

A MODEL OF THE SODIUM AND MEMBRANE POTENTIAL DEPENDENCE OF HIGH AFFINITY GLUTAMIC ACID TRANSPORT IN RAT CORTICAL SYNAPTOSOMES

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Abstract

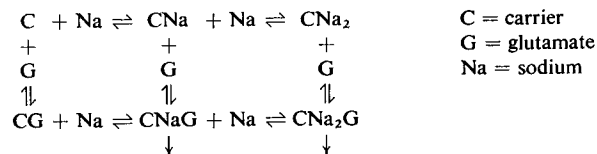
The effect of extrasynaptosomal potassium concentration ($[K]_o$) and membrane potential (E_m) on the initial velocity of uptake and steady state distribution ratio of L-glutamic acid has been examined in rat cortical synaptosomes. A fluorescent dye technique has been utilized to estimate membrane potential as a function of $[K]_o$, thus allowing transport to be correlated with membrane potential. Uptake of glutamic acid has been found to be electrogenic—depolarization of the membrane accompanies transport. With a $[Na]_o$ of 27 mM, initial velocity of glutamate uptake as a function of $[K]_o$ gives a good fit to the Goldman equation. Thus at this $[Na]_o$, $[K]_o$'s effect on transport can be attributed to its effect on E_m . However, at 120·8 mM $[Na]_o$, $[K]_o$'s effect on transport cannot be attributed totally to alteration of E_m . Steady state glutamate distribution ratio is found to be proportional to the electrochemical potential energy gradient.

The results of the present study have been compared to those from previous kinetic studies. Where direct comparisons can be made, predictions from previous studies are supported by the results of the present study. In addition, further deductions can now be made about the mechanism of sodium dependent glutamate transport. These deductions have been incorporated with those from previous studies, and a unified model is presented which offers considerable insight into the nature of glutamate's function in synaptic transmission. Storage of glutamate in the resting pre-synaptic neuron, release upon depolarization, and re-uptake with repolarization are all logical consequences of the operation of the model presented. A mechanism for altering post-synaptic excitability independently of classical transmitter function is suggested by the electrogenicity of the glutamate transporter. It is concluded that this model may have significant implications in understanding the role of glutamic acid in synaptic function.

1. Introduction

Several studies have been made in this (Wheeler, 1979*a*, 1980*a,b*, 1981; Wheeler and Hollingsworth, 1978; Wheeler *et al.*, 1980) and other laboratories (Navon and Lajtha, 1969; Bennett *et al.*, 1973; Takagaki, 1976) of the sodium dependence of high affinity glutamic acid transport in rat cortical synaptosomes. We find uptake to be essentially totally dependent on the presence of sodium ions in the incubation medium. Kinetic studies have been made in which initial velocity of uptake has

been measured as a function of both glutamic acid and sodium concentration in the incubation medium. Computer optimization techniques were then used to fit these data to various models which could account for the sodium dependence of transport. The following minimal best fit model emerged from the optimization process:



Thus the model predicts that there are two pathways for uptake—as CNaG and as CNa₂G. The coupling ratio (Na/G) is one for uptake as CNaG and two for uptake as CNa₂G. Although the model predicts that the percentage of total uptake by each of the two pathways varies with the sodium concentration, at a physiological [Na]_o of 140 mM, 84% of the uptake should take place as CNa₂G.

In solution at pH 7.4, glutamic acid has a net negative charge. Co-transport of two sodium ions with each glutamate suggests that the operation of the glutamic acid transport mechanism might be electrogenic, with consequent alteration of the resting membrane potential. Conversely, alteration of the resting potential might be expected to alter the rate of glutamic acid uptake and the steady state distribution ratio. In fact, in a study of glutamic acid uptake in peripheral nerve (Wheeler, 1979b), glutamate uptake was found to be inhibited by both increasing extracellular [K] and by passage of depolarizing currents across the axonal membranes.

The present report concerns the function of the transmembrane potential in the operation of the sodium dependent glutamic acid transporter.

2. Materials and methods

Preparation of synaptosomes

Adult male Long-Evans rats were used in all experiments. Following decapitation with a guillotine, the brain was removed and placed on a filter paper moistened with Krebs-Henseleit solution (Lockwood, 1961) which was kept cold by placing on a petri dish filled with ice. The cortex was rapidly dissected free, weighed, and placed in 20 vol of ice cold 0.32 M-sucrose in a 15-ml Potter-Elvehjem mortar fitted with a Teflon pestle. The clearance between mortar and pestle was 0.004–0.006 in. The homogenizer was placed in an icewater bath and the tissue homogenized for 1 min, utilizing six 10-s strokes with a speed of 840 rev./min. The homogenate was centrifuged for 10 min at 1085g at a temperature of 2°C to remove nuclei and cellular debris. The supernate was removed and centrifuged at 27,000g for 15 min to sediment the synaptosomes. The supernate from this procedure was decanted and discarded, and the synaptosomes were resuspended in 20 vol of ice cold 0.32 M-sucrose.

Subfractionation of crude synaptosomal preparations by using discontinuous sucrose gradient centrifugation has shown that 83% of total L-glutamate uptake

is confined to the synaptosomal fraction (Takagaki, 1976). In such subfractionation studies, distribution of uptake parallels the distribution of marker enzymes for synaptosomes. Thus one can reasonably conclude that most, if not all, L-glutamate uptake by crude synaptosomal preparations represents uptake by nerve terminals.

Measurement of uptake

The uptake of ¹⁴C-labelled L-glutamic acid was measured by incubation of synaptosomes in a modified Krebs-Henseleit solution at 30°C. L-Glutamic acid (U.L., specific activity of 244 mCi/mmol) was obtained from the New England Nuclear Corporation, Boston, MA, and was at least 99% pure. Non-labelled L-glutamic acid was obtained from Sigma Chemical Company, St. Louis, MO. All incubations were carried out in a Dubnoff metabolic shaker. Unless otherwise stated, the modified Krebs-Henseleit used consisted of 127.2 mM NaCl, 5mM KCl, 2.7 mM CaCl₂, 1.3 mM MgSO₄, 25 mM Tris buffer, and 11.1 mM glucose, at a pH of 7.4. Concentration of sodium and potassium in the incubation medium was varied by replacement of NaCl or KCl with equimolar Tris buffer. The concentration of glutamic acid in the final incubation medium was 1 × 10⁻⁵ M, including 0.02 μCi/ml labelled isotope. Incubation was begun by pipetting 0.2 ml of the synaptosomal suspension (equivalent to 10 mg of cortical tissue) into the scintillation vial containing 3.8 ml of incubation medium.

Following incubation, synaptosomes were separated from incubation medium by rapid filtration, utilizing a Millipore 30-place manifold and Millipore filters (HAWPO2500). The synaptosomes were then washed with a 5-ml aliquot of Krebs-Henseleit (0 Na) at 30°C. Previous studies indicate that such a wash procedure results in little loss of label from within the synaptosomes (Wheeler, 1978). Following completion of all incubations, each filter was removed, the bottom side blotted on filter paper, placed in a scintillation vial, and 1 ml of distilled water added. After standing for 1 h, scintillation fluid was added; 24 h later, the vials were counted for radioactivity in a Packard Model 3320 liquid scintillation spectrometer until the error was less than 1% at the 95% confidence level. Disintegrations per minute were calculated from c.p.m. by use of an external standard to determine counting efficiency.

Blanks

In order to correct for filter retention of label when incubation medium is poured through them, blanks were run with each experimental solution. A 3.8-ml aliquot of incubation medium was added to a well of the filtration manifold, followed by the usual wash procedure. Fifteen replications were made. Initial velocity measurements were corrected by subtracting the blank value.

Fluorescence measurements

Synaptosomal membrane potential was estimated by measuring the fluorescence of the potential sensitive dye, 3,3'-dipropyl-2,2'-thiodicarbocyanine, which is

designated diS-C₃- (5) (Sims *et al.*, 1974). Fluorescence was measured with an Aminco-Bowman spectrofluorometer with 1.0 mm slit widths and excitation and emission wave length settings of 622 nm and 670 nm, respectively. Unless otherwise stated, experiments were conducted at 30°C, which was maintained with a Lauda K-2/R circulating water bath (Brinkman Instruments). The diS-C₃- (5) was obtained in crystalline form from Dr. Alan Waggoner, Amherst College, and a stock solution of 1.22 mM was prepared in absolute ethanol and stored at -20°C. For each experiment a dye solution of 0.1 mM in H₂O was freshly prepared.

