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GLYCINE FLUXES IN SQUID GIANT AXONS

BY P. C. CALDWELL AND T. J. LEA

From the Department of Zoology, University of Bristol, Bristol and the Laboratory of the Marine Biological Association of the U.K., Plymouth

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SUMMARY

1. The influx of a number of amino acids into squid giant axons has been studied. Particular emphasis has been placed on glycine and to a lesser extent glutamate.

2. To facilitate the study of the uptake of ^{14}C-labelled amino acids a technique was devised in which the ^{14}C taken up was measured directly in the intact axon with a glass scintillator fibre. This technique gave results similar to the usual technique in which the axoplasm was extruded for the assay of radioactivity.

3. The changes in glycine influx with extracellular glycine concentration suggests that two saturating components are present, one with high affinity and one with low affinity.

4. The glycine influx does not seem normally to be sensitive to the removal of extracellular sodium by replacement with choline. A Na-sensitive component appeared, however, after a period of immersion in artificial sea water. There was also some depression of glycine influx if Na were replaced by Li.

5. Glutamate uptake was greatly reduced by removal of extracellular Na in confirmation of work by Baker & Potashner (1973). Orthophosphate uptake was also greatly reduced by removal of extracellular Na.

6. CN reversibly inhibited glycine uptake after a delay, indicating that part of the uptake mechanism may require ATP.

7. ^{14}C-labelled glycine injected into squid axons was found not to exchange to any serious extent with other compounds over periods of a few hours. The glycine efflux could therefore be studied. This was found to be markedly increased by extracellular glycine and by certain other neutral amino acids applied extracellularly in the artificial sea water.

8. The enhanced glycine efflux in extracellular glycine was not affected by ouabain and CN.

9. It is suggested that glycine uptake in squid axons involves two components. One is sensitive to CN and ouabain and probably derives energy from ATP breakdown. The other is probably an ATP independent exchange diffusion system in which other amino acids as well as glycine can exchange for glycine. Both these systems are independent of extracellular Na concentration. A third Na-dependent system may appear under certain conditions.

INTRODUCTION

Much work is at present being carried out on the movement of amino acids and other substances across cell membranes but there is as yet no comprehensive and detailed description of the transport mechanisms and their relationships in a single cell. The squid giant axon offers some advantages for such a study, as it has for studies of cation transport, and recently some information has been obtained about the transport of glycine and glutamate (Caldwell & Lea, 1973, 1975; Baker & Potashner, 1973) and also cysteine (Hoskin & Brande, 1973). This paper describes a fairly detailed investigation of glycine transport in squid giant axons although the uptake of a number of other amino acids is also considered. ^{14}C-labelled glycine has been used in studies of both the influx and efflux of glycine. Extensive use has been made in the influx experiments of an intracellular glass scintillator technique for the measurement of weakly active radio-isotopes in the intact axon which has already been briefly described (Caldwell & Lea, 1973). One of the objects of the investigation has been to evaluate this technique by comparison of the results obtained with those obtained by the conventional method which involves extrusion of the axoplasm at the end of the uptake period.

METHODS

Material

Hindmost stellate giant axons were dissected from the squid *Loligo forbesi*, which were usually decapitated after being caught and then kept in refrigerated sea water during transportation to the laboratory. Sometimes squid which had been kept alive in laboratory tanks for a few hours were killed and used immediately.

Salines

The compositions of the salines used are given in Table 1.

TABLE 1. Compositions of saline and liquid scintillation mixtures

	Concentration (mg ions/l. solution)							
Salines	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	Choline	Li^+
Artificial sea water	462·5	10·4	11	55	602	2·5	—	—
zero Na, choline sea water	—	10·4	11	55	599·5	2·5	460	—
zero Na, Li sea water	—	10·4	11	55	559·5	2·5	—	460

pH was 7·8–8·0. Adjustments were made with HCl or Tris base.

	Liquid scintillation mixtures					
	Toluene	*p*-dioxane	Methanol	PPO	POPOP	Naphthalene
	(ml.)			(g)		
Influxes	1000	—	—	4·0	0·1	—
Effluxes	400	400	200	5·0	0·1	60

Influx measurements

Two methods were used to measure amino acid influx. The first was essentially the extrusion method first used by Bear, Schmitt & Young (1937). It has recently been used for the study of orthophosphate uptake (Caldwell & Lowe, 1970). Uncleaned pair axons from the same animal were tied off at each end and immersed in artificial sea waters (ASWs) containing ^{14}C-labelled amino acids (0·05 μC ml.$^{-1}$). Of each pair of axons from a squid, one was treated as a control

(usually with 1 mM-amino acid in the ASW) and the other was exposed to a test condition. After a given time, usually 40 min, the axons were removed and washed in non-radioactive saline. Part of each axon, 1 cm from the end, was cleaned and cut, and the axoplasm from the middle length of axon was extruded onto a weighed polyethylene square, which was then re-weighed. The axoplasm was left to soak in 0·1–0·2 ml. distilled water for 12 h. Scintillation cocktail with the composition given in Table 1 was then added and ^{14}C activity measured using a Packard Tricarb liquid scintillation spectrometer. Checks were made for the effects of quenching. The radioactivity in a measured volume of artificial sea-water was also measured.

Amino acid influx was calculated from the relation

$$\text{influx (f-mole cm}^{-2}\text{ sec}^{-1}) = \frac{M_s C_a d\rho}{4 C_s W t} \times 10^{15}, \quad (1)$$

where M_s is the amino acid concentration in the sea water (M). C_s is counts per minute (cpm) per litre of sea water, C_a is cpm for the axoplasm, W is its weight (g), t is time of immersion (sec), d is axon diameter (cm), ρ is density of axoplasm (assumed 1·0).

