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THE ROLE OF METABOLIC ENERGY IN THE TRANSPORT OF GLUTAMATE BY INVERTEBRATE NERVE

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SUMMARY

The Na^+-dependent component of glutamate influx in both crab nerve and squid giant axons is influenced by the metabolic state of the cell. In crab nerve, the only kinetic parameter that seems to be under metabolic control is the affinity for glutamate. Lowering the ATP level increases the apparent K_m for glutamate but seems to have no effect either on V or on the affinity for external Na^+. In squid axons, glutamate efflux is also reduced in fully poisoned cells. The effects of metabolic poisons on glutamate influx in squid axons are not, and in crab are unlikely to be, secondary to changes in membrane potential or internal Na^+, K^+ or Ca^{2+}.

INTRODUCTION

Despite an adverse electrical gradient, glutamate is concentrated inside many invertebrate nerves[1]. Thus walking leg nerves of the spider crab *Maia squinado* contain 40–50 mmoles glutamate/kg cell water whereas there is less than 1 mmole glutamate/l of blood[2]. The exact concentration of glutamate in *Maia* blood has not been determined but in the shore crab *Carcinus maenas* there is only 0.2 mmole glutamate/l of crab blood, and of this only 0.01–0.06 mmole/l seems to be free in the plasma[3]. An essentially similar distribution of glutamate is found in squid axons.

If glutamate is distributed purely passively, it should be present in lower concentrations inside the cells. The observed high intracellular level of glutamate seems to be maintained, in part, by a Na^+-dependent uptake process[2,4,5]. Of particular interest is the observation that in both crab nerve and squid axons the kinetics of activation by external Na^+ are consistent with two Na^+ co-operating in the uptake of each molecule of glutamate. In crab, but not squid axons, the activation by Na^+ is inhibited competitively by external K^+. Provided the activating Na^+ are also transported into the cell and provided glutamate is transported in the form bearing a single negative charge, the glutamate gradient that can theoretically be produced is given by

$$\frac{[Glu]_o}{[Glu]_i} = \frac{[Na^+]_i^2}{[Na^+]_o^2} e^{VF/RT} \quad (1)$$

Abbreviation: EGTA, ethyleneglycol-bis(aminoethyl)tetraacetic acid.

As $[Na^+]_i/[Na^+]_0$ is roughly 1/10 and E is about -60 mV, the participation of two Na^+ can concentrate glutamate about 1000-fold inside the cell. This calculation suggests that in crab nerve and squid axons there is enough energy available in the Na^+ gradient to maintain the observed intracellular concentration of glutamate. In view of this, it is of interest that transport of glutamate also depends upon the metabolic state of the cell: the rate of Na^+-dependent glutamate uptake being reduced in poisoned cells. The kinetic data on crab nerve described in this paper suggest that metabolism exerts its influence primarily on the affinity of the transport system for glutamate and glutamate transport in this tissue does not seem to have an obligatory requirement for energy-rich phosphate compounds.

METHODS

Material

Two preparations were used. Nerve bundles were dissected from the walking legs of the spider crab *Maia squinado* as described by Baker[6] and hindmost stellar giant axons were obtained from the squid *Loligo forbesi*. Crabs were kept in an aquarium before use. The squid were usually killed at sea by decapitation and brought to the laboratory in refrigerated sea water. The axons were dissected within 2–6 h of capture. Living squid were used in some experiments.

Solutions

External solutions were artificial sea waters. The composition of the main solutions used is given in Table 1. Chemicals were Analar grade where available.

Flux measurements

Influxes were determined by including L-[U-^{14}C]glutamate in the external solutions. Tracer influx was linear for up to 1 h in crab nerve and for up to 2 h in squid axons[4,5]. It follows that in short incubations the uptake of tracer should give a good measure of influx. After a suitable time in the tracer, normally 10 min at 16 °C for crab nerve and 30–60 min at 20 °C for squid axons, the extracellular radioactivity was removed by washing over 10–15 min in 5 separate 20-ml aliquots of ice-cold artificial sea water. Subsequent treatment depended on the kind of nerve. With crab nerves the middle section was taken and dissolved in Nuclear Chigaco Solubilizer (NCS). With squid axon, the ganglion end of the giant axon was cleaned of adhering small nerve fibres, the end of the axon was cut and axoplasm extruded from the giant axon through the cleaned cut end. Axoplasm from within 1 cm of each end of the axon was rejected to avoid sampling from a depolarized region and only axoplasm from the

TABLE I

THE IONIC COMPOSITION OF THE ARTIFICIAL SEA WATERS

When sea waters were adjusted to pH 6.8, 5 mM Tris was used as a buffer.

Solution	*Concentration (mmole/l solution)*						
	Na^+	$Choline^+$	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-
Na^+-containing artificial sea water	460	nil	10	11	55	600	2.5
$Choline^+$-containing artificial sea water	nil	460	10	11	55	600	2.5

middle of the axon was taken for counting. It was weighed and dissolved in NCS. L-[U-^{14}C]Glutamate was estimated by liquid scintillation counting and the counts were corrected for quenching. Under the experimental conditions used, more than 95% of the counts taken up by the nerve were recoverable as glutamate.

Efflux measurements were made on both preparations. Crab nerves were loaded with L-[U-^{14}C]glutamate by soaking for 1 h in Na^+-containing artificial sea water containing 2.5 μCi carrier-free L-[U-^{14}C]glutamate. The nerve was subsequently washed well for 40 min in 8 changes of sea water at 20 °C to remove extracellular radioactivity. Cleaned squid axons were loaded by injection directly into the interior of the cell, by means of a fine glass capillary attached to a Hamilton microsyringe[7]. Thus in the squid preparation only the contents of the axon were labelled with L-[U-^{14}C]glutamate, whereas in crab nerve all the cellular components presumably took up some label and an appreciable amount of this may have become attached to extracellular structures.

For efflux measurements the nerves were normally transferred to a small glass tube (6 cm long $\times$ 0.3 cm internal diameter) attached to a 1-ml disposable syringe. This enabled adequate mixing while keeping to a minimum the volume of fluid used. Efflux samples were counted by liquid scintillation and the counts were corrected for quenching. At the end of the experiment the nerve was dissolved in NCS and counted. The efflux was expressed as that fraction of the total radioactivity lost per min. The identity of the radioactivity effluxing from crab nerve was checked in a few instances. Although the bulk of the radioactivity (50–70%) was recoverable as glutamate, an appreciable fraction (20–30%) was present as CO_2. This fraction was rather constant and no attempt was made to correct for it.

Na^+ and K^+ determinations

Na^+ and K^+ were measured by flame photometry.