Fluorescence measurements were made after addition of diS-C₃- (5) to a suspension of synaptosomes in modified Krebs-Henseleit (identical to that used for measurement of glutamate uptake). Osmolarity was kept constant at 400 mOsm/l with Tris buffer. To 1.9 ml of prewarmed incubation medium, 25 µl of synaptosomal preparation was added and mixed by pipetting. The cuvette was immediately placed in the fluorometer, and after 1 min, 20 µl of dye solution was added to obtain a final concentration of 1.03 µM. Valinomycin, 10⁻⁴ M (Sigma Chemical Co.), was prepared in ethanol and in some experiments, 2 µl was added and mixed to obtain a final concentration of 0.103 µM. To test the electrogenecity of glutamate transport, 2 µl of 2 × 10⁻² M L-glutamic acid was added and mixed to obtain a final concentration of 2.08 × 10⁻⁵ M.

Computer optimization

In order to fit the data to the several equations used, a parameter optimization approach was used to minimize the following function:

$$J = \sum_{i=1}^n \left(\frac{V_{\text{model}} - V_{\text{data}}}{V_{\text{data}}} \right)^2.$$

J , the objective function, is the sum of the squared fractional errors between the values predicted by the equations and those observed experimentally. The parameter optimization algorithm used in this research was based on the Flexible Polyhedron approach of Nelder and Mead (1965). The algorithm was coded in BASIC and run on a Challenger III microcomputer (Ohio Scientific Instruments) fitted with 48K bytes of memory and a dual drive floppy disk.

To use the BASIC program, the appropriate equation and experimental data were first added to the optimization portion of the program. Initial guesses for all constants were then entered, and program operation was begun. After the algorithm returned the optimal parameters, the program was restarted several times to insure that the minimum possible error had been achieved. Finally, the program printed the various experimental values paired with the corresponding values predicted by the equations.

3. Results

Measurement of fluorescence changes as an index of changes in membrane potential

Changes in fluorescence of potential sensitive dyes have been shown to be reliable indicators of membrane potential in synaptosomes (Blaustein and Goldring, 1975; Kamino and Inouye, 1978). The fluorescent dye, 3,3'-dipropyl-2,2'-thiocarbocyanine (diS-C₃- (5); Sims *et al.*, 1974) was used to demonstrate the relation of E_m to the extra-synaptosomal potassium concentration ($[K]_o$). Addition of synaptosomes to incubation medium containing dye reduces the fluorescence of the system, since entry of dye into the synaptosomes probably results in formation of dye aggregates with a resultant decrease in fluorescence (Sims *et al.*, 1974). The amount of dye taken up is proportional to the membrane potential. Hence, fluorescence increases as E_m decreases. Figure 1 shows fluorescence changes which

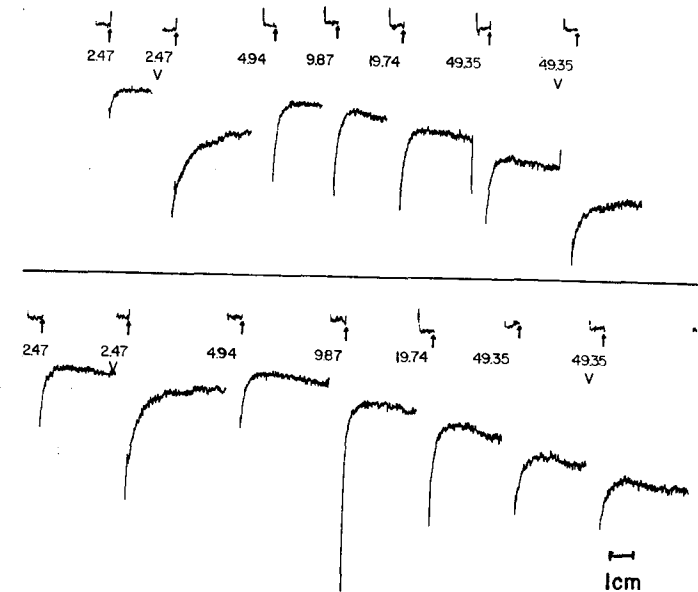


Fig. 1. Fluorescence measurements as a function of $[K]_o$. Synaptosomes were added to the incubation medium, mixed, allowed to stabilize for one minute, and the fluorescence was recorded (upper trace). At the point indicated by the arrow, the recorder was turned off, dye added to the cuvette, mixed, and the recorder turned on again. The recording was then allowed to stabilize and the process repeated for the next $[K]_o$. Vertical distance between fluorescence recorded prior to dye addition and that after the trace had stabilized was measured and taken as fluorescence in arbitrary units. Final $[K]_o$ (mM) is indicated on each trace. Total osmolarity was maintained at 400 mOsm/liter with Tris buffer. V denotes presence of 0.1 mM veratridine. Paper speed is 1 cm/min. Upper panel shows data for $[Na]_o = 125.5$ mM, while lower panel is for $[Na]_o = 28.05$ mM.

occur when dye is added to synaptosomal suspension containing 2.47, 4.94, 9.87, 19.74, or 49.35 mM $[K]_o$. Fluorescence increases as the $[K]_o$ is increased, thus indicating depolarization as $[K]_o$ is increased, with the consequent uptake of less dye. Figure 2 shows a plot of fluorescence versus $\ln[K]_o$ for 125.5 mM (high) and 28.05 mM (low) $[Na]_o$. For both high and low $[Na]_o$, fluorescence increases rapidly (E_m declines) with the \ln of the $[K]_o$. This is consistent with the interpretation that K^+ predominates in determining the membrane potential. However, at

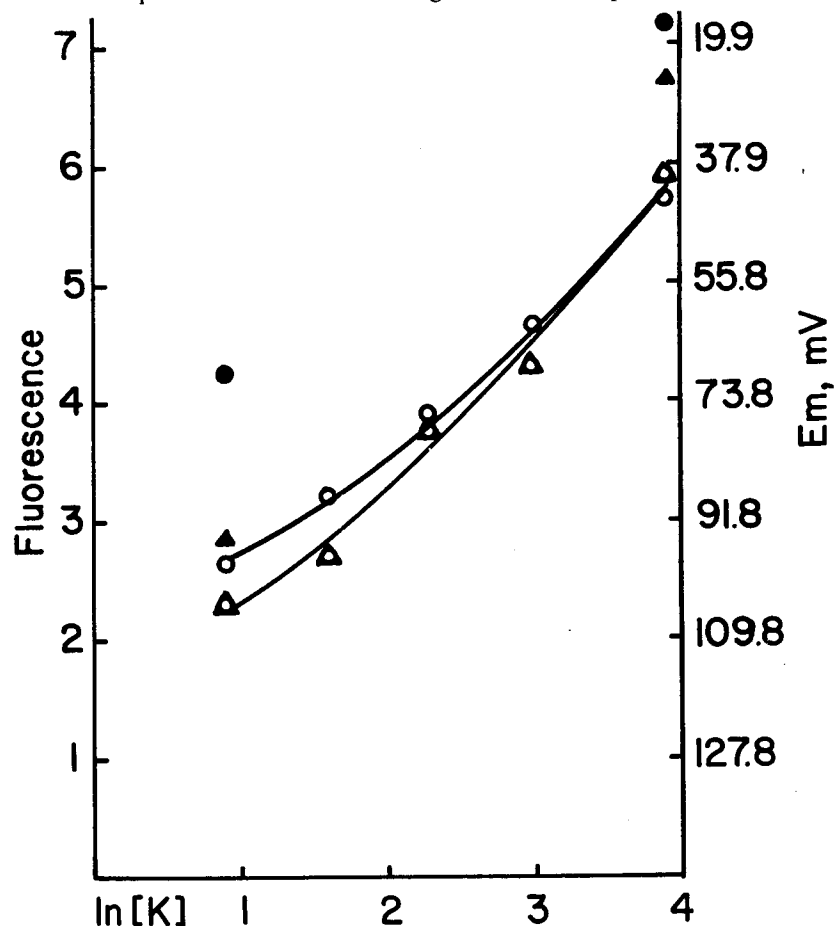


Fig. 2. Relationship of fluorescence and E_m to external potassium concentration ($[K]_o$). Symbols indicate data points: Δ = 28.05 mM $[Na]_o$, \circ = 125.5 mM $[Na]_o$; \blacktriangle , \bullet indicate presence of 0.1 mM veratridine in the incubation medium. Solid lines show the computed fit of all data to equation (1). Scale to the left gives fluorescence in arbitrary units, while that to the right gives resting potential (E_m) in millivolts. 25 μ l of synaptosomal suspension was added to 1.90 ml of incubation medium. Final $[K]_o$ was 2.47, 4.94, 9.87, 19.74 or 49.35 mM. Similar results were seen in three experiments.