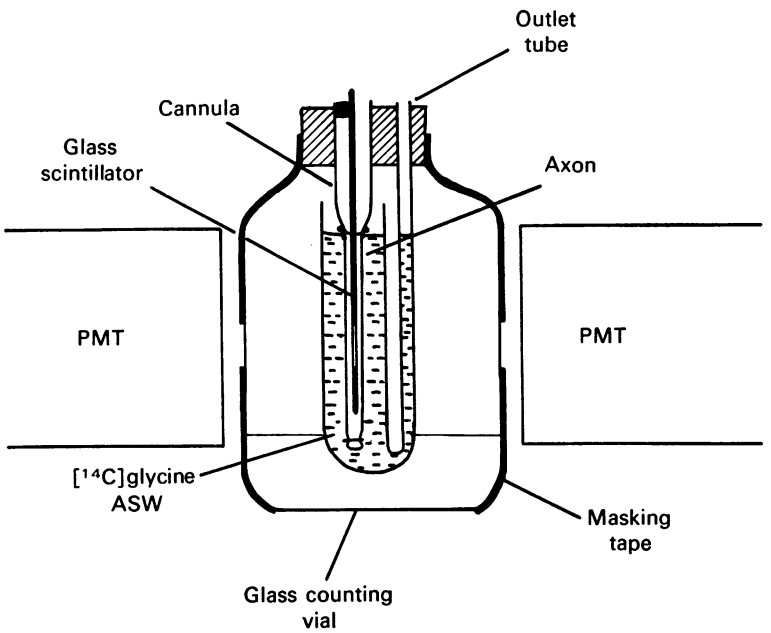

Fig. 1. The experimental assembly for the measurement of the influx of [^{14}C]glycine and other ^{14}C-labelled amino acids into a squid axon by an intracellular glass scintillator. PMT = photomultiplier tube; ASW = artificial sea water.

The second method used involved scintillation counting in intact axons with an intracellular glass scintillator probe. The experimental arrangement is shown in Fig. 1. A cleaned 5 cm length of axon was cannulated at one end and tied off at the other. A 125–175 μm diameter glass scintillator fibre (Koch-Light Laboratories Ltd., glass scintillator type (GSF 1) was inserted longitudinally into the axon, in a similar way to a capillary electrode (Hodgkin & Huxley, 1945). The axon was then immersed in about 1 ml. saline containing ^{14}C-labelled amino acid (2–26 μC ml.$^{-1}$) in a glass tube fixed vertically in a glass counting vial. The vial was placed in the counting well of a Packard Tricarb spectrometer and the counts for successive 5 or 10 min intervals were recorded and plotted against time. The counts per unit time interval were found to increase linearly with time of immersion for at least 4 hr as [^{14}C]amino acid entered the axon across the axolemma and was detected by the glass scintillator (see Fig. 3). The resulting scin-

tillations were detected by the photomultiplier tubes. By measurement of the slope of the plot and calibration of the glass scintillator in the external saline at the end of the experiment, it was possible to calculate a value for the amino acid influx using the relationship

$$\text{influx (f-mole cm}^{-2}\text{ sec}^{-1}) = \frac{M_s Y_a d}{4 Y_s t}\, 10^{12}, \tag{2}$$

where Y_a is counts/unit time for a given length of glass scintillator in the axon. Y_s is counts/unit time for the same length in external [^{14}C]amino acid saline. This relationship was derived from eqn. (1), using the observation that for a given length of glass scintillator in saline containing [^{14}C]glycine, Y_s is proportional to the ^{14}C activity of 1 l. saline C'_s,

$$\text{i.e. } Y_s = \frac{EC'_s}{1000}, \tag{3}$$

where E is efficiency (cpm/disintegrations per minute (dpm) per ml.). A similar relationship is assumed to hold for the probe in the axon:

$$Y_a = EC'_a \rho / W. \tag{4}$$

At the spectrometer discrimination settings employed in the experiment (EHT = 1200 V, gain = 20 %, discriminator 10 to ∞), a 1·0 cm length of glass scintillator of diameter 160 μm had a value for E of about 2×10^{-5} cpm/dpm ml.$^{-1}$, i.e. it gave 440 cpm in [^{14}C]ASW containing 10 μC/ml. This compared with a background counting rate (i.e. due to an empty counting vial) of 20 cpm. Neither the presence of the [^{14}C]ASW nor the scintillator probe in ASW had any significant effect on the background rate.

The counts given by the glass scintillator probe in the axon are assumed to result almost entirely from β particles which have been emitted from [^{14}C]amino acid in the axoplasm. The glass scintillator detection range for [^{14}C]β particles in ASW was investigated by enclosing a 1 cm length of 160 μm diameter glass scintillator in different diameter glass capillaries filled with [^{14}C]glycine ASW (100 μC/ml.) and then counting the resulting scintillation rates. The count rate rose to a plateau level as the capillary diameter was increased. This is shown in Fig. 2*B* in which cpm are plotted against the mean radial distance of the glass scintillator surface from the capillary wall (r^1). Fifty per cent of the scintillations produced by the glass scintillator originate from disintegrations which are within 40 μm of the scintillator's surface, and 90 % of the scintillations originate from disintegrations within 100 μm of the surface. Those β particles originating at distances greater than 150 μm are barely detected. This means that a glass scintillator of diameter 150 μm if centrally placed within axons of diameters greater than 450 μm should be unable to detect β disintegrations occurring outside the axon in the external saline (the axons used in the experiments described had diameters of 660–935 μm).

The assumption that E has the same value in saline as in the axon implies that the glass scintillator's range of detection in axoplasm is the same as that in ASW and that there is no absorption of the scintillator's light output by the axon. One of the main factors governing the detection range is the penetration of the β particles in the medium surrounding the glass scintillator: this in turn depends on the density of the medium and the energy of the β particle. Since the density of axoplasm is approximately 1·0 g.cm^{-3} (Keynes & Lewis, 1951) it is unlikely that the glass scintillator's range of detection in axoplasm will differ from that in ASW. In water or muscle, [^{14}C]β particles with maximum energy ($\epsilon_{max.}$ = 0·156 MeV) can penetrate about 300 μm. However, the majority of [^{14}C]β particles are less energetic: particles with average energy (ϵ_{av} = 0·05 MeV) can penetrate only 34 μm (American Institute of Physics Handbook, editor D. W. Gray, 1972). It may be coincidence but this figure agrees well with the experimentally found distance within which 50 % of the detected scintillations arise. No quantitative relation between the detection range and the β particle penetration distance has been attempted. The amount of absorption of the light output of the glass scintillator was not measured. Experiments with giant barnacle muscle fibre (C. C. Ashley & T. J. Lea, unpublished) indicate that in this case it is less than 10 % and it is probably also less than 10 % for the squid giant axons.