ATP determinations

ATP concentrations were measured by the firefly luciferin–luciferase technique. About 20–40 mg of nerve was homogenized in 2 ml of 5% trichloroacetic acid at 0 °C. The homogenate was allowed to stand for 20 min at 0 °C before being spun at 700 $\times$ *g* for 3 min. The supernatant was extracted three times with 3 vol. of diethyl ether to remove trichloroacetic acid and and the last traces of ether were blown off by passing a stream of cold air over the solution for 5 min at 0 °C. 1-ml aliquots were taken for assay of ATP. The results were expressed as mmoles ATP/kg cell water. Separate determinations showed that 30% of the wet weight of crab nerve is extracellular space[6] and the dry weight is 13.8 $\pm$ 0.3% of the wet weight.

RESULTS

Experiments on crab nerve

Choice of metabolic poison. Previous attempts to poison crab nerves have met with very variable success[6]. In the present experiments, after a 30-min pre-soak period in Na^+-containing artificial sea water containing the inhibitor, the rate of ATP consumption was increased by stimulating the nerves electrically, thus accelerating the rate of ATP utilization by the Na^+ pump[6]. These axons were then soaked for a further period of 30 min in the presence of the inhibitor. In some experiments a Na^+-free sea water was used for the second soak.

TABLE II

THE EFFECT OF VARIOUS INHIBITORS ON THE CELLULAR ATP CONCENTRATION AND GLUTAMATE INFLUX IN CRAB NERVE

Nerves kept in Na^+-containing artificial sea water (pH 7.8) were blotted, transferred to the media indicated below and allowed to soak for 30 min at 16 °C. They were then stimulated (1-ms pulses 30 V, 30 Hz for 10 min at room temperature) and soaked for a second 30-min period at 16 °C. Cellular ATP levels were then determined in deacidified trichloroacetic acid extracts of the nerves. Influx of 0.1 mM L-[U-^{14}C]glutamate (spec. act. $4 \cdot 10^3$ cpm/nmole) was also assayed for 10 min at 16 °C, in several of the media below. Results expressed as mean ± S.E. of the mean, with the number of experiments in parentheses.

External medium	*Cellular ATP concentration (mmoles/kg cell water)*	*Glutamate influx (mmoles/kg nerve per h)*
Na^+-containing artificial sea water (pH 7.8)	1.80 ±0.12 (9)	
+ 1 mM sodium fluoroacetate	1.81 ±0.30 (3)	
+50 mM sodium arsenate	1.93 ±0.17 (3)	
+10 mM sodium fluoride	1.33 ±0.27 (3)	
+ 1 mM iodoacetamide	1.30 ±0.14 (3)	
+10 mM sodium iodide	1.11 ±0.01 (3)	
+ 2 mM sodium cyanide	0.62 ±0.05 (3)	
+ 2 mM sodium azide	0.49 ±0.02 (3)	
Na^+-containing artificial sea water (pH 6.8)	1.82 ±0.11 (3)	$0.17_3 \pm 0.01_4$ (8)
+ 2 mM iodoacetamide	1.29 ±0.19 (3)	$0.04_4 \pm 0.00_4$ (6)
+ 0.2 mM 2,4-dinitrophenol	0.41 ±0.06 (3)	$0.10_5 \pm 0.01_4$ (6)
+ 2 mM iodoacetamide	$0.05_1 \pm 0.01_2$ (5)	$0.03_3 \pm 0.00_3$ (6)
+10 mM 2-deoxy-D-glucose	$0.23_4 \pm 0.01_1$ (3)	$0.08_4 \pm 0.01_7$ (6)
+20 mM 2-deoxy-D-glucose	$0.05_5 \pm 0.00_1$ (3)	$0.04_2 \pm 0.00_3$ (7)
+20 mM 2-deoxy-D-glucose*	$0.01_7 \pm 0.00_2$ (2)	$0.03_3 \pm 0.00_5$ (3)
$Choline^+$-containing artificial sea water (pH 6.8)**	—	$0.020 \pm 0.00_1$ (3)

* $Choline^+$ replaced Na^+ in the post-stimulation soak medium.
** $Choline^+$ replaced Na^+ in the influx assay.

The results obtained with a number of different inhibitors are shown in Table II. The lowest ATP levels were found in axons pretreated with a mixture of 2,4-dinitrophenol and either iodoacetamide or 2-deoxy-D-glucose. By varying the amount of iodoacetamide or 2-deoxy-D-glucose, it proved possible to produce a number of stable intermediate concentrations of ATP (Fig. 1). The effect of these inhibitors on the influx of glutamate is also included in Table II. In general the glutamate influx is reduced in parallel with the reduction in ATP, the exception being iodoacetamide which inhibited glutamate uptake to a similar extent at markedly different ATP concentrations. Because of the inhibitory effect of iodoacetamide *per se*, mixtures of 2,4-dinitrophenol and 2-deoxy-D-glucose were used in the subsequent experiments.

Corroborative evidence for the effectiveness of 2,4-dinitrophenol *plus* 2-deoxy-D-glucose in reducing the cellular ATP is provided by Fig. 2. The ouabain-sensitive uptake of ^{42}K was measured at different ATP concentrations and the results show an appreciable inhibition at low levels of ATP.

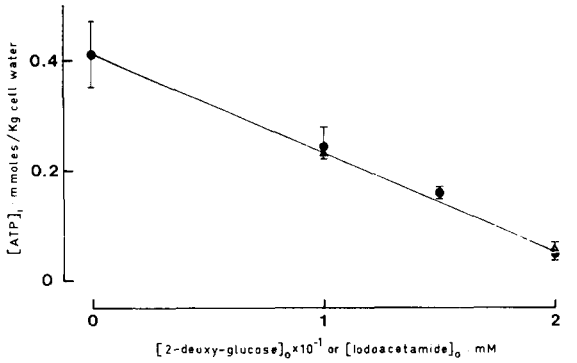

Fig. 1. The effects of 2-deoxy-D-glucose and iodoacetamide in 2,4-dinitrophenol-containing sea water on cellular ATP levels. Nerves were soaked for 30 min at 16 °C in Na^+-containing artificial sea water (pH 6.8) containing 0.2 mM 2,4-dinitrophenol and the indicated concentrations of 2-deoxy-D-glucose or iodoacetamide. They were then stimulated electrically (see Table II) and allowed to soak for a further 30 min. Cellular ATP levels were then determined. (●) iodoacetamide, (▲) 2-deoxy-D-glucose.