any given $[K]_o$, fluorescence is slightly greater (E_m less) in high $[Na]_o$ than in low $[Na]_o$. This result is to be expected if sodium ions have a low, but finite, permeability compared to that of potassium ions. In order to quantitate the relationship between fluorescence and $[K]_o$, the data for both 125.5 mM and 28.05 mM $[Na]_o$ were simultaneously fitted to the following equation:

$$(1) \quad \text{Fluorescence} = K_1 \ln([K]_o + K_2[Na]_o + K_3[Cl]_i) + K_4.$$

Note that this equation is analogous in form to the Goldman equation for E_m as a function of $[K]_o$, $[Na]_o$, and $[Cl]_i$, where $K_2 = p_{Na}/p_K$ and $K_3 = p_{Cl}/p_K$:

$$(2) \quad E_m = -(RT/F) \ln([K]_o + (p_{Na}/p_K)[Na]_o + (p_{Cl}/p_K)[Cl]_i) + C.$$

The solid lines in Figure 2 show the computed fit of the data to equation (1). The data give an excellent fit to the equation, with an average per cent difference between calculated and experimental values of only 2.66% per data point. Best fit constants were found to be as follows: $K_1 = 1.4524$, $K_2 = 0.0175$, $K_3[Cl]_i = 1.7003$, and $K_4 = 0.00144$. Now K_2 is analogous to p_{Na}/p_K in the Goldman equation. Thus the predicted ratio of sodium permeability to potassium permeability in this preparation is $0.0175/1.000$ or $p_K/p_{Na} = 57.1$. Mean value for p_{Na}/p_K was found to be 0.0156 in three experiments. Thus the synaptosomal membrane has retained its differential permeability properties. Hence an increase in p_{Na} should have a depolarizing effect. This is verified in Figures 1 and 2; for both high and low $[Na]_o$, veratridine, which increases p_{Na} (Levi *et al.*, 1980), caused an increased in fluorescence, which indicates depolarization. As expected, the effect of veratridine is more pronounced for high $[Na]_o$ than for low $[Na]_o$.

Determination of null point with valinomycin

Since fluorescence is proportional to E_m , fluorescence measurements show what the shape of a plot of E_m versus $\ln[K]_o$ should look like; however, in order to convert fluorescence to E_m , the curves must be calibrated. If potassium ions are at equilibrium for a given set of $[K]_i$ and $[K]_o$, then increasing K^+ permeability would not alter K^+ flux and hence would not alter the membrane potential. The ionophore valinomycin increases potassium permeability (Carvalho and Carvalho, 1979). Figure 3 shows the change in fluorescence when valinomycin is added to a synaptosomal suspension in Krebs-Henseleit containing a given concentration of K_o . At the low end of the $[K]_o$'s used, addition of valinomycin results in a decrease in fluorescence (hyperpolarization), while at the higher $[K]_o$'s, an increase in fluorescence results (depolarization). At the point where the curves cross, valinomycin caused no change in fluorescence (and hence potential); thus at this value of $[K]_o$, potassium is at equilibrium, and the membrane potential may be calculated from the Nernst equation for K^+ .

$$(3) \quad E_m = E_K = (RT/F) \ln([K]_i/[K]_o) = 84.2 \text{ mV},$$

$$[K]_i = 167.2 \text{ mM (Wheeler unpublished),}$$

$$[K]_o = 6.63 \text{ mM.}$$

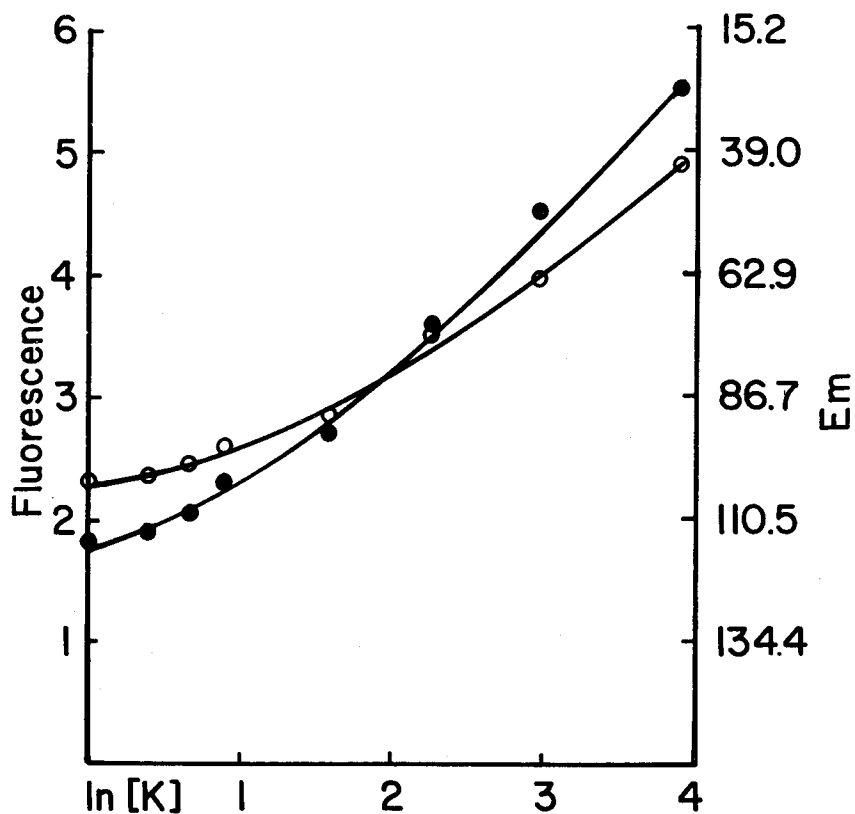


Fig. 3. Effect of valinomycin on fluorescence and membrane potential. Symbols show experimental data points: \circ = control, \bullet = valinomycin. 25 μ l of synaptosomal suspension was added to 1.9 ml of incubation medium followed 1 min later by addition of dye. After steady state fluorescence was attained, valinomycin was added and the change in fluorescence recorded. Final concentration of Na^+ in the incubation medium was 125.5 mM. Solid lines show computed fit to equation (1). E_m (mV) was calculated from equation (2). The two curves cross at $[\text{K}]_o = 6.63$ mM.