The measurement of influx was restricted to a length of 1–2 cm in the middle of a 4 cm length of axon by coating the ends of the scintillator fibre with shellac. About 50 μm of shellac were found to be sufficient to almost block out the light emission of the scintillation fibre. The glass counting vial was marked with black tape, leaving a window at the level of the non-coated

scintillator. Solution changes could be made by removing the vial from the counting well and emptying the saline vessel using a pipette via the outlet tube. Care was necessary in this operation to ensure that the position of the scintillator in the axon with respect to the photomultiplier tubes of the spectrometer was not altered.

This was facilitated by fixing the scintillator to the cannula with wax, and having the cannula in a rigid Perspex holder in the glass vial.

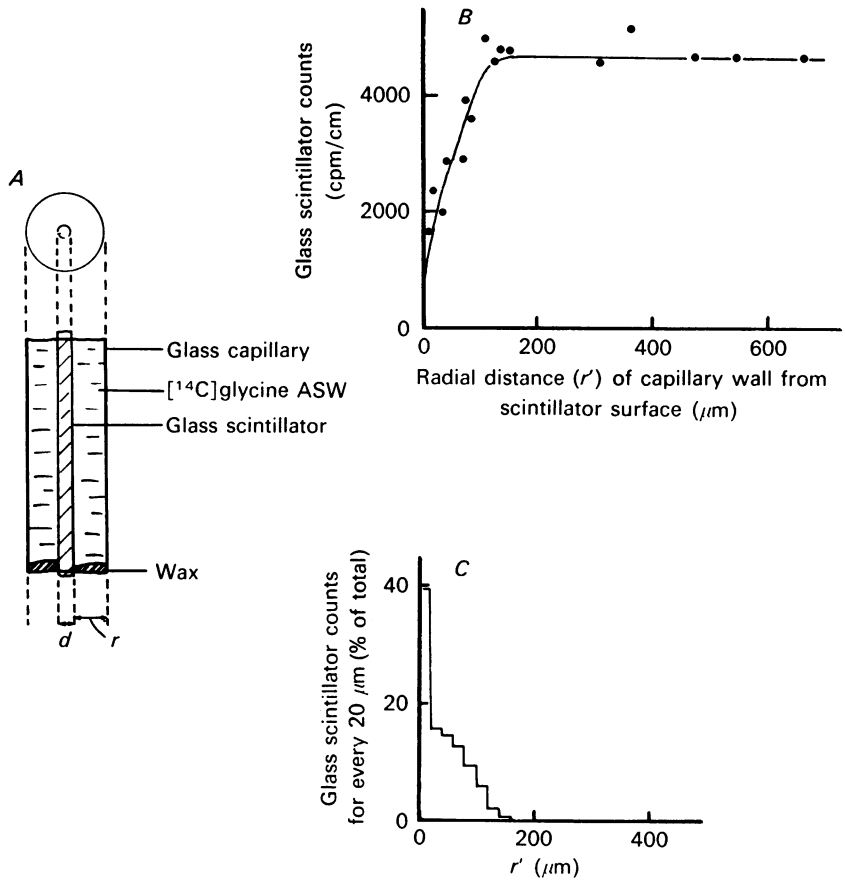

Fig. 2. Investigation of the range of detection of ^{14}C activity by the glass scintillator. *A*, a 1 cm length of glass scintillator (diameter 160 μm) was enclosed in glass capillaries containing [^{14}C]glycine ASW. *B*, the scintillation output (cpm per cm length) of the glass scintillator as a function of the mean radial distance (r^1) of the inside wall of the capillary from the scintillator's surface. *C*, the differentiated form of the curve in *B* showing the individual contributions (%) to the total scintillation output for concentric annuli of saline (20 μm width) surrounding the glass scintillator in a capillary of diameter 1450 μm.

Efflux measurement

A cleaned squid axon of 6 cm length was cannulated at one end and about 1 μl. ~ 1 mM-[^{14}C]glycine (0·1 μC; specific activity = 114 mC/m-mole) was introduced into the axon by means of a fine glass capillary attached to a Hamilton microsyringe (Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969). The injected length was 1 cm in the middle of the axon. Efflux measurements were made by transferring the axon with both ends tied off, to a small glass tube (6 cm long × 0·3 cm inner diameter) which was attached to a disposable syringe and

which contained about 0·2 ml. saline. The saline could be replaced at regular intervals and the effluxing radioactivity was counted by liquid scintillation. At the end of the experiment the whole axon was dissolved in Nuclear Chicage Solubilizer and its radioactivity counted. The composition of the mixture used for liquid scintillation counting is given in Table 1.

Identification of the effluxing ^{14}C material

Axons were injected as before and immersed in 0·05–0·1 ml. saline for 4–5 hr. After checking that the axons were excitable the axoplasm was extruded. Axoplasm and saline samples were stored at −20 °C in 5% ethanol for analysis. High-voltage paper electrophoresis was used to separate the individual amino acids, using 2% formic acid/8% acetic acid buffer (pH 2·1) with the following conditions: 5 kV; 30 min; 40–100 mA; Whatman 20 paper. This produced a good separation of glycine from the other amino acids. The paper was stained with 0·25% ninhydrin/2% 2,4,6-collidine in absolute ethanol. A Berthold scanner with a 2π detector was used to localize ^{14}C activity when it was high enough. Active parts were then cut out in $1 \times \frac{1}{2}$ in. strips, soaked in 0·2 ml. distilled water and counted in 10 ml. cocktail by liquid scintillation. Separation of amino acids in the saline sample was impaired by the salt; some of the samples were desalted by passing them through a 15 × 0·5 cm column of Zeokarb 225 cation exchange resin and eluting the amino acids with 2 N-NH_3 solution (Smith, 1960). This also removed proteins, non-electrolytes, taurine and some arginine from the samples. Recoveries of glycine were measured by initially adding [^{3}H]glycine to the samples and counting both ^{3}H and ^{14}C in the desalted samples.

