradient (external/internal) gives a slope of 1.51. The mean (±SD) of 20 experiments was found to be 1.5 ± 0.2. This is likely to be an

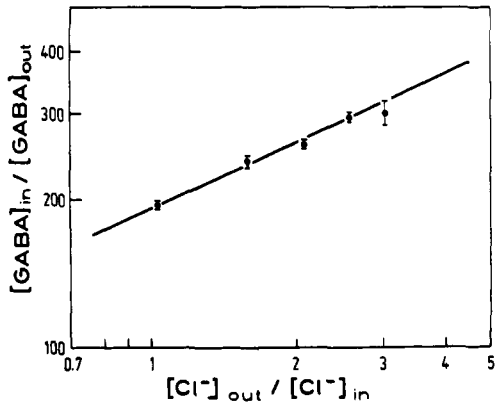

FIGURE 4: Dependence of GABA gradient on chloride gradient. Membrane vesicles (10 μL, 38 μg of protein) loaded with 40 mM KP_i, pH 6.8, + 50 mM KCl were diluted with 18 volumes of influx solution containing mixtures of NaCl and sodium glucuronate (sum 150 mM) plus 0.14 μM [2,3-$^3H_2$]GABA (36.9 Ci/mmol) and also containing 2.5 μM valinomycin. The final ratio of external to internal chloride was that shown on the abscissa. Reactions were stopped at 5 min.

underestimate. At 100 mM NaCl the initial rate of $^{22}Na^+$ influx is about 150 nanoequiv of Na^+/(min·mg of protein) (B. I. Kanner, unpublished experiments). Thus it is very likely that at t = 3 min, when the steady-state level of GABA accumulation is reached, the sodium ion gradient has partly dissipated. Thus if the actual sodium ion concentration gradient at t = 3 min would have been known and plotted, a higher value for the stoichiometry would have been found. Further aspects of the method are discussed below. It has been verified that at all Na^+ concentrations the steady state is indeed reached. In this context it should be remembered that although at lower sodium concentrations the rates of GABA uptake are lower, the steady state of GABA accumulation is even a steeper function of the sodium concentration. Furthermore, at t = 5 min the slope is found to be 1.43 ± 0.07 (n = 3), which is the same—within experimental error—as that at t = 3 min. Thus the slopes are not influenced by kinetic effects.

Chloride Ion/GABA Stoichiometry. The principles described above are used but now the chloride ion gradient is varied, while keeping the other parameters constant (Figure 4). In this experiment a coupling ratio of 0.47 is found. The mean (±SD) of four experiments gave a value of 0.47 ± 0.02. It has been verified that under the conditions of this experiment, probably due to the combination of K^+ and valinomycin, the TPP^+ concentration gradient remains constant (data not shown). Furthermore, it has also been verified here that the steady state indeed has been reached.

Effects of Membrane Potential: Number of Charges Moving per Translocation Cycle. GABA transport in synaptic plasma membrane vesicles from rat brain appears to be an electrogenic process (Kanner, 1978). This is further emphasized by the experiment displayed in Figure 5. In one experiment with the same vesicle preparation, the effects of the ionophores valinomycin and CCCP on GABA (Figure 5A) as well as on TPP^+ (Figure 5B) uptake are measured. The potassium ionophore valinomycin is expected under the given conditions ($[K^+]_{in} > [K^+]_{out}$) to enhance the magnitude of the membrane potential (interior negative), and this is indeed observed (Figure 5B). Parallel to this, GABA transport is also enhanced (Figure 5A). On the other hand, the proton ionophore CCCP is expected to decrease the magnitude (no pH gradient present—$[H^+]_{in} = [H^+]_{out}$). Although the membrane potential is not totally dissipated, a significant inhibition of TPP^+ uptake is observed (Figure 5B), and this is paralleled