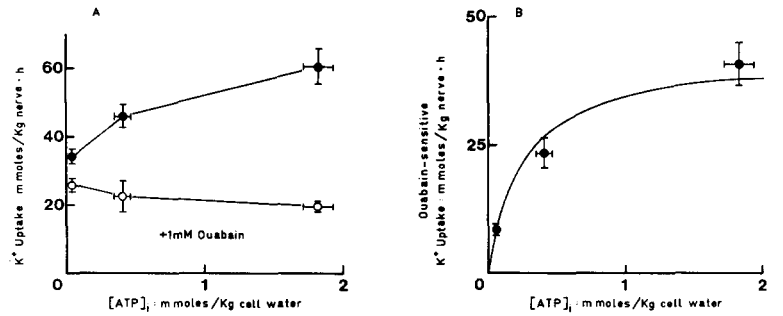

Fig. 2. The effect of internal ATP levels on K^+ influx. Conditions were identical to those described for Table III except that the influx of 10 mM ^{42}K (spec. act. $3 \cdot 10^3$ cpm/μmole) was measured for 10 min at 16 °C in Na^+-containing artificial sea water and Na^+-containing artificial sea water + 1 mM ouabain. The values for the ouabain-sensitive influx were obtained by subtracting the values for the influx in Na^+-containing artificial sea water + 1 mM ouabain from those in ouabain-free sea water. The smooth curve (B) was calculated from the kinetic parameters derived from the corresponding double reciprocal plot (not shown). The apparent K_m for ATP was 0.25 mM and the V was 43.25 mmoles/kg nerve per h.

Treatment with 2,4-dinitrophenol reversibly increased the efflux of glutamate from crab nerve (Fig. 3). The efflux was roughly doubled after exposure to 2,4-dinitrophenol for 1 h. This increase was not much affected by the ionic environment, by the presence of up to 20 mM 2-deoxy-D-glucose nor by electrical stimulation. It was not mimicked by cyanide which produced a small fall in efflux. Possible reasons for the increase will be discussed later; but the existence of an increased efflux implies that during poisoning with 2,4-dinitrophenol there might be a rise in the concentration of glutamate immediately external to the axon membrane. An increased leak of unlabelled glutamate might, by decreasing the specific activity, produce an apparent inhibition of glutamate influx. The data shown in Fig. 4 and discussed in the next section

make it unlikely that changes in the specific activity of glutamate contributed to the inhibition observed in the present experiments (see p. 129). In absolute terms the efflux in the presence of 2,4-dinitrophenol was very small and could not, by loss of radio activity, have resulted in any significant error in the influx measurements.

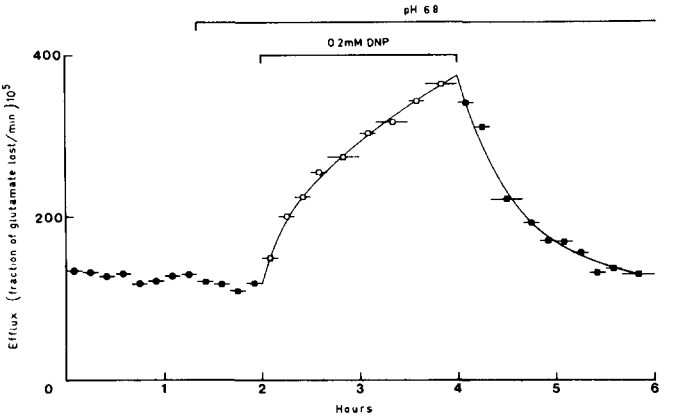

Fig. 3. The effect of 2,4-dinitrophenol (DNP) on glutamate efflux. Nerves were loaded with glutamate as described in Methods. Efflux was subsequently assayed for 10-min periods over several hours at 20 °C. (●), Na^+-containing artificial sea water (pH 7.8); (■), Na^+-containing artificial sea water (pH 6.8); (□), Na^+-containing artificial sea water (pH 6.8) containing 0.2 mM 2,4-dinitrophenol. The lines between the points were drawn by eye.

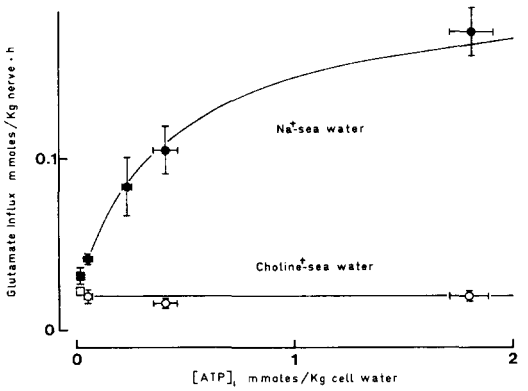

Fig. 4. The effect of internal ATP levels on glutamate influx from Na^+ and choline$^+$ sea waters. Conditions were as in Table II. 2-deoxy-glucose and 2,4-dinitrophenol were used as metabolic inhibitors. External L-[U-^{14}C]glutamate was 0.1 mM. The solid square represents the values obtained when choline$^+$ replaced external Na^+ in the post-stimulation soak medium, while the hollow square shows the value when choline$^+$ replaced Na^+ in the influx assay as well as in the post-stimulation soak medium. The smooth curve was calculated from the kinetic parameters derived from a double reciprocal plot of Na^+-sensitive glutamate influx *versus* ATP concentration. The apparent K_m for ATP was 0.41 mM. The V was 0.179 mmole/kg nerve per h.

Dependence of Na^+-sensitive glutamate influx on ATP. The relation between glutamate influx and intracellular ATP is shown in Fig. 4. There are two striking features of this figure. In the absence of external Na^+, changes in ATP have no effect on the

glutamate influx. This observation makes it unlikely that changes in specific activity (see previous paragraph) are interfering with the influx assay. In the presence of external Na^+, the glutamate influx increases with the intracellular ATP concentration and the relation between Na^+-sensitive glutamate influx and intracellular ATP can be fitted by a rectangular hyperbola.

These observations show that at an external glutamate concentration of 100 μM, the Na^+-sensitive glutamate influx can be almost completely inhibited in fully poisoned axons. It should be stressed that this effect is unlikely to be secondary to a run-down of the Na^+ and K^+ gradients because appreciable gradients still exist at the end of the presoak period (Table III). In one series of experiments, following stimulation the nerves were soaked for 30 min in Na^+-free choline$^+$-containing sea water in the presence of 2,4-dinitrophenol and 2-deoxy-D-glucose, before assay of glutamate influx. Although after this treatment the intracellular Na^+ was close to its value in unpoisoned axons (Table III), the Na^+-sensitive glutamate influx was still strongly inhibited (Fig. 4).

TABLE III

THE EFFECT OF SOAKING IN VARIOUS INHIBITORS ON THE LEVELS OF CELLULAR Na^+ AND K^+ IN CRAB NERVE

Nerves kept in Na^+-containing artificial sea water (pH 7.8) were blotted, transferred to the media indicated below and allowed to soak for 30 min at 16 °C. They were then stimulated as described in Table II and soaked for a second 30-min period at 16 °C After washing over 10 min in 5 changes of ice-cold choline$^+$-containing artificial sea water, the nerves were blotted and digested in conc. nitric acid. Na^+ and K^+ were estimated in the diluted digest using an EEL flame photometer.