In order to test for $^{14}CO_2$ in the effluxing material aliquots of the saline in which the axons had been immersed were divided into two portions. The radioactivity in one portion was counted directly by liquid scintillation. To the other portion was added excess 1 N-HCl, and after an hour the sample was evaporated to dryness in a vacuum dessicator to remove dissolved CO_2. 0·2 ml. distilled water was added and the remaining radioactivity was counted by liquid scintillation and expressed as a percentage of the counts in the control portion. Aliquots of axoplasm elutant were treated in a similar way.

Radioactive isotopes

[U-^{14}C]amino acids were obtained from the Radiochemical Centre, Amersham, Bucks. Specific activities of the amino acids mainly used were: glycine, 114 mc/m-mole; glutamate, 265 mc/m-mole; arginine, 318 mc/m-mole; alanine, 159 mc/m-mole; aspartate, 224 mc/m-mole.

Temperature

The temperature during the experiments was 17–22 °C.

RESULTS

Influx values for some representative amino acids

Table 2 gives the results of a survey of the influxes into squid axons of some representative amino acids obtained by the extrusion method. The axons were all bathed in artificial sea water. The amino acid concentrations were all 1 mM since Deffner (1961) found that the concentrations of most of the amino acids in squid blood are in the range of 0·1–1·0 mM. It will be seen that glycine, alanine, leucine, serine and proline have relatively high influx values, phenylalanine and tyrosine have intermediate values while glutamate, aspartate and arginine have low values.

A check was made that the uptake of ^{14}C label by an axon immersed in [^{14}C]glycine ASW was in fact a measure of the uptake of glycine and not of one of its metabolites. Axoplasm was extruded from a cleaned axon which had been immersed in [^{14}C]-glycine ASW for several hours. This was dispersed in water, samples of which were then analysed by high voltage paper electrophoresis. The glycine spot was found to contain 95% of the total ^{14}C activity.

TABLE 2. Influx of some amino acids into squid axons

Amino acid (1 mM)	Influx (f-mole cm^{-2} sec^{-1})	
Glycine	498 ± 38	(27)
Alanine	437 ± 117	(5)
Leucine	509 ± 81	(5)
Serine	383 ± 34	(5)
Proline	367 ± 69	(5)
Phenylalanine	147 ± 35	(3)
Tyrosine	128 ± 14	(5)
Glutamate	44 ± 4	(10)
Aspartate	24 ± 3	(8)
Arginine	21 ± 2	(6)

Axons were bathed in artificial sea water containing 1 mM of the ^{14}C-labelled L-amino acid. Uptake was measured by extrusion of the axoplasm at the end of the soaking period. ± S.E. of mean shown with number of axons in brackets.

Comparison of the two methods for the measurement of amino acid influxes

Table 3 shows that influx values calculated for glycine and L-glutamate with an internal scintillator are in good agreement with those obtained by measurement of the radioactivity in the extruded axoplasm.

The counting rates from axons with an internal scintillator, immediately after immersion in [^{14}C]glycine sea water were on average higher than the background counting rate, obtained with the counting vial but without an axon and scintillator. Initial counts due to the axon and scintillator at $t = 0$ min were 20–69 cpm (mean 30 cpm) compared with the background rate of 20 cpm. It seems likely therefore that in some experiments the internal scintillator was detecting some ^{14}C activity outside the axon. This probably arose from non-central positioning of the scintillator in the axon and the fact that squid axons are not uniformly cylindrical (see Methods). The extra initial counts were low in comparison with the counts recorded after 1–2 hr immersion of the axon in [^{14}C]amino acid sea water and represented only 0–4·1% (mean 1·8%) of the counts which the scintillator gave when directly in contact with the labelled sea water. Since the over-all count rate due to the scintillator in the axon increased linearly with time when in radioactive sea water and influx values calculated from the rate of increase are in good agreement with values from the extruded axoplasm method, it appears that the size of this extra component in the counts stays constant with time. For some purposes it can be neglected. If necessary the background including this component can be obtained by extrapolation of the initial part of the uptake curve back to zero time.

The presence of the glass scintillator fibre in the axon had no effect on the excitability of the axon. The efflux of [^{14}C]glycine was investigated with the internal scintillator by replacement of the radioactive saline with tracer free artificial seawater after a 3 hr influx experiment. The count rate showed no significant changes over an hour (Fig. 3) and although it was impossible to estimate the efflux rate constant accurately it did not seem to be greater than 10^{-5} min^{-1}, the figure obtained in other experiments by the injection of [^{14}C]glycine and the measurement of the rate of ^{14}C appearance in the saline.