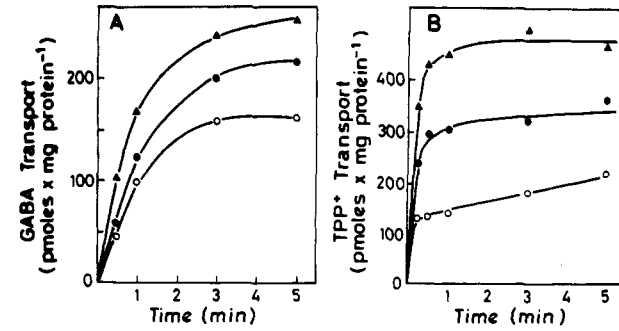

FIGURE 5: Effects of valinomycin and CCCP on GABA and TPP^+ uptake. Membrane vesicles (10 μL, 54 μg of protein) loaded with 0.1 M KP_i, pH 6.8, + 1 mM $MgSO_4$ were diluted with 190 μL of external medium containing (A) 0.15 M NaCl + 1 mM $MgSO_4$ + 0.136 μM [2,3-$^3H_2$]GABA (29.3 Ci/mmol) or (B) 0.15 M NaCl + 1 mM $MgSO_4$ + 4.5 μM [^{3}H]TPP^+ (888 Ci/mol). Transport was measured at different times as described under Methods. Additions: (●) none; (○) 5 μM CCCP; (▲) 2.5 μM valinomycin.

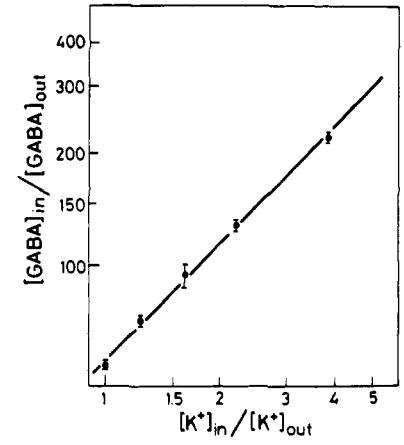

FIGURE 6: Relationship between membrane potential and GABA gradient. Membrane vesicles (15 μL, 75 μg of protein) loaded with 25 mM KP_i + 75 mM LiP_i, pH 6.8, were diluted with 10 volumes of 0.1 M NaCl containing 0.16 μM [2,3-$^3H_2$]GABA (29.3 Ci/mmol) plus a total of 25 mM sodium and potassium phosphate, pH 6.8, such that the final ratio of internal to external potassium was that shown on the abscissa. Valinomycin (2.5 μM) was also added. Reactions were stopped at 5 min as described under Experimental Procedures.

by an inhibition of GABA transport (Figure 5A). The following values for the membrane potential (interior negative) can be calculated from the data of Figure 5B: control, −59 mV; +valinomycin, −66 mV; +CCCP, −45 mV.

The number of charges entering per translocation cycle ($n - m$, eq 2) can be determined as well. Under the conditions of a constant sodium and chloride ion gradient, the membrane potential is varied by varying $[K^+]_{in}/[K^+]_{out}$ in the presence of valinomycin (Figure 6). Under these conditions it is expected that $\Delta\psi = -(RT/F) \ln ([K^+]_{in}/[K^+]_{out})$. A plot of the logarithm of the GABA concentration gradient (internal/external) vs. the logarithm of the potassium ion gradient (internal/external) in the presence of valinomycin yields a straight line with a slope of 1. The mean (±SD) for four experiments was found to be 0.90 ± 0.08. It is of interest to note that when in the presence of valinomycin the logarithm of the TPP^+ concentration gradient (internal/external) is plotted vs. the logarithm of the potassium concentration gradient, a straight line with a slope of 0.8 ± 0.07 is obtained (data not shown).

Discussion

It has been suggested in the past that the stoichiometry of sodium ions/GABA transported by the GABA transporter from rat brain is 2 or 3 (Martin & Smith, 1972; Blaustein &

King, 1976). This was based on the observation that the sodium dependence of GABA uptake in rat brain synaptosomes can be described by a sigmoid curve. Although this is not proof, these results are consistent with the idea. Studies with isolated synaptic plasma membrane vesicles provided evidence that chloride is cotransported as well by the transporter and that the overall process is electrogenic (Kanner, 1978; Kanner & Kifer, 1981). Further support for this suggestion is presented in this paper (Figure 5). If one assumes that the predominant form of GABA at neutral pH—the zwitterion—is transported, the prediction for the stoichiometry is $n\mathrm{Na}^+$:$m\mathrm{Cl}^-$:GABA with $n > m$. The simplest stoichiometry would be $n = 2$ and $m = 1$. The prediction is in harmony with the results described in this paper. The stoichiometry measurements indicate respectively 1.5 ± 0.2 Na^+ (Figure 3) and 0.47 ± 0.02 Cl^- (Figure 4) transported per GABA. The difference between the two numbers would be the net number of charges translocated in the overall process. Independent measurements of this parameter yield 0.9 ± 0.08 (Figure 6), which falls within the experimental limits of the prediction. Thus the assumption that the translocated form of GABA is the zwitterionic species is highly suggestive.