External medium	$[Na^+]_i$ *(mequiv/kg cell water)*	$[K^+]_i$ *(mequiv/kg cell water)*	$[ATP]_i$ *(mmoles/kg cell water)*
Na^+-containing artificial sea water (pH 6.8)	50.3 ± 11.6 (3)	262.2 ± 11.8 (2)	1.82 ± 0.11 (3)
+ 0.2 mM 2,4-dinitrophenol	108.8 ± 4.7 (3)	234.2 ± 9.3 (3)	0.41 ± 0.06 (3)
+20 mM 2-deoxy-D-glucose	114.1 ± 2.5 (3)	212.7 ± 4.0 (3)	$0.05_5 \pm 0.00_1$ (3)
+20 mM 2-deoxy-D-glucose*	67.7 ± 2.0 (3)	148.8 ± 6.7 (3)	$0.01_7 \pm 0.00_2$ (2)

* Choline$^+$ replaced Na^+ in the post-stimulation soak medium.

A more detailed kinetic analysis of the dependence of the Na^+-sensitive glutamate influx on ATP is shown in Figs 5A and 5B. The Na^+-sensitive glutamate influx was determined at three ATP levels and three concentrations of external glutamate. The Lineweaver–Burk plot (Fig. 5B) shows that reducing the intracellular concentration of ATP decreases the apparent affinity for glutamate without affecting the maximum rate of Na^+-sensitive glutamate influx. The dependence of the K_m for glutamate on ATP is shown in Fig. 6.

Although changes in internal ATP affect the affinity for glutamate they seem to be without effect on the affinity for external Na^+ (Figs 7A, 7B).

Figs 8A, 8B, 9A and 9B show that the apparent affinity for internal ATP is unaffected by the external concentrations of either glutamate or Na^+.

To summarize, the kinetic analysis of the Na^+-sensitive influx of glutamate in

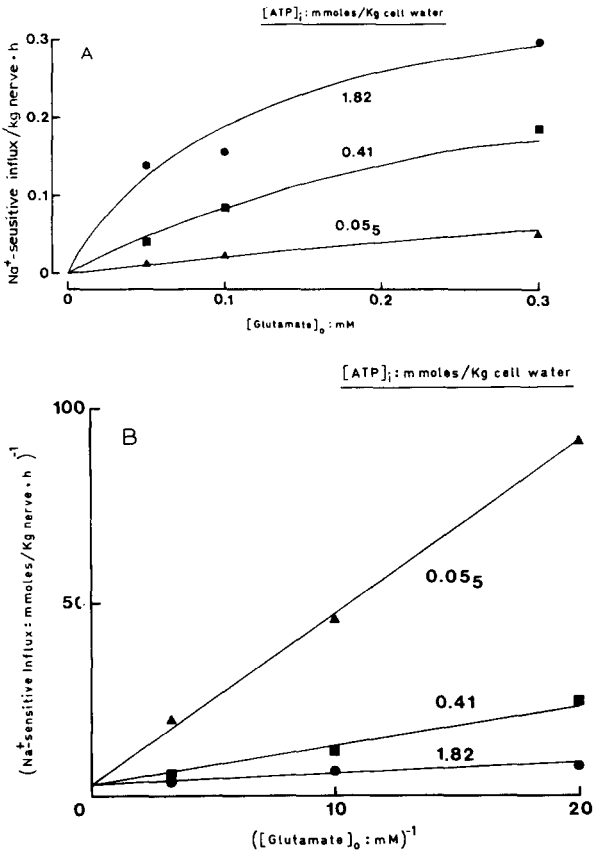

Fig. 5. The effect of internal ATP levels on Na^+-sensitive glutamate influx. Conditions were identical to those in Table III except that the influx of three different concentrations of L-[U-^{14}C]-glutamate was measured from Na^+-containing artificial sea water and choline$^+$-containing artificial sea water for 10 min at 16 °C. (B) A double reciprocal plot of the data shown in (A). The ordinate in (A) is the Na^+-sensitive influx obtained by subtracting the influx in choline$^+$ artificial sea water from that in Na^+ artificial sea water. The smooth curves in (A) were calculated using the apparent kinetic parameters derived from (B): K_{Glu} the apparent affinity for glutamate, was 0.11 mM, 0.37 mM and 1.82 mM when $[ATP]_i$ was 1.82, 0.41 and 0.055 mM, respectively. V was 0.4 mmole/kg nerve per h.

crab nerve shows that the only kinetic parameter under metabolic control is the apparent affinity for glutamate. Although the level of ATP has been used as an indicator of the metabolic state of the nerves, there is no proof that the effects of poisoning are due to changes in ATP levels *per se*. They could, for instance, be due to changes in the concentrations of other phosphate esters or be secondary to the large changes in ionized Ca^{2+} levels that occur in poisoned cells[8].

Experiments on squid axons

Effects of metabolic poisons on glutamate influx and efflux. Table IV summarizes data on the effects of various metabolic poisons on the fluxes of glutamate in squid axons. Cyanide, azide and 2,4-dinitrophenol reduce the Na^+-sensitive glutamate

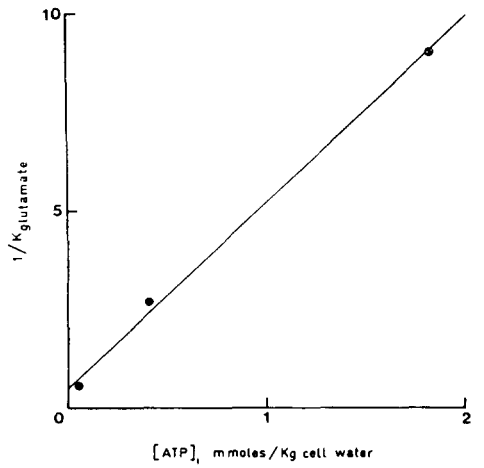

Fig. 6. The effect of internal ATP levels on the K_m for glutamate. Conditions as in Fig. 5. The K_{Glu} values were derived from Fig. 5B.