We can now calculate the value for C in equation (2):

$$E_m = -(RT/F)\ln([\text{K}]_o + (p_{\text{Na}}/p_{\text{K}})[\text{Na}]_o + (p_{\text{Cl}}/p_{\text{K}})[\text{Cl}]_i) + C,$$

$$E_m = 84.2 \text{ mV},$$

$$R = 8.315 \text{ joules/}^\circ\text{K mole},$$

$$T = 303^\circ\text{K},$$

$$F = 96,500 \text{ coulombs/mole},$$

$$[\text{K}]_o = 6.63 \text{ mM},$$

$$p_{\text{Na}}/p_{\text{K}} = 0.0175; (p_{\text{Cl}}/p_{\text{K}})[\text{Cl}]_i = 1.7003,$$

$$[\text{Na}]_o = 125.5 \text{ mM}.$$

A value of 145.7 was obtained for C . We now have equations for both E_m (2) and fluorescence (1) as a function of $[\text{K}]_o$. Both E_m and fluorescence were calculated for each $[\text{K}]_o$; for the data in Figure 2, the linear relationship between E_m and fluorescence is shown in Figure 4. This relationship was then used to calibrate Figure 2 in terms of E_m as a function of $\ln[\text{K}]_o$. For 125.5 mM $[\text{Na}]_o$, E_m declines from 97.4 mV at $[\text{K}]_o = 2.47$ mM to 41.9 mV at $[\text{K}]_o = 49.35$ mM. Presence of veratridine depolarizes the membrane from 97.4 mV to 69.3 mV with $[\text{K}]_o = 2.47$ mM, and from 41.9 mV to 17.2 mV with 49.35 mM. For 28.05 mM $[\text{Na}]_o$, E_m declines from 105.5 mV at $[\text{K}]_o = 2.47$ mM to 42.8 mV at $[\text{K}]_o =$

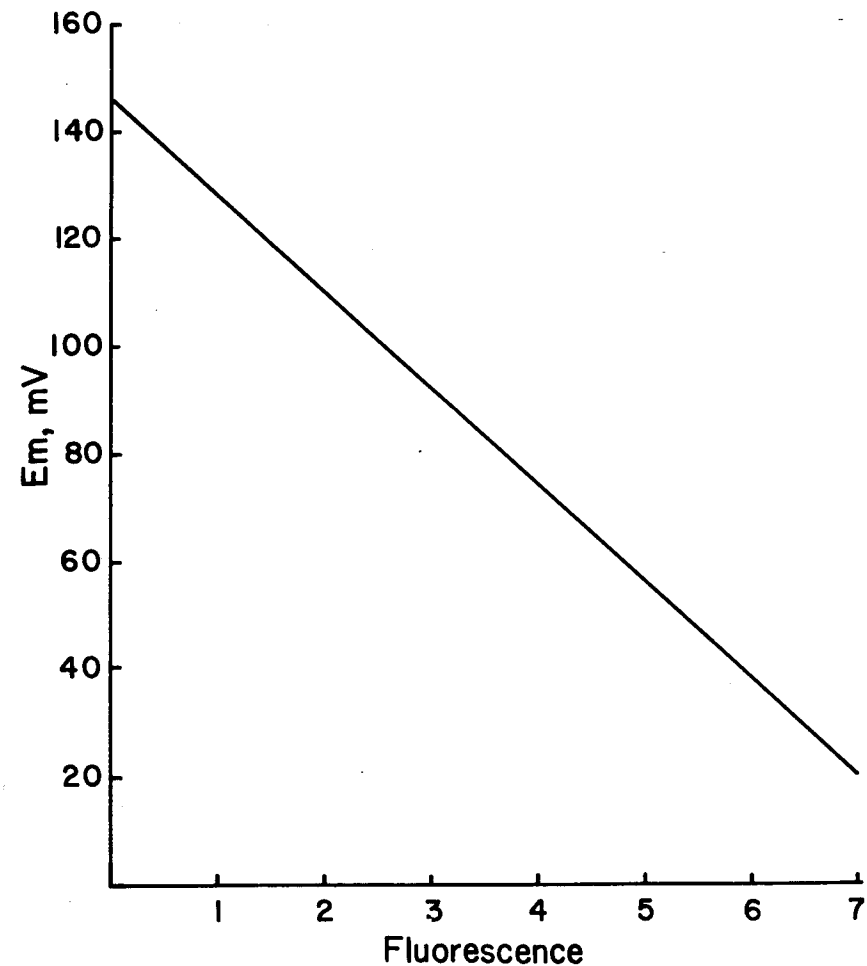


Fig. 4. Relationship between E_m and fluorescence for the data shown in Fig. 2 and a null point of $[\text{K}]_o = 6.63$ mM at an E_m of 84.2 mV. E_m is given in millivolts.

49.35 mM. Veratridine further depolarizes from 105.5 to 94.5 mV with 2.47 mM $[K]_o$, and from 42.8 to 25.3 mV with 49.35 mM $[K]_o$. At a normal $[K]_o$ of about 5 mM, $E_m = 88.6$ mV with 125.5 mM $[Na]_o$ and 94.2 mV with 28.05 mM $[Na]_o$.

Time course of glutamate uptake

Figure 5 shows a plot of glutamate uptake expressed as synaptosomal concentration/medium concentration (distribution ratio, DR) as a function of time. Uptake reaches a plateau at a DR of about 1840 after about 25 minutes of incubation and remains at this level for the duration of the experiment (60 minutes). Maintaining a DR of 1840 for glutamic acid requires a very large energy expenditure, since not only must energy be provided to maintain a concentration gradient, but must also be provided to oppose the electrical gradient of the membrane potential, since glutamate is negatively charged. The minimum energy requirement for transport can be utilized to gain insight into the minimum participation of sodium ions in the transport process. If the energy for transport of glutamate comes from the sodium gradient and the membrane potential, then the increase in free energy as a result of the glutamate concentration gradient can be no greater than the decrease in free energy from the interaction of the sodium gradient and the membrane potential. Therefore, it can be shown that (Aronson, 1981):

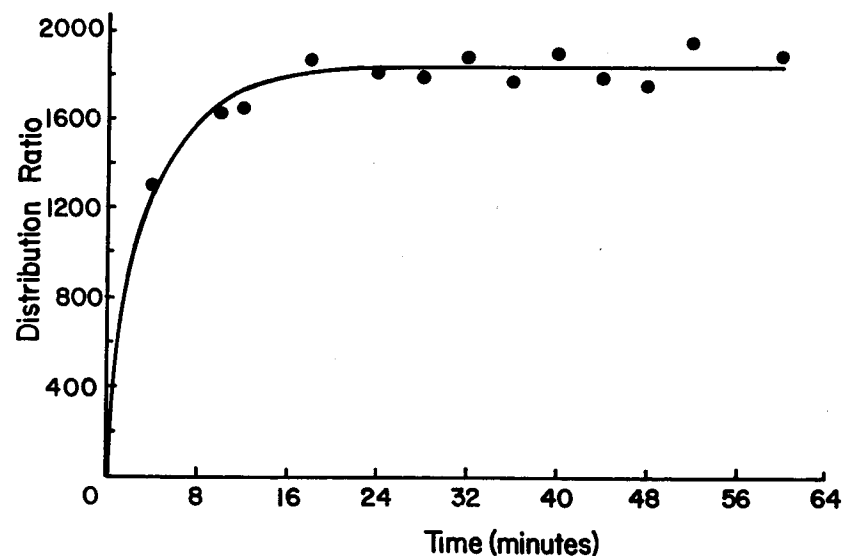


Fig. 5. Distribution ratio (C_{in}/C_{out}) as a function of incubation time. D.R. was calculated by dividing disintegrations recovered from synaptosomes per milliliter intrasynaptosomal water by disintegrations per milliliter incubation medium. Synaptosomes were incubated in Krebs-Henseleit (300 mOsm/l) containing 1×10^{-5} M L-glutamic acid, including 0.02 μ Ci/ml labelled isotope. Previous estimates of synaptosomal volume were used to calculate C_{in} (Wheeler, unpublished). Similar results were seen in three experiments.

$$(4) \quad [G]_i/[G]_o \leq ([Na]_o/[Na]_i)^n e^{(FE_m/RT)(Z_G + nZ_{Na})}$$

- n = coupling ratio, Na/glutamate,
 E_m = membrane potential ≈ 89 mV,
 F = Faraday's constant = 96,500 coulombs/mole,
 R = universal gas constant = 8.315 joules/ $^{\circ}$ K mole,
 T = absolute $T = 303^{\circ}$ K,
 Z_G = charge on glutamate = -1 ,
 Z_{Na} = charge on sodium = $+1$.