The effect of external glycine concentrations on the glycine influx

Figs. 4*A* and 5 show how the size of the glycine influx varies with extracellular glycine concentration. The results in Fig. 4 were obtained using the pair axon extrusion method and required twenty-seven pairs of axons. The results shown in Fig. 5 came from a single axon using the internal scintillator method. It can be concluded from both influx–concentration curves that the size of the glycine influx tends to saturate at high glycine concentrations. This would indicate that most of

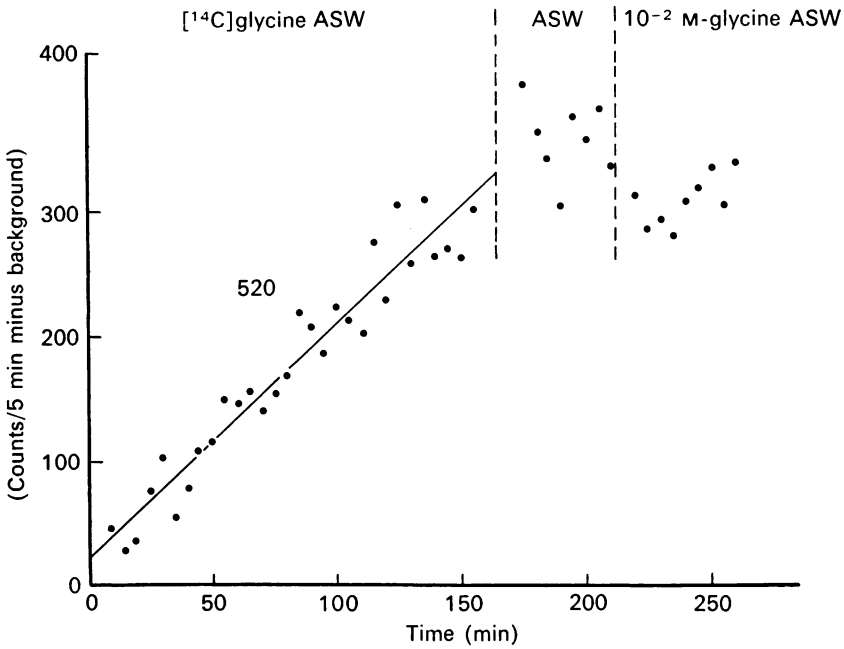

Fig. 3. A typical curve for the continuous measurement by a glass scintillator of the influx of [^{14}C]glycine into an axon in ASW. Glass scintillator output (counts per 5 min minus background) is plotted against time of incubation in [^{14}C]glycine. External glycine concentration 1 mM. Influx rate for glycine (f-mole cm^{-2} sec^{-1}) is shown alongside the curve, as it is in subsequent Figures. In ASW and 10 mM-glycine ASW ([^{14}C]-glycine-free), the output showed no significant changes with time. The reduction in output observed on transfer to 10 mM-glycine is probably due to a change in the scintillator's position. Axon diameter = 850 μm.

the glycine which enters the axon does so by a carrier mechanism rather than by passive diffusion. Other evidence described later shows that diffusion probably comprises only a very small fraction of the total influx. The influx at an extracellular glycine concentration of 1 mM in the intracellular scintillator experiment on a single axon (Fig. 5*B*) was about half the mean value found in the pair axon experiments (Fig. 4*A*). The influx in Fig. 5*B* was, however, within the range of values normally encountered when glycine influxes at this extracellular concentration are measured by entrusion of the axoplasm.

A plot of the reciprocal of glycine influx against the reciprocal of glycine concentration does not give a very good straight line (Fig. 4*B*). Nor does a plot using the

reciprocal of the square of glycine concentration. The curve in Fig. 4*B* can be approximated to two straight lines, which suggests that the influx consists of two saturable components. One possible model for the total influx J_T therefore consists of two components J_1 and J_2 each of which can be described by Michaelis-Menten kinetics, with different apparent affinity constants. The total influx is then given by the relation

$$J_T = J_1 + J_2 = \frac{J_{(max)_1}(gly)_o}{K_1 + (gly)_o} + \frac{J_{(max)_2}(gly)_o}{K_2 + (gly)_o}, \quad (5)$$

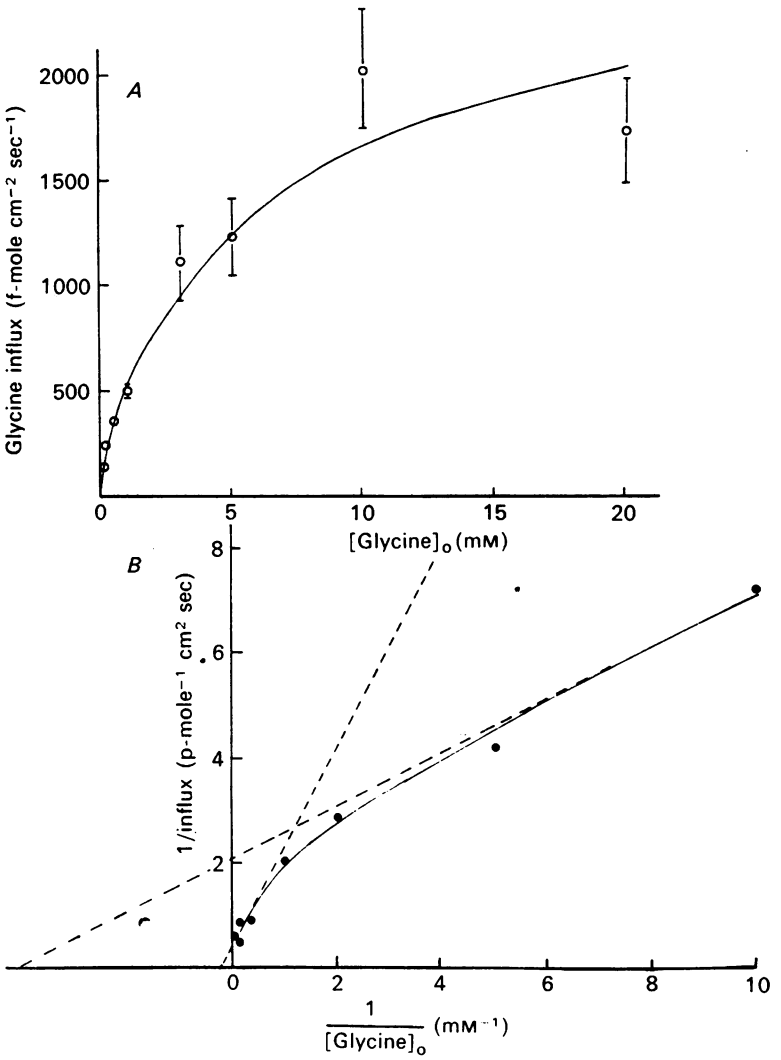

Fig. 4. *A*, glycine influx, measured by the pair axon method, plotted against external glycine concentration in ASW. Each point is the mean from three or four axons: vertical lines are ± S.E. of mean. Axon diameter 470–813 μm. Curve drawn according to eqn. (5) (see text). *B*, a double-reciprocal plot of the data in Fig. 4*A*. The curve is drawn according to eqn. (5) with the same constants as in 4*A*. The dashed lines indicate, only approximately, the straight lines predicted for the two individual components of the influx, J_1 and J_2.