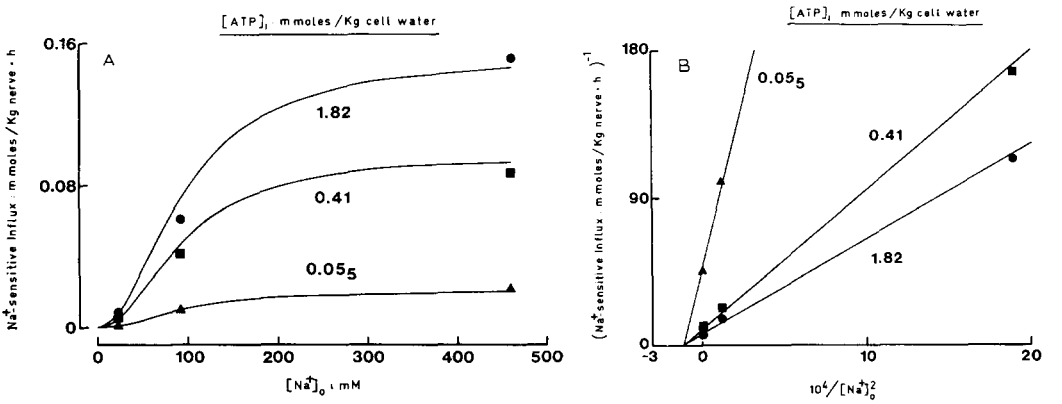

Fig. 7. The effect of internal ATP levels on the Na^+ activation of glutamate influx. Conditions were identical to those in Table III except that the influx of 0.1 mM L-[U-^{14}C]glutamate was measured for 10 min at 16 °C at the concentrations of external Na^+ indicated below as well as in choline$^+$ artificial sea water. The values for the Na^+-sensitive influx were obtained by subtracting the influx in choline$^+$ artificial sea water from those in Na^+-containing artificial sea water. The smooth curves in (A) were calculated from the apparent kinetic parameters in (B): V was 0.152, 0.097 and 0.021 mmole/kg nerve per h when [ATP]$_i$ was 1.82, 0.41 and 0.05_5 mM, respectively. The K_m for Na^+ was 93 mM.

influx and the inhibitory effects of cyanide and azide are reversible. No attempt was made to reverse the inhibition produced by 2,4-dinitrophenol because, in squid axons, there is a very poor recovery of the ATP levels after removal of 2,4-dinitrophenol[9]. As in crab nerve, the inhibitory effect of metabolic poisons was confined to the Na^+-sensitive component of the glutamate influx. It should also be pointed out that changes in the concentration of internal Na^+ were minimized by pretreating with the metabolic poison in Na^+-free choline$^+$-containing sea water.

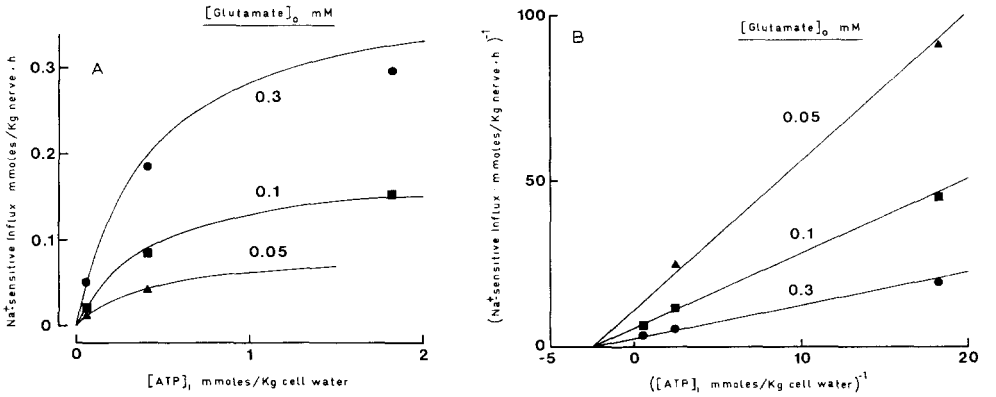

Fig. 8. The effect of external glutamate on the ATP activation of the Na^+-sensitive glutamate influx. The conditions were identical to those in Fig. 5. The smooth curves in (A) were calculated from the apparent kinetic parameters in (B): V was 0.400, 0.182 and 0.089 mmole/kg nerve per h when the concentration of external glutamate was 0.3, 0.1 and 0.05 mM, respectively. K_m for ATP was 0.41 mM.

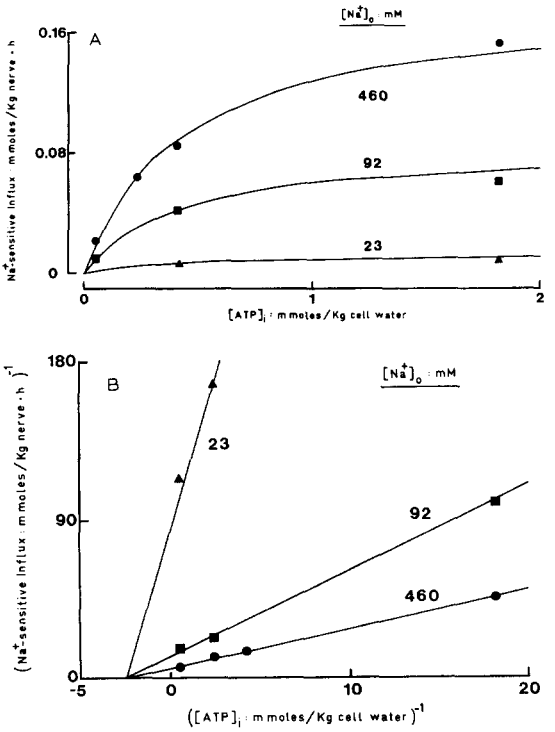

Fig. 9. The effect of external Na^+ on the ATP activation of the Na^+-sensitive glutamate influx. Conditions were identical to those in Fig. 7. The smooth curves in (A) were calculated from the apparent kinetic parameters in (B); V was 0.179, 0.085, 0.012 mmole/kg nerve per h when the concentration of Na_o^+ was 460, 92 and 23 mM, respectively. The K_m for ATP_i was 0.41 mM.

TABLE IV

CUMULATIVE DATA OF THE EFFECT OF VARIOUS METABOLIC INHIBITORS ON GLUTAMATE FLUXES IN GIANT AXONS OF *LOLIGO FORBESI*

Influx: Uncleaned axons were placed in choline-sea waters containing the metabolic inhibitors indicated below and allowed to soak for 90 min at room temperature. Control nerves were placed in inhibitor-free sea water. Some fibres were then transferred to the corresponding Na^+-sea waters for 30 min, the rest remained in the choline$^+$ media. The influx of 0.1 mM L-[U-^{14}C]glutamate (spec. act. $3 \cdot 10^3$ cpm/nmole) was then assayed in fresh medium for 1 h at 20 °C. In some experiments inhibited axons were washed in 12 successive changes of inhibitor-free Na^+-containing artificial sea water over 2 h and the influx was again assayed and found to be near its unpoisoned value. Efflux: Cleaned axons were injected axially with 0.2 μCi L-[U-^{14}C]glutamate over a 1-cm length near the midpoint of the fibre. The efflux was assayed over 10-min periods for several hours at 20 °C. The steady-state values are quoted below. Axon diameter 680–850 μm.