If the coupling ratio, n , is 1, then

$$[G]_i/[G]_o \leq [Na]_o/[Na]_i.$$

A reasonable value for $[Na]_o/[Na]_i$ is about 10. Thus the distribution ratio must be no greater than 10. Therefore, we can rule out a coupling ratio of 1 from energetic considerations. If, however, the coupling ratio is 2,

$$[G]_i/[G]_o \leq ([Na]_o/[Na]_i)^2 e^{FE_m/RT}$$

$$\ln([G]_i/[G]_o) \leq 2 \ln([Na]_o/[Na]_i) + FE_m/(RT)$$

$$[G]_i/[G]_o \leq 3,023.$$

With a coupling ratio of 2, a distribution ratio of 1840 is quite possible. Thus energetic considerations support the predictions of the previous modelling studies that a major portion of the uptake of glutamic acid is accompanied by two sodium ions (Wheeler, 1979a).

Effect of $[K]_o$ on glutamic acid uptake

We have shown that the membrane potential can be altered over a wide range by varying the $[K]_o$ between about 2.5 mM and 50 mM. Therefore, the effect on glutamate uptake of varying $[K]_o$ was studied in two sets of experiments.

In the first set of experiments, initial velocity of uptake was measured in Krebs-Henseleit solutions containing 2.375, 4.75, 9.5, 19, or 47.5 mM $[K]$. Figure 6 shows a plot of initial velocity as a function of the natural logarithm of the external $[K]$ for 120.8 mM and 27 mM $[Na]_o$. For 120.8 mM $[Na]_o$, as $[K]_o$ is increased from 2.375 mM to 9.5 mM, initial velocity increases slightly (6.8%) with increased $[K]_o$. Although E_m is decreasing as $[K]_o$ is increasing (Figure 2), rate of uptake increases. Thus it appears that potassium ions in this concentration range are exerting an effect on rate of glutamic acid uptake by some means in addition to an effect on membrane potential. Above 9.5 mM $[K]_o$, there is a rapid linear decline in initial velocity as $[K]_o$ increases. Uptake with 47.5 mM $[K]_o$ is 70.93% of that with 9.5 mM $[K]_o$. The effect of the depolarizing agent veratridine was tested at two $[K]_o$'s (Table 1). Presence of 0.1 mM veratridine in the incubation medium reduces initial velocity. The reduction in uptake at 2.375 mM $[K]_o$ is greater than that with 47.5 mM $[K]_o$. This is what one would expect if the effect

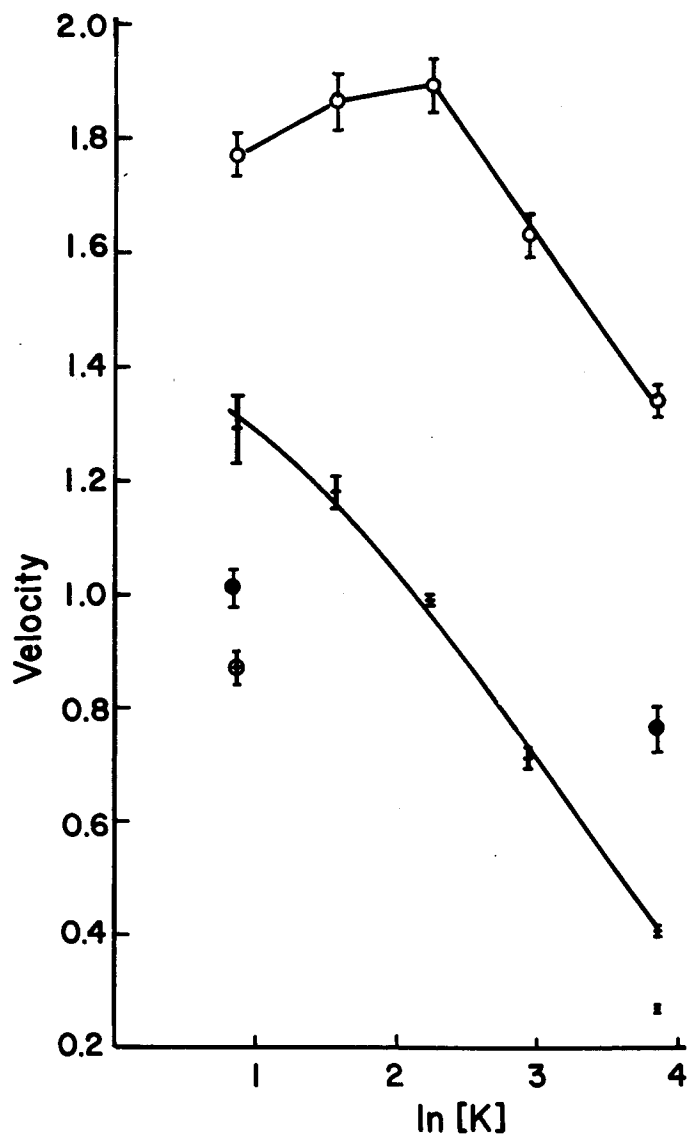


Fig. 6. Initial velocity of uptake as a function of the natural log of the external K^+ concentration. Synaptosomes were incubated for 1 min in a modified Krebs-Henseleit solution containing either 120.8 or 27 mM $[Na]_o$, 1×10^{-5} M L-glutamic acid (including $0.02 \mu Ci/ml$ labelled isotope), and either 2.375, 4.75, 9.50, 19.0 or 47.5 mM $[K]$. Osmolarity was maintained at about 400 mOsm/l by the addition of Tris buffer. Symbols show mean \pm SEM of two measurements on each of six animals. $\circ = 120.8$ mM $[Na]_o$; $+ = 27$ mM $[Na]_o$; $\bullet, \oplus =$ veratridine.

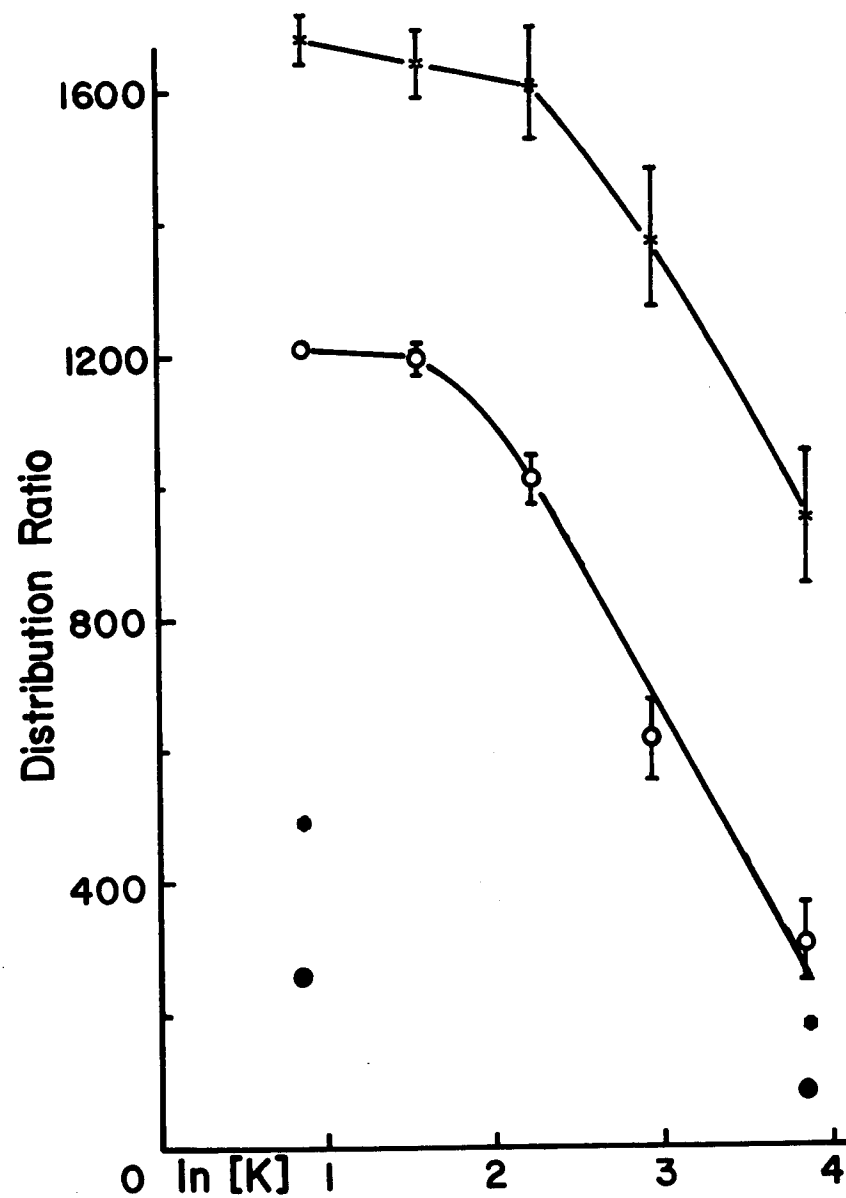


Fig. 7. Distribution ratio as a function of the natural log of $[K]_o$. Conditions are identical to those for Fig. 6, except that incubation time is 30 min. Previous estimates were used along with the assumption that synaptosomes behave as osmometers (Carvalho and Carvalho, 1979) to calculate intrasynaptosomal volume. Symbols ($*$, $\bullet = 120.8$ mM $[Na]_o$; $\circ, \oplus = 27$ mM $[Na]_o$) show mean \pm SEM of two measurements on each of six animals. Filled symbols indicated presence of veratridine.