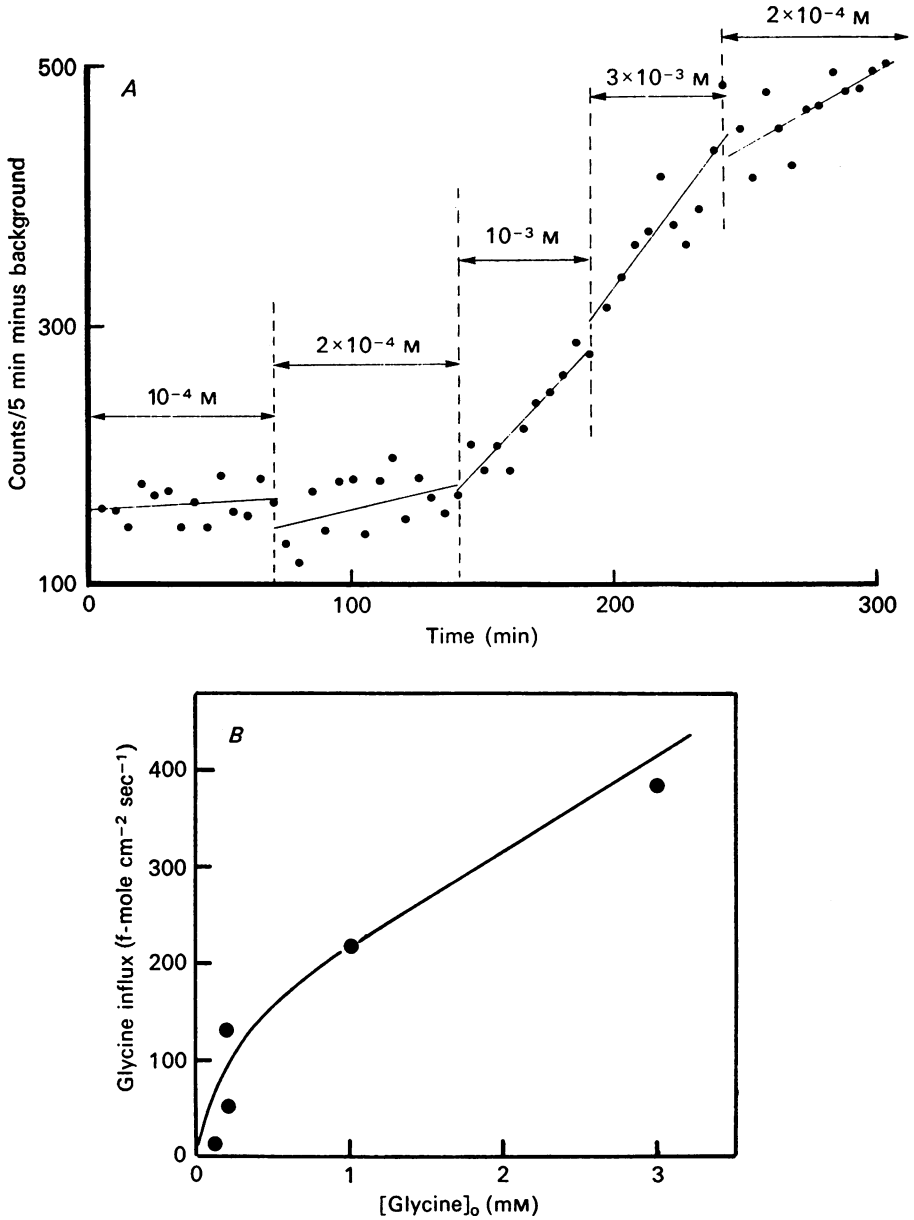

Fig. 5. *A*, effect of external glycine concentration on glycine influx of a single axon, diameter 865 μm, measured by a glass scintillator. The axon was immersed in a series of sea waters containing different concentrations of glycine labelled with [^{14}C]glycine at a specific activity of 10 μC μM^{-1}. Scintillator output (counts/5 min minus background) plotted against time. Straight lines calculated by linear regression. *B*, glycine influxes, calculated from the lines in Fig. 5*A*, plotted against external glycine concentration. Curve drawn according to eqn. (5) with $J_{(max)_1}$ = 125 f-mole cm^{-2} sec^{-1}, $J_{(max)_2}$ = 1003, K_1 and K_2 as in Fig. 4.

where $[gly]_o$ is the external glycine concentration, K_1 and K_2 are the two half-saturation constants, and $J_{(max)_1}$ and $J_{(max)_2}$ are the two maximum rates of influx. The curves in Fig. 4 were calculated from this equation with the following values for the constants: $K_1 = 0{\cdot}16$ mM, $K_2 = 7{\cdot}4$ mM, $J_{(max)_1} = 283$ f-mole $cm^{-2}\,sec^{-1}$, $J_{(max)_2} = 2380$ f-mole $cm^{-2}\,sec^{-1}$. They give a better fit to the experimental points than do curves calculated on the assumption that the influx consists of only one