For a long time the fear that the synaptic plasma membrane preparation may contain a large proportion of vesicles that do not have a GABA transporter has repressed attempts to determine the stoichiometry by which GABA, sodium, and chloride ions are translocated through the carrier, especially due to the fact that a kinetic approach is not feasible in this case. However, it has been shown that the intravesicular volume to which GABA has access in a sodium-dependent fashion is 3.8 μL/mg of protein while the total internal volume is 7.4 μL/mg of protein (Kanner, 1978). Furthermore, more than 50% of the GABA entrapped inside the vesicles in a random process can be released in a sodium- and chloride-dependent process (Kanner & Kifer, 1981). Thus at least 50% of the vesicles contain a functional GABA transporter.

In the present paper we found that the conventional thermodynamic approach can be applied. This approach is based upon a comparison of the concentration gradients of the solutes at steady state. Although it is easy to determine directly the GABA concentration gradient, there are problems with sodium and chloride. The common problem is that not all the vesicles of the population can transport GABA, and comparison of the concentration gradients should be performed only on the very vesicles that transport GABA. Moreover, with chloride we have encountered the technical problem that radioactive chloride is leaking out of the vesicles during the washing procedure of the filters. In the method that we chose to determine the stoichiometry, these problems are circumvented since we monitor GABA only while varying the other parameters one at a time. As indicated under Results, the problem of this is that we use the concentration gradients of ions that were present initially, while the GABA gradient is determined at steady state. This will lead to underestimates of the stoichiometric ratios, since—due to dissipation—the actual concentration gradients of the ions are smaller than those calculated for the onset of transport. An inherent assumption is that when one parameter (for instance the sodium ion gradient) is varied, the others, which are kept constant (chloride ion gradient and membrane potential), remain constant. For the membrane potential this was directly demonstrated (TPP^+ gradient remained constant as the sodium gradient was varied). In the case of chloride the membrane potential also remained constant as the chloride gradient was varied. With regard to the direct measurements of the membrane potential, these would suffer from the objection that TPP^+ gradients give an average of all the vesicles and would not necessarily reflect the exact situation in the GABA-transporting vesicles. However, from the results described in Figure 5 it is clear that this is not the case.

It is possible to estimate the correctness of using the potassium gradient in the presence of valinomycin (Figure 1) as an indicator of the membrane potential. Furthermore, a plot of the logarithm of the TPP^+ gradient vs. the logarithm of the K^+ gradient (present at the time of the dilution) gives a straight line with a slope of 0.8 ± 0.07. Ideally, the slope would have been 1. The slope of 0.9 ± 0.08—the number of changes translocated per cycle—obtained in Figure 6—is also expected to be a minimal estimate (see above).

In conclusion, although other stoichiometries cannot be excluded, the simplest stoichiometry for the transporter is $2\mathrm{Na}^+$:Cl^-:GABA. It is of interest to note that, while this work was being reviewed, a study was published that used a similar approach to determine the stoichiometry of the GABA transporter in intact synaptosomes (Pastuszko et al., 1982). These authors reached different conclusions on the dependence of the process on both chloride and the membrane potential. On the other hand, their data indicate a stoichiometry of $2\mathrm{Na}^+$/GABA, which is in harmony with our findings. We are now in the process of purifying the reconstitutingly active GABA transporter (Agmon & Kanner, 1980). Hopefully, proteoliposomes inlaid with the purified GABA transporter will allow accurate determination of the stoichiometry by the kinetic as well as the thermodynamic approach.

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Registry No. GABA, 56-12-2; Na, 7440-23-5; chloride, 16887-00-6.