of veratridine on uptake is through a membrane depolarization, since at 47.5 mM $[K]_o$, the membrane is already considerably depolarized.

Table 1
Effect of veratridine of initial velocity and DR

$[Na]_o$,mM	$[K]_o$,mM	Control	Veratridine	Δ	% Δ
<i>Initial velocity*</i>					
120.8	2.375	1.768	1.012	0.756	42.76
120.8	47.5	1.340	0.762	0.578	43.13
27	2.375	1.292	0.867	0.425	32.89
27	47.5	0.404	0.268	0.136	33.66
<i>Distribution ratio</i>					
120.8	2.375	1674.8	489.3	1185.5	70.8
120.8	47.5	948.0	185.7	762.3	80.4
27	2.375	1198.2	254.2	944.0	78.8
27	47.5	300.4	77.1	223.3	74.3

* Units of initial velocity are n-mol/10 mg wet wt. min.

The results with 27 mM $[Na]_o$ are significantly different from those for 120.8 mM (Figure 6). Rate of uptake declines as $[K]_o$ is increased; when $[K]_o$ is increased from 2.375 mM to 47.5 mM, rate of uptake declines so that uptake at 47.5 mM is only 31.26% of that at 2.375 mM, an even more pronounced effect than that seen with 120.8 mM $[Na]_o$. The shape of the curve is very similar to that seen for E_m (Figure 2). Therefore, the velocity data were fitted to the following equation, which is similar in format to equation (2) for E_m :

$$(5) \quad v = -K_1 \ln([K]_o + (p_{Na}/p_K)[Na]_o + (p_{Cl}/p_K)[Cl]_i) + K_2.$$

The data give an excellent fit to the equation, with an average error per data point of only 2.19% (p_{Na}/p_K and $(p_{Cl}/p_K)[Cl]_i$ were taken from the fit of the data shown in Figure 2). Thus, in contrast to that seen for 120.8 mM $[Na]_o$, the effect of K^+ at low $[Na]_o$ may be entirely through an alteration of E_m . The effect of veratridine with 27 mM $[Na]_o$ was also tested (Table 1). As with 120.8 mM $[Na]_o$, the reduction in uptake in the presence of veratridine is greater with 2.375 mM $[K]_o$ than with 47.5 mM $[K]_o$. At a given $[K]_o$, the effect of veratridine is more pronounced with high $[Na]_o$ than with low $[Na]_o$. Again, since veratridine acts to depolarize by increasing sodium conductance, and thus causes a greater change in E_m with 120.8 mM $[Na]_o$ than with 27 mM $[Na]_o$, this is the expected result if the veratridine effect on transport is mediated through a reduction in E_m .

In a second set of experiments, steady state glutamate distribution ratio was measured as a function of $[K]_o$ for 120.8 mM and 27 mM $[Na]_o$ (Figure 7). With 120.8 mM $[Na]_o$, DR declines slowly from 1674.8 to 1597.3 as $[K]_o$ is increased from 2.375 to 9.5 mM. As was seen with initial velocity, above 9.5 mM $[K]_o$, there is a sharp change in slope of the curve; DR falls from 1597.3 to 948.0 as

$[K]_o$ is increased from 9.5 to 47.5 mM. Presence of 0.1 mM veratridine reduced DR from 1674.8 to only 489.3 with 2.375 mM $[K]_o$, and from 948.0 to 185.7 with 47.5 mM $[K]_o$. With 27 mM $[Na]_o$, the shape of the plot of DR versus $\ln[K]_o$ (Figure 7) is very similar to that seen for initial velocity. DR declines from 1198.2 with 2.375 mM $[K]_o$ to 1185.9 with 4.75 mM $[K]_o$; above 4.75 mM $[K]_o$, DR falls off sharply with $\ln[K]_o$, from 1185.9 with 4.75 mM $[K]_o$ to 300.4 with 47.5 mM $[K]_o$. Presence of 0.1 mM veratridine reduced DR from 1198.2 to 254.2 with 2.375 mM $[K]_o$, and from 300.4 to 77.1 with 47.5 mM $[K]_o$. Again, the effect of veratridine is consistent with expectations if the effect is through an alteration in E_m .

Is the transport of glutamic acid electrogenic?

If the uptake of a major portion of the glutamic acid involves co-transport of two sodium ions, then the operation of the transport mechanism might be electrogenic—that is, result in a net flow of current into the synaptosome. In order to answer this question, glutamic acid was added to incubation medium containing dye, and the fluorescence monitored. As can be seen from Figure 8, addition of glutamic acid resulted in an increase in fluorescence, thus indicating a depolarization of E_m as a result of net positive current inward across the synaptosomal membrane. It appears that there is an initial fast depolarization of about 5 mV over the first 10–15 seconds, followed by a slower rate of depolarization of about 12–15 mV over the ensuing 3–4 minutes. Thus the results are again consistent with the predictions of the previous modelling studies—namely, that two sodium ions are co-transported with each glutamate by one of the translocation pathways.

4. Discussion

The hypotheses proposed, namely, that operation of the glutamate transporter should be electrogenic and that velocity and distribution ratio should be related to E_m have been confirmed. With low extracellular Na^+ , inhibition of glutamate uptake with increasing concentrations of medium potassium can be attributed primarily to an alteration of resting membrane potential. However, at high $[Na]_o$, the effect of $[K]_o$ on transport does not parallel the effect of $[K]_o$ on E_m . Thus, at high $[Na]_o$ the relationship of uptake to $[K]_o$ is influenced by factors in addition to $[K]_o$'s effect on E_m .

The results of the measurement of E_m with the fluorescent dye technique confirm the conclusions of previous investigators (Blaustein and Goldring, 1975; Kamino and Inouye, 1978) that such measurements provide a reliable indicator of membrane potential. In all cases, the results are consistent with expectations based on this assumption. The relationship of fluorescence to $[K]_o$ is indeed predictable from the Goldman equation, not only at a single $[Na]_o$, but also when data for both high and low $[Na]_o$ are simultaneously fitted to this equation. The ratio of p_{Na} to p_K (0.016) predicted from this fit is consistent with the interpretation that the synaptosomal membrane has retained its differential permeability proper-

ties. The depolarizing effect of veratridine and the effect of valinomycin (hyperpolarization at low $[K]_o$, depolarization at higher $[K]_o$, see Figure 3) are also consistent with expectations.