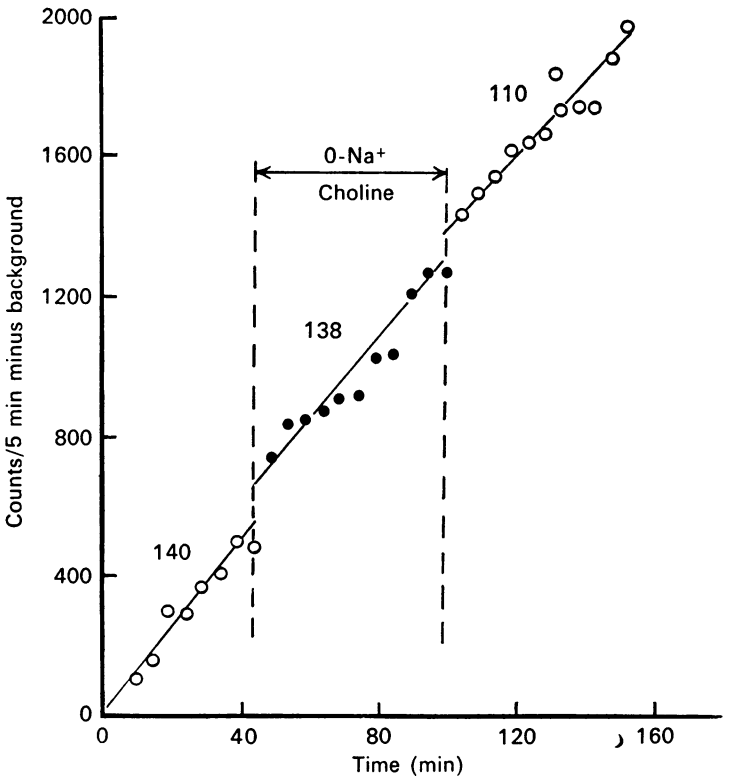

Fig. 6. Effect of replacing external Na by choline on the influx of glycine for an axon (diameter 595 μm) in 0·2 mM-glycine ASW. Influxes, calculated from slopes of straight lines through points, are shown. Solution changes (at vertical dashed lines) were followed by a period of 30 min washing in the appropriate saline, from which [^{14}C]-glycine was omitted. Time scale is for time in [^{14}C]glycine only.

saturable process describable by Michaelis–Menten kinetics. The methods used for the separation of the influx into two components were those of Neame & Richards (1972). Agreement with the experimental data was less good at concentrations above 3 mM than at lower concentrations. The scatter between values for the influxes at the higher concentrations was such that differences between mean influx values at 3 and 5 mM, and at 10 and 20 mM were not significant at $P < 0{\cdot}05$ (Student's t test). According to the two component model, 46·3% of the total glycine influx at 10^{-3} M-glycine is the high affinity component J_1 and 53·7% is the low affinity component J_2.

The effects of the removal of external Na on the glycine influx

Choline was used to replace Na completely in the artificial sea-water and the effects on glycine influx are shown in Fig. 6 and summarized in Table 4. The results of pair axon and internal scintillator experiments show that at 1 mM-glycine there normally appears to be no obviously identifiable Na-sensitive component of the

TABLE 3. Amino acid influxes measured with the internal scintillator and after extrusion of the axoplasm

Amino acid	Influx (f-mole cm^{-2} sec^{-1})	
	Extrusion of axoplasm	Internal scintillator
Glycine (1 mM, various salines)	574 ± 121 (10)	498 ± 98 (10)
Glycine (1 mM, ASW)	498 ± 38 (27)	547 ± 98 (10)
Glutamate (0·1 mM, ASW)	23* ± 3 (10)	18 ± 3 (7)

± S.E. of the mean is given with number of axons in brackets. The first set of influx values for glycine in various salines was obtained from a single group of axons, the influx being first measured for each axon with an internal scintillator and then measured a second time by determination of the radioactivity in the extruded axoplasm. The other influx values are for independent groups of axons.

* From Baker & Potashner (1973).

TABLE 4. The effects of Na removal on glycine influx

Glycine concentration	Influx method	Na^+ substitute	Influx (f-mole cm^{-2} sec^{-1})		
			Normal Na^+	Na^+-free	Na^+-free/Na^+
1 mM	PA	Choline	596 ± 79	594 ± 89	1.00 ± 0.05 (5)
1 mM	PA*	Choline	931 ± 93	536 ± 83	0·57 ± 0·05 (4)
1 mM	IS	Choline	816 ± 244	908 ± 464	0·99 ± 0·37 (3)
0.2 mM	IS	Choline	115 ± 31	128 ± 34	1·12 ± 0·02 (3)
5 mM	IS	Choline	561	498	0·98
1 mM	PA	Li^+	657 ± 133	448 ± 89	0·70 ± 0·09† (5)

PA, pair axon; IS, internal scintillator. ± S.E. of mean shown, with number of axons in brackets. In pair axon experiments the test axon was agitated in Na-free sea water for 30 min before immersion in [^{14}C]glycine, Na-free sea water for 40 min. In internal scintillator experiments, the axon was washed with six successive changes of Na-free sea water over 30 min before exposure to [^{14}C]glycine, Na-free sea water.

* Axon pairs were pretreated by 3 hr immersion in ASW or 1 mM-glycine ASW.

† This ratio is significantly less than 1·0 ($P < 0·02$).

influx. This was also true for 0·2 mM-glycine, and probably 5 mM-glycine although only one experiment was tried at this concentration. It was thought possible that the failure to detect an obvious Na-sensitive component was the result of poor equilibration of the axon extracellular space with the choline sea-water. This was tested by looking at the effect of Na replacement on glutamate influx since with 0·1 mM-glutamate outside, about 90% of the glutamate influx is known to be Na-sensitive (Baker & Potashner, 1973). Fig. 7 shows that when the sodium in artificial sea water was replaced by choline the glutamate influx was markedly reduced. A further test to show that replacement of Na by choline depresses sodium sensitive