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Interaction of 4-Azido-2-nitrophenyl Phosphate, an Inorganic Phosphate Photoreactive Analogue, with Chloroplast Coupling Factor 1†

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ABSTRACT: 4-Azido-2-nitrophenyl phosphate (ANPP), a photoreactive derivative of inorganic phosphate (P_i) [Lauquin, G., Pougeois, R., & Vignais, P. V. (1980) *Biochemistry 19*, 4620–4626], was used to study the P_i binding site in purified spinach chloroplast coupling factor 1 (CF_1). Upon photoirradiation with visible light, [^{32}P]ANPP bound covalently to CF_1 and inactivated the enzyme. The labeling stoichiometry was measured in the presence of either EDTA, Mg^{2+}, or Ca^{2+}. In all cases, extrapolation to 100% photoinactivation yielded values close to 1 mol of [^{32}P]ANPP/mol of CF_1. P_i fully protected the enzyme against both photoinactivation and radiolabeling whereas ADP and ATP protected partially. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of [^{32}P]ANPP-labeled CF_1 followed by fluorography revealed only one radioactive peptide that was, in all cases, characterized as the β subunit. Photoirradiation carried out with the nonphosphorylated precursor, 4-azido-2-nitrophenol, had no effect on the enzymatic activity. The same results were obtained when heat-activated CF_1 was used instead of native CF_1.

The membrane-bound coupling factor CF_1[1] from spinach chloroplasts contains the catalytic site responsible for the synthesis of ATP in chloroplasts; its latent ATPase activity can be activated by heat, dithiothreitol, or trypsin [for reviews, cf. McCarty (1978) and Nelson (1976)]. Although the interaction of nucleotides with CF_1 has been studied in many laboratories [for a review, cf. Shavit (1980)], the binding of P_i has not been investigated; to our knowledge, only mention was made that CF_1 does not bind P_i (Tiefert et al., 1977). In fact, as shown here, CF_1 binds P_i poorly. Consequently, we have investigated the putative P_i binding site of CF_i by using a photoaffinity derivative of P_i, ANPP. It has been previously reported (Lauquin et al., 1980) that ANPP labels the β subunit of the mitochondrial F_1 and that the binding of 1 mol of ANPP to 1 mol of F_1 fully inactivates the enzyme. In this paper, we show that ANPP also binds to CF_1 and that ANPP binding is prevented by P_i. As in the case of mitochondrial F_1, the ANPP binding site on CF_1 is located on the β subunit and full inactivation requires 1 mol of ANPP/mol of enzyme, native or heat activated.

Materials and Methods

Materials

Carrier-free [^{32}P]P_i was purchased from the Commissariat à l'Energie Atomique (Saclay, France); dilutions of the isotope were stored in plastic containers. [^{14}C]NEM (40 mCi/mmol) was purchased from New England Nuclear. ADP and ATP were obtained from Boehringer. ANP, ANPP, and [^{32}P]-ANPP were synthesized and purified as described (Lauquin et al., 1980). All other chemicals were of reagent-grade quality. Trypsin (treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone) was obtained from Worthington.

Methods

Enzyme. Solubilized CF_1 was prepared from fresh spinach leaves by either EDTA or chloroform extraction (Lien & Racker, 1971; Younis et al., 1977). Both preparations gave exactly the same results. CF_1 was stored as a precipitate in 2 M ammonium sulfate, 10 mM Tris-HCl, 1 mM EDTA, and 0.5 mM ATP, pH 7.2, at 4 °C.

Preparation of heat-activated CF_1 was performed as described (Holowka & Hammes, 1977). CF_1 (2 mg/mL) in 40 mM Tris-HCl and 2 mM EDTA, pH 8.0, was heat activated at 60 °C for 4 min after addition of ATP to 40 mM and dithiothreitol to 6 mM and then cooled immediately at 22 °C. The enzyme was precipitated with an equal volume of saturated ammonium sulfate and the centrifuged protein pellet was resuspended in 40 mM Tris-HCl–2 mM EDTA, pH 7.1. The redissolved enzyme was then desalted as described hereafter.

[^{32}P]P_i Binding Assays. CF_1 (5 mg/mL) was desalted by the elution–centrifugation method described by Penefsky (1977). The Sephadex (G-50 fine) column was equilibrated

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[1] Abbreviations: CF_1, chloroplast ATPase; F_1, beef heart mitochondrial ATPase; P_i, inorganic phosphate; ANPP, 4-azido-2-nitrophenyl phosphate; ANP, 4-azido-2-nitrophenol; Tris, tris(hydroxymethyl)-aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide.

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