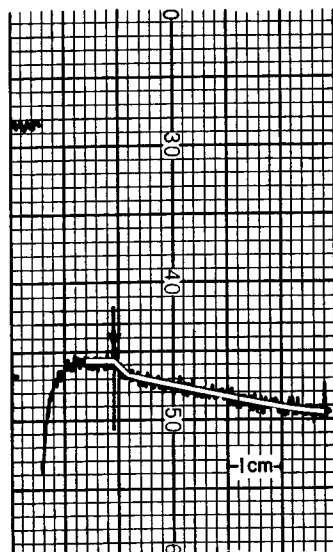
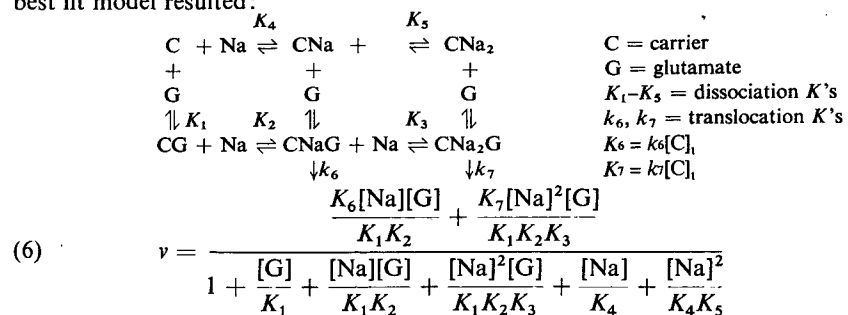


Fig. 8. Electrogenicity of glutamate uptake. 25 μ l of synaptosomal suspension was added to 1.90 ml of Krebs-Henseleit containing 5 mM $[K]$ and 127 mM $[Na]$ (osmolarity = 400 mOsm/l). At arrow, recorder was turned off, 2 μ l of 2×10^{-2} M L-glutamic acid was added, mixed, and recorder turned on. White line shows slope of fluorescence trace. Paper speed is 1 cm/min. Similar results were seen in three experiments.

Comparison of present results with those from previous studies

In previous studies, initial velocity of glutamate uptake was measured as a function of both glutamate and sodium concentration, and computer optimization techniques were utilized to fit these kinetic data to various models which would account for the role of sodium in the transport process. The following minimal best fit model resulted:



$$(7) \quad v = \frac{\left\{ \frac{K_3K_6[Na] + K_7[Na]^2}{K_2K_3 + K_3[Na] + [Na]^2} \right\} [G]}{\left\{ \frac{K_1K_2K_3K_4K_5 + K_1K_2K_3K_5[Na] + K_1K_2K_3[Na]^2}{K_2K_3K_4K_5 + K_3K_4K_5[Na] + K_4K_5[Na]^2} \right\} + [G]} = \frac{V_a[G]}{K_i + [G]}$$

The best fit constants associated with the model are shown in Table 2.

Table 2
Constants giving best fit

n	K_n	n	K_n
1	3.7132×10^{-5}	5	8.3615×10^{-3}
2	10.4482	6	1.5827×10^3
3	4.3981×10^{-5}	7	2.6678
4	3.3579×10^{-1}		

$K_6 = k_6[C]_i$; $K_7 = k_7[C]_i$; Units for K_6, K_7 are n-mol/10 mg tissue minute.

The assumptions implicit in the model and the predictions from the fit of the model to the data are as follows:

- (1) there is a fixed total concentration of carrier available;
- (2) there is no fixed order of combination of sodium and glutamate with the carrier;
- (3) reactions of sodium and glutamate with the carrier may be treated as equilibria;
- (4) translocation may occur as either CNaG or as CNa₂G;
- (5) as a consequence of (4), the coupling ratio is both 1 and 2;
- (6) uptake is 100% dependent on the presence of sodium in the incubation medium;
- (7) V_a , the apparent maximal velocity, is dependent on $[Na]_o$;
- (8) K_i , the apparent Michaelis-Menten constant, is also dependent on $[Na]_o$;
- (9) Sodium becomes inhibitory to uptake when $[Na]_o$ exceeds a critical value;
- (10) the relative rates of uptake by each of the two pathways ($v_1 =$ through CNaG; $v_2 =$ through CNa₂G) depend on the $[Na]_o$: $v_2/v_1 = K_7[Na]_o/(K_3/K_6)$ —thus the mean coupling ratio changes with $[Na]_o$.

The fit of the experimental data to the above model is excellent; the mean error between experimental data points and model predictions is only 1.26%.

We can use some of the results of the present study to check previous predictions from the above model. First of all, the model predicts that glutamate uptake via one of the translocation pathways (CNa₂G) is associated with two sodium ions. Since in solution at pH 7.4 glutamic acid has a net negative charge of 1, one might expect that glutamate uptake along with two Na⁺ ions would result in a net inward positive current. The present study confirms the electrogenicity of the operation of the glutamate carrier. Addition of glutamate to a synaptosomal

suspension (final concentration $\approx 2 \times 10^{-5}$ M) resulted in a depolarization of some 12–15 mV. The widespread depolarizing effect of glutamate has been attributed to transmitter-like effects of applied glutamate (Krnjevic, 1974); that is, depolarization as a consequence of increased conductance to other ions. However, these results raise the possibility that much of the glutamate induced depolarization might be related to glutamate uptake.

Second, the model predicts that at 27 mM $[\text{Na}]_o$, the ratio of the uptake as CNa_2G to that as CNaG should be 1.035; thus the average coupling ratio should be $((1.035 \times 2) + 1)/2.035 = 1.51$. We can use the results of the present study to test this prediction. The results of the initial velocity studies are consistent with the interpretation that the effect of K_o^+ on transport may be primarily mediated through an alteration of membrane potential if the extrasynaptosomal $[\text{Na}]$ is low. If the extrasynaptosomal $[\text{Na}]$ is high, then K_o^+ must be influencing transport through mechanisms in addition to that of altering membrane potential. Therefore, it seems probable that at low $[\text{Na}]_o$, steady state distribution ratio is proportional to the electrochemical potential gradient (equation (4)). By substituting equation (2) for E_m into equation (4) and re-arranging the equation, we obtain:

$$(8) \ln([G]_i/[G]_o) = -(n-1)/n([\text{K}]_o + (p_{\text{Na}}/p_{\text{K}})[\text{Na}]_o + (p_{\text{Cl}}/p_{\text{K}})[\text{Cl}]_i) + C,$$

G = glutamate,

n = coupling ratio,

$[\text{Na}]_o = 27$ mM,

C = constant,

$p_{\text{Na}}/p_{\text{K}} = 0.0175$,

$(p_{\text{Cl}}/p_{\text{K}})[\text{Cl}]_i = 1.7003$.

The distribution ratio data for $[\text{Na}]_o = 27$ mM were fitted to the above equation. A relatively good fit results, with an average error between experimental data points and calculated values of only 1.78%. The predicted value for n, which is the ratio of Na uptake to glutamate uptake, is 1.63. Thus on the basis of these data, we conclude that at 27 mM $[\text{Na}]_o$, 1.6 Na ions are taken up with each glutamate. Considering the fact that predictions from previous studies were based on initial velocities, while present conclusions were derived from studies of the relationship between membrane potential and distribution ratio, the agreement between the two studies is remarkably good.

Third, we can test the accuracy of previous estimates of kinetic constants by comparing steady state distribution ratios calculated from these constants with those predicted from results of the present study. If we assume that when the membrane potential is zero, the carrier mechanism is symmetrical, then at steady state $V_{o \rightarrow i} = V_{i \rightarrow o}$

$$(9) \frac{k_{6(o \rightarrow i)}[\text{Na}]_o[\text{G}]_o}{K_1 K_2} + \frac{k_{7(o \rightarrow i)}[\text{Na}]_o^2[\text{G}]_o}{K_1 K_2 K_3} = \frac{k_{6(i \rightarrow o)}[\text{Na}]_i[\text{G}]_i}{K_1 K_2} + \frac{k_{7(i \rightarrow o)}[\text{Na}]_i^2[\text{G}]_i}{K_1 K_2 K_3}$$

$$k_{6(o \rightarrow i)} = k_{6(i \rightarrow o)},$$

$$k_{7(o \rightarrow i)} = k_{7(i \rightarrow o)}.$$

We know from the above model that

$$K_6 = k_6[\text{C}]_i = 1.5826 \times 10^3,$$

$$K_7 = k_7[\text{C}]_i = 2.6678,$$

$$k_6/k_7 = 593.3.$$

Substituting the values from Table 2 for the several constants and solving for DR we obtain

$$[G]_i/[G]_o = (2.609 \times 10^{-2}[\text{Na}]_o + [\text{Na}]_o^2)/(2.609 \times 10^{-2}[\text{Na}]_i + [\text{Na}]_i^2).$$