	Glutamate influx (pmoles/cm² per h)			*Glutamate efflux (fraction of glutamate lost/min × 10⁶) into*	
	Na^+-containing artificial sea water	*Choline$^+$-containing artificial sea water*	*Na^+-sensitive*	*Na^+-containing artificial sea water*	*Choline$^+$-containing artificial sea water*
Control pH 7.8	82.30 ± 11.93 (10)	9.97 ± 3.57 (5)	72.32 ± 11.92 (10)	154.7 ± 29.1 (12)	158.9 ± 6.9 (3)
+2 mM NaCN	49.76 ± 8.72 (5)	10.37 ± 2.02 (2)	39.39 ± 8.72 (5)	17.6 ± 2.4 (10)	19.6 ± 0.9 (2)
+0.1 mM NaN_3	20.92 ± 2.82 (5)	10.64 ± 0.66 (2)	10.28 ± 2.82 (5)	18.1 (1)	—
then recovered	70.92 ± 24.62 (2)	—	—	168.7 ± 15.9 (5)	—
Control pH 6.8	72.12 ± 5.79 (2)	—	—	153.3 ± 26.2 (8)	—
+0.2 mM 2,4-dinitrophenol	24.87 ± 5.51 (3)	—	—	29.0* (1)	—

* Inhibition is usually preceded by a period during which the efflux is increased above control levels.

Table IV also shows that exposure to cyanide, azide or 2,4-dinitrophenol produces a reversible fall in the efflux of glutamate from squid axons. The response to cyanide and azide was always a fall, cyanide reducing the efflux on average to 12% of its initial value; but 2,4-dinitrophenol always produced an initial increase and on some occasions almost doubled the efflux before any inhibition became apparent. The inhibition in the presence of 2,4-dinitrophenol was most apparent in axons that had recovered from pretreatment with cyanide. Application of 2,4-dinitrophenol to an axon that was already fully poisoned with cyanide had no effect on the efflux, an observation that makes it unlikely that 2,4-dinitrophenol is simply altering the permeability to glutamate. The initial reponse to 2,4-dinitrophenol resembles that seen in crab nerve (Fig. 3).

Is inhibition of glutamate fluxes by metabolic poisons secondary to changes in internal ionic levels? The inhibitory effects of metabolic poisons on both influx and efflux of glutamate might be due to changes in energy-rich phosphate compounds or could be secondary to changes in internal ions or membrane potential. A change in membrane potential seems unlikely because Hodgkin and Keynes[10] showed that poisoning has little effect on the resting potential of squid axons; but poisoning can produce changes in the levels of Na_i^+, K_i^+ and Ca_i^{2+}.

As mentioned in the previous section, cyanide inhibits the Na^+-sensitive glutamate influx even in axons that have been poisoned in Na^+-free sea water, and Fig. 10 shows that the extent and time-course of inhibition of the glutamate efflux by cyanide is independent of the Na^+ concentration in the external medium. These experiments

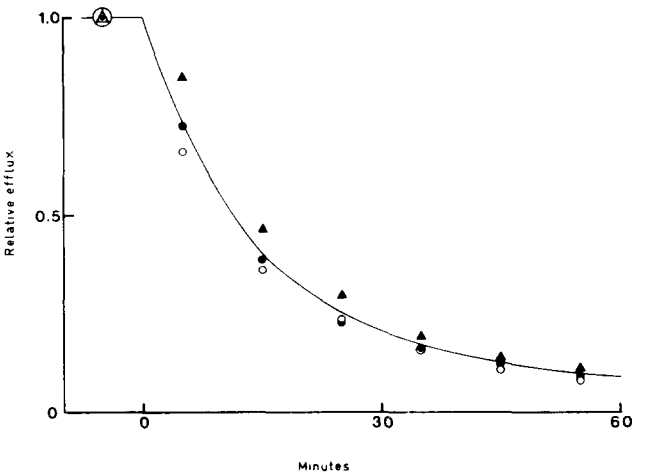

Fig. 10. The effect of internal EGTA and external Na^+ on the inhibition of glutamate efflux from squid axons by 2 mM NaCN. Cleaned axons were either (a) axially injected with 0.1 M K^+–EGTA (pH 7.2) over their entire length to give a final concentration of about 3 mM and injected again with 0.2 μCi L-[U-^{14}C]glutamate but only over a 1-cm length in the middle of the axon, or (b) injected only with the L-[U-^{14}C]glutamate. Glutamate efflux from EGTA-containing axons into Na^+-containing artificial sea water ± 2 mM NaCN (●) and from EGTA-free axons into Na^+-containing artificial sea water ± 2 mM NaCN (○) as well as choline$^+$-containing artificial sea water ± 2 mM NaCN (▲) was assayed over 10-min periods for several hours at 20 °C. The values were normalized against the steady state efflux into the cyanide-free sea waters. The poison was added at zero time. The smooth curve joining the points was drawn by eye.

seem to rule out the possibility that inhibition of glutamate fluxes is secondary to changes in internal Na^+ levels. Similar experimental evidence to rule out changes in the concentration of internal K^+ is less easy to obtain, but changes in intracellular K^+ levels are very small (10–20%) under the experimental conditions used. A more likely candidate is Ca^{2+}, the ionized concentration of which is known to increase up to 30 times in fully poisoned axons[8]. In order to examine this possibility, a number of measurements of influx and efflux were made in axons that had been pre-injected with the Ca^{2+}-chelating agent ethyleneglycol-bis(aminoethyl)tetraacetic acid (EGTA) to buffer the internal Ca^{2+} at or below its physiological level. Injection of EGTA to give a final concentration of about 3 mM had no appreciable effect on the glutamate efflux and did not alter the response to metabolic inhibitors (Fig. 10). Injection of EGTA increased influx of glutamate over that in uninjected controls, but working with pairs of injected axons, application of cyanide *plus* azide reduced the influx, and the size of this reduction was not significantly different from that produced by the same inhibitors in axons that had not been injected with EGTA (Table V).

TABLE V

THE EFFECT OF INTERNAL EGTA ON THE INHIBITION OF GLUTAMATE INFLUX BY 2 mM CYANIDE *PLUS* 0.1 mM AZIDE

EGTA-free axon pairs were treated as described in the influx section of the legend to Table IV except that the metabolic inhibitors were 2 mM NaCN *plus* 0.1 mM NaN_3, and all pairs were transferred to Na^+-sea water (with or without inhibitor) for the final 30 min of their pre-treatment. These axons were not injected. Pairs of axons for injection of EGTA were cleaned and placed either in choline$^+$-sea water containing 2 mM NaCN *plus* 0.1 mM NaN_3 or in inhibitor-free choline$^+$-sea water, for 1 h at room temperature. The axon pairs were then returned to the corresponding Na^+-sea waters, injected axially over their entire length with 0.1 M K^+–EGTA (pH 7.2), and allowed to complete their 2-h pre-treatment in Na^+-sea waters. Influx into both EGTA-free and EGTA-containing axon pairs was assayed for 1 h at 20 °C in Na^+-containing artificial sea water ± (2 mM NaCN + 0.1 mM NaN_3) containing 0.1 mM L-[U-^{14}C]glutamate (spec. act. $4 \cdot 10^3$ cpm/nmole).