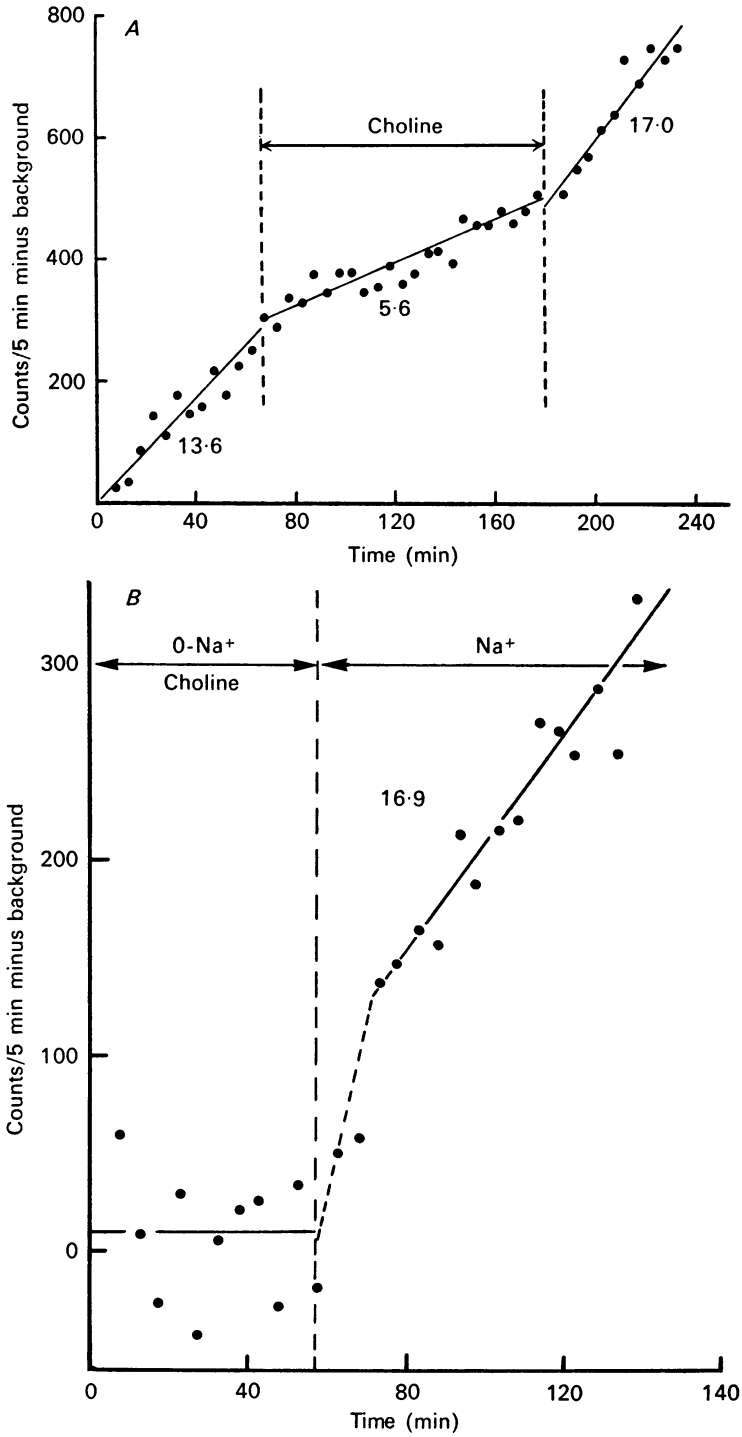

Fig. 7. The effects of replacing external Na^+ by choline on the influx of L-glutamate for two axons of diameters *A*, 700 μm and *B*, 763 μm, bathed in 0·1 mM-glutamate ASW. Other experimental details as in Fig. 6. The relatively high rate in 0 Na in *A* may have been due to deterioration of the axon.

fluxes was carried out on the uptake of ^{32}P-labelled orthophosphate. The pair axon method was used as in a previous study (Caldwell & Lowe, 1970). The results, given in Table 5, show that the orthophosphate uptake is greatly reduced when Na^+ is replaced by choline or Li^+, and that in this orthophosphate uptake in squid axons is similar to that in rabbit vagus nerve (Anner, Ferrero, Jirounek & Straub, 1975). It can be concluded therefore that the method used to replace Na by choline is satisfactory, and that in fact under normal conditions in artificial sea water there is no Na-sensitive glycine influx.

TABLE 5. The effect of Na removal on ^{32}P-labelled orthophosphate influx

	Orthophosphate influx (f-mole cm^{-2} sec^{-1})	
Normal Na^+	Na-free; choline chloride	Na^+-free/Na
2·8	0·16	0·06
2·2	0·38	0·17
7·3	0·27	0·04
12·0	1·1	0·09
10·0	1·4	0·14
	Na.-free; LiCl	
9·6	1·4	0·15
5·7	0·7	0·12
Mean ± S.E. 7·1 ± 1·4	0·77 ± 0·2	0·11 ± 0·02

The pair axon method was used throughout. Extracellular orthophosphate concentration was 0·1 mM.

Two other results helped to complicate this picture, however. It was found that immersion of the uncleaned axons in ASW or 1 mM-glycine ASW for 3 hr at room temperature before the measurement of influx by the pair axon method caused an increase in the glycine influx of about 50% and the appearance of a Na-sensitive component which was approximately the same size as the increase in influx (row 2, Table 4). This result was confirmed by dividing a pair of axons into two pairs and then measuring the glycine influx of one pair immediately and that of the other pair after 3 hr. A possible explanation for this result is that a Na-sensitive component of the glycine uptake exists but that it only becomes activated after the axon has been exposed for a prolonged period to media, such as artificial sea-water, which lack the amino acids present *in vivo*.

The second interesting result was that if Na^+ were replaced by Li^+ instead of choline, the glycine influx at 1 mM-glycine decreased to about 70% (row 6, Table 4). In view of the apparent absence of a Na-sensitive component under normal conditions, it is tentatively concluded that Li may have a direct inhibitory effect on a component of glycine influx.

The effect of cyanide on glycine influx

The metabolic inhibitor CN is well known as an inhibitor