If we assume that $[\text{Na}]_o/[\text{Na}]_i = 10$, we obtain a DR of 38.5 for $[\text{Na}]_o = 120.8$ mM and 18.4 for $[\text{Na}]_o = 27$ mM. Recall that the presumption in deriving this equation is that $E_m = 0$. When $E_m = 0$, equation (4) reduces to

$$[G]_i/[G]_o \leq ([\text{Na}]_o/[\text{Na}]_i)^n.$$

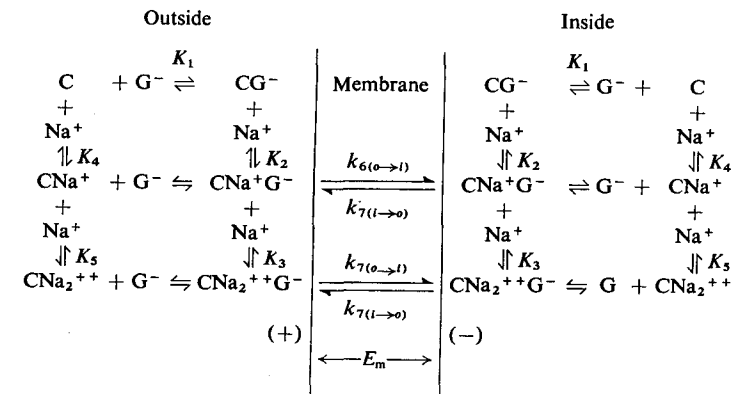
At $[\text{Na}]_o = 120.8$ mM, $n = 1.82$ and $[G]_i/[G]_o \leq 66.1$.

At $[\text{Na}]_o = 27$ mM, $n = 1.51$ and $[G]_i/[G]_o \leq 32.4$.

The values calculated from equation (9) above are below those limits in both instances. Thus equation (9) is consistent with the present results.

A model which is consistent with all results thus far

We can use the results of the present study along with those from previous studies (Wheeler, 1979a, 1980b) to put together a rather comprehensive picture of a possible mechanism for sodium dependent glutamate transport in rat cortical synaptosomes. The following model reconciles the results of all studies thus far:



The fundamental assumption in the model is that we have symmetry with regard to carrier concentration and dissociation constants on the two sides of the

membrane. Under normal resting conditions, asymmetry of electrochemical potential is introduced by two factors. First, the operation of the Na-K pump introduces chemical asymmetry in the Na^+ concentrations such that $[\text{Na}]_o \gg [\text{Na}]_i$. This factor alone will result in a steady state $[\text{G}]_i/[\text{G}]_o > 1$. Second, the imposition of membrane potential results in electrical asymmetry, and consequent asymmetry in $k_{7(o \rightarrow i)}/k_{7(i \rightarrow o)}$. This electrical asymmetry in turn multiplies the chemical asymmetry in $[\text{G}]_i/[\text{G}]_o$ caused by the sodium gradient. The net result is a distribution ratio for glutamate that is potentially enormous—greater than 3,000/1.

We can now use this model to make some further deductions.

(1) In the steady state,

$$\text{DR} = C([\text{Na}]_o/[\text{Na}]_i)^n e^{(FE_m/RT)(n-1)}$$

see equation (4).

A. In the presence of a membrane potential

$$\text{DR} = -(n-1) \ln \left(\frac{([\text{K}]_o + (p_{\text{Na}}/p_{\text{K}})[\text{Na}]_o + (p_{\text{Cl}}/p_{\text{K}})[\text{Cl}]_i)}{([\text{K}]_i + (p_{\text{Na}}/p_{\text{K}})[\text{Na}]_i + (p_{\text{Cl}}/p_{\text{K}})[\text{Cl}]_o)} \right) + n \ln([\text{Na}]_o/[\text{Na}]_i) + C.$$

Thus the glutamate DR depends on both E_m and the sodium concentration ratio.

B. In the absence of E_m , we conclude from present studies that

$$\text{DR} = C([\text{Na}]_o/[\text{Na}]_i)^n.$$

From previous studies, we conclude that (see equation (9))

$$\text{DR} = 2 \cdot 609 \times 10^{-2} [\text{Na}]_o^2 + [\text{Na}]_o^2 / (2 \cdot 609 \times 10^{-2} [\text{Na}]_i + [\text{Na}]_i^2).$$

Are these two relationships for DR reconcilable? We calculated from equation (9) and $[\text{Na}]_o = 27$ mM that $\text{DR} = 18 \cdot 44$. If we insert $18 \cdot 44$ for DR and $n = 1 \cdot 51$ (calculated previously) into equation (10) and solve for C , we obtain $C = 0 \cdot 571$. At $[\text{Na}]_o = 120 \cdot 8$ mM, previous studies have predicted a coupling ratio of $1 \cdot 82$. Substituting this value for n and $0 \cdot 571$ for C in equation (10), we calculate a DR of $37 \cdot 7$, compared to $38 \cdot 48$ calculated from equation (9) above. Thus the results are in agreement whether we use equation (10), which was derived from the present studies, or equation (9), which was derived from previous kinetic studies, to calculate DR.

(2) In the non-steady condition,

A. In the presence of a membrane potential, initial velocity is proportional to v_m . This would follow if CNa_2G has a net positive charge so that the rate constant $k_{7(o \rightarrow i)}$ is proportional to E_m . Initial velocity is dependent on both $[\text{CNaG}]$ and $[\text{CNa}_2\text{G}]$, which in turn are complexly dependent on the $[\text{Na}]_o$ as given by equation (6).

B. In the absence of E_m , $k_{6(o \rightarrow i)} = k_{6(i \rightarrow o)}$; $k_{7(o \rightarrow i)} = k_{7(i \rightarrow o)}$. Initial velocity depends on the concentration of CNa_2G and CNaG only; CNa_2G and CNaG in turn are complexly related to $[\text{Na}]$ as given by equation (6).

Glutamic acid is thought to serve as a neural transmitter (Krnjevic, 1974). The above model has important implications in this synaptic function of glutamate. First, glutamate must be stored in the presynaptic terminal to be available for

stimulus-triggered release. Second, depolarization of the nerve terminal must be accompanied by net efflux from the terminal. Third, glutamate must be removed from the synaptic cleft in preparation for succeeding impulses. Thus the above model might provide insight into all three of these processes. Under normal resting conditions, the electrochemical potential energy gradient is directed inwardly. Thus glutamate is concentrated in the nerve terminal. Upon depolarization, the electrical component of the energy gradient is abolished or directed outwardly (overshoot of the action potential); thus glutamate efflux would occur. Upon repolarization, the inward electrochemical potential energy gradient is re-established, glutamate influx occurs, synaptic glutamate concentration is reduced, and terminal glutamate concentration is re-established. The terminal is now ready for the next cycle.

The results are also important in understanding the post-synaptic effects of glutamate. If the post-synaptic cell also has a glutamate transporter similar to that present in synaptosomes, then part of the depolarizing effect of glutamate may result from operation of the electrogenic transport mechanism, rather than from glutamate induced conductance increases to other ions.

In conclusion, the model offers a mechanism for control of synaptic transmission. The dependence of glutamate movement, both in terms of amount and direction, on E_m , offers the possibility of extremely fine control of synaptic excitability, since the direction and amount of glutamate movement would cycle with the cycle of depolarization and repolarization of the invading action potential. This presumes that the glutamate transporter has a sufficiently short response time to follow membrane electrical events. If this is not the case, synaptic excitability could still be controlled, since the time-averaged membrane potential of the presynaptic terminal would control the amount and direction of glutamate movement. The results also suggest the possibility that glutamate might serve as a synaptic modulator in some instances, rather than function as a transmitter in the classical sense. The operation of a glutamate transporter in the post-synaptic cell would produce membrane depolarization; the amount of such depolarization would be related to the concentration of glutamate in the synapse. Thus excitability of the post-synaptic cell could be controlled by presynaptic events, in the absence of any specific receptors in the classical sense—that is, receptors which respond to transmitter with an increased conductance to other ions.

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