	Axon pairs	*Influx (pmoles/cm² per h) from*		
		Na^+-containing artificial sea water	*Na^+-containing artificial sea water + 2 mM NaCN + 0.1 mM NaN_3*	*Difference*
EGTA-free	A	55.93	11.52	44.41
	B	51.75	24.75	33.00
	C	80.54	23.01	57.53
	D	97.75	27.63	70.12
	E	72.35	17.70	54.65
	$\bar{x} \pm$ S.E.	72.86 ± 6.91	20.92 ± 2.55	51.94 ± 5.60*
EGTA-containing	F	170.30	127.24	43.06
	G	235.59	199.74	35.85
	H	161.07	172.46	−11.39
	I	371.25	190.25	181.00
	J	152.41	123.07	29.34
	$\bar{x} \pm$ S.E.	218.12 ± 16.40	162.55 ± 6.36	55.57 ± 29.29*

* The difference between these two numbers is not significant ($P \ll 90\%$).

These experiments provide strong evidence that the inhibition of both influx and efflux of glutamate by metabolic poisons does not result either from changes in membrane potential or from changes in the levels of intracellular Na^+, K^+ or Ca^{2+}.

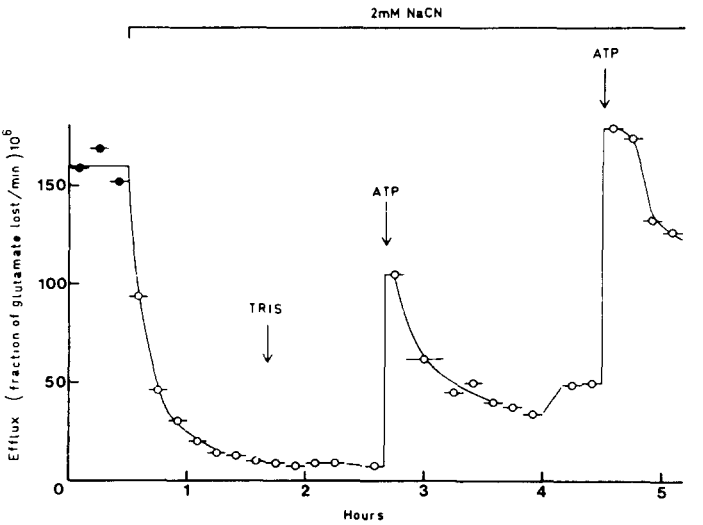

Fig. 11. The effect of ATP injection on the inhibition of glutamate efflux by 2 mM NaCN. The axon was cleaned and injected axially with 0.2 μCi L-[U-^{14}C]glutamate over a 1-cm length near its midpoint. The micro-injector was removed, refilled with 0.3 M K^+–Tris (pH 7.2) and reinserted down the entire length of the axon. Efflux into Na^+-containing artificial sea water ± 2 mM NaCN was measured and the Tris injected once the steady state of the cyanide inhibition had been achieved. The injector was again removed, refilled with 0.1 M ATP in 0.3 M Tris (pH 7.2) and reinserted down the entire length of the fibre. After establishing that the efflux was unchanged the ATP was injected. The ATP injection was repeated a second time. Axon diameter 950 μm. Temperature 20 °C. The smooth curves joining the points were drawn by eye.

Injection of ATP. Fig. 11 shows the effect of injecting ATP into a poisoned axon to give a final ATP concentration of about 3 mM inside the axon. Two consecutive injections of ATP produced transient rises in glutamate efflux whereas a control injection of Tris was without effect. The influence of ATP injection on the glutamate influx in poisoned axons was examined in only one experiment. The injection of an ATP-regenerating system (9 munits of creatine phosphokinase *plus* 0.2 μmole of creatine phosphate) into a cyanide-poisoned axon increased the influx to a value somewhat higher than that in an unpoisoned control axon injected with the same amount of enzyme.

DISCUSSION

Despite their differences in origin, crab nerve and squid axons have remarkably similar transport systems for glutamate[4,5]. In both preparations the influx of glutamate is activated by two Na^+ and in both preparations the Na^+-dependent component of the glutamate influx is inhibited by metabolic poisons. The most noticeable difference between the two preparations is that in crab nerve the activation by external Na^+ is competitively inhibited by external K^+ whereas there is evidence that a similar

effect does not exist in squid axons. There is no evidence for glutamate–glutamate exchange in either preparation.

As mentioned in the Introduction, provided two Na^+ enter the cell with each negatively charged glutamate molecule, in theory there seems to be enough energy in the Na^+ gradient to produce the intracellular concentration of glutamate found in crab nerve and squid axons. It is, therefore, particularly interesting to find that glutamate transport is also strikingly influenced by the metabolic state of the cell.

In both preparations studied, glutamate influx is reduced by a variety of metabolic poisons and this effect seems unlikely to be secondary to changes in membrane potential or the gradient of Na^+, K^+ or Ca^{2+}. This is most clear in squid axons where inhibition occurs with little change in either membrane potential or the intracellular concentrations of K^+, Na^+ or Ca^{2+}. In crab nerve, external K^+ leaking from the cells during metabolic inhibition might accumulate immediately external to the axolemma and inhibit glutamate influx; but this is most unlikely as more than 100 mM K^+ would be required to cause appreciable inhibition[2]. A similar argument cannot be applied to squid axons where the influx is insensitive to external K^+[5]. Although these observations rule out some obvious possibilities for the mechanism of inhibition of glutamate influx by metabolic inhibitors, other possibilities exist and, at this stage, it is not clear what is the link between metabolism and glutamate transport.

The experiments on crab nerve provide a fairly precise kinetic description of the influence of metabolism on glutamate influx. The only kinetic parameter that seems to be under metabolic control is the affinity for glutamate. Both the affinity for external Na^+ and the maximum rate of transport seem to be unaffected by the metabolic state of the cell. The implication of this analysis is that although the affinity for glutamate depends either directly or indirectly on the intracellular concentration of ATP, glutamate transport can still occur in the absence of ATP. This observation seems to rule out the possibility of an obligatory stoichiometric participation of energy-rich phosphate compounds in glutamate transport. The evidence is consistent with the affinity for glutamate being under metabolic control, but the energy for glutamate transport being provided by the ion gradients. As phosphate ions were present inside the poisoned cells, it is conceivable that the inward movement of Na^+ might generate a phosphorylated intermediate that effects the transport of glutamate, but the particular scheme put forward by Kimmich[11] seems unlikely because the external K^+ concentration used in the present experiments should have been adequate to inhibit Na^+–Na^+ exchange through the Na^+ pump.

The observations with metabolic inhibitors are consistent with the presence of two interconvertible forms of the glutamate carrier: a high affinity form (Y) and a low affinity form (X) (Fig. 12). When the ATP concentration is high, Y predominates, but gives way to X as the ATP level falls. The mechanism of interconversion of X and Y is not clear, but various possibilities exist including reversible adsorption of ATP or a derivative of it; reversible phosphorylation by ATP or a derivative of it or interaction with some other intracellular substance, the concentration of which changes during metabolic inhibition.

As the low affinity form of the carrier is present in fully-poisoned axons, the model illustrated in Fig. 12 predicts that glutamate efflux ought also to be decreased by metabolic inhibitors. This is clearly the case in squid axons exposed to cyanide and azide both of which inhibit the efflux of glutamate. But in both squid axons and crab

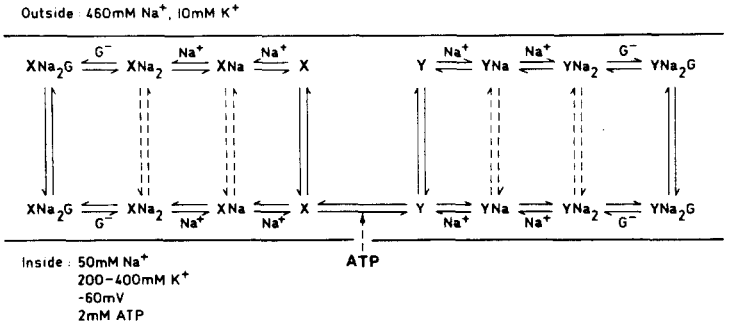

Fig. 12. A possible model for the observed dependence of glutamate transport on intracellular ATP. ATP is presumed to convert a membrane carrier with a low affinity for glutamate (X) into one with a high affinity (Y); in both conditions the energy for glutamate transport being obtained from the Na^+ gradient.

nerve, 2,4-dinitrophenol produces an initial stimulation of glutamate efflux. In squid axons the rise usually gives way to inhibition but the inhibitory effect was not observed in crab nerve. The explanation for this transient effect of 2,4-dinitrophenol is not clear.

Crab nerve seems to be in a steady state when the nerves are immersed in Na^+-containing artificial sea water containing 1–10 mM glutamate. Under these conditions influx and efflux are about equal. In the crab preparation the measurements of efflux are likely to be overestimated because it was not possible to exclude counts emerging from the damaged ends of the nerves. The net effect of poisoning depends on the poison used; but in general influx is reduced to a somewhat greater extent than efflux. It follows that under these conditions glutamate will be moving out of the nerve. The direction of net glutamate movement can be reversed by raising the external glutamate concentration which increases influx without changing efflux. Thus, even in fully poisoned nerves it is possible to effect a net uptake of glutamate against its electrochemical gradient.

As there is enough energy available in the ion gradients to account for the accumulation of glutamate, it is surprising that metabolism exerts a direct influence on glutamate transport. The most likely explanation is that metabolism, by maintaining the transport system in its high affinity form, enables a faster rate of glutamate transport at low concentrations of external glutamate. It follows that the overall effect of metabolism will be to facilitate a more rapid approach to the limiting glutamate gradient that can be produced from the existing Na^+ gradient. As there is an appreciable downhill movement leak of glutamate out of the cell, an increase in the rate of Na^+-dependent glutamate uptake will, in the steady state, lead to a greater accumulation of glutamate inside the cell. As glutamate is probably a transmitter substance in invertebrate nerve[12], the observation that metabolism increases the affinity for glutamate may be of significance in ensuring a rapid rate of removal of glutamate after nervous activity.

The transport of glutamate seems not to have been as intensively studied as that of other amino acids, but the results discussed in this paper suggest that glutamate transport in invertebrate nerve has many features in common with other amino acid transport systems[13–16]. There is disagreement over the relative importance of metabolism and ion gradients in the transport of amino acids[14–19]. Working with Ehrlich

ascites cells, Potashner and Johnstone[14] concluded that ATP is essential for the transport of glycine and methionine. Their kinetic analysis indicated that K_m for the amino acid but not for Na^+ was under metabolic control. Eddy *et al.*[18] have also obtained evidence for an effect of ATP of glycine transport over and above its role in generating the ion gradients. They interpret their results in terms of a role of ATP in maintaining a high membrane potential[19]; but they did not exclude the possibility that the affinity of the carrier system for the amino acid is also affected. The present results do, however, contrast quite sharply with the results of Vidaver[13] on glycine transport in avian red cells. This system is of interest because it resembles the glutamate transport system discussed in this paper in requiring two Na^+ for activation, but unlike the glutamate system, Vidaver[13] could find no dependence on metabolism down to ATP concentrations as low as 0.08 mM.

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REFERENCES

1 Lewis, P. R. (1952) *Biochem. J.* 52, 330
2 Baker, P. F. and Potashner, S. J. (1971) *Biochim. Biophys. Acta* 249, 616
3 Evans, P. D. (1972) *J. Expt. Biol.* 56, 501
4 Baker, P. F. and Potashner, S. J. (1973) *J. Physiol.*, in the press
5 Baker, P. F. and Potashner, S. J. (1973) *J. Physiol.*, in the press
6 Baker, P. F. (1965) *J. Physiol.* 180, 383
7 Baker, P. F., Blaustein, M. P., Keynes, R. D., Manil, J., Shaw, T. I. and Steinhardt, R. A. (1969) *J. Physiol.* 200, 459
8 Baker, P. F. (1972) *Prog. Biophys. Mol. Biol.* 24, 177
9 Caldwell, P. C. (1960) *J. Physiol.* 152, 545
10 Hodgkin, A. L. and Keynes, R. D. (1955) *J. Physiol* 128, 28
11 Kimmich, G (1970) *Biochemistry* 9, 3669
12 Johnson, J. L. (1972) *Brain Res.* 37, 1
13 Vidaver, G. A. (1964) *Biochemistry* 3, 662
14 Potashner, S. J. and Johnstone, R. M. (1971) *Biochim. Biophys. Acta* 91, 233
15 Schafer, J. A. (1972) in *Na^+-linked Transport of Organic Solutes* (Heinz, E., ed.), p. 68, Springer-Verlag, Berlin
16 Shultz, S. G. and Curran, P. F. (1970) *Physiol. Rev.* 50, 637
17 Johnstone, R. M. (1972) *Biochim. Biophys. Acta* 282, 366
18 Eddy, A. A., Mulcahy, M. F. and Thomson, P. J. (1967) *Biochem. J.* 103, 863
19 Gibb, L. E. and Eddy, A. A. (1972) *Biochem. J.* 129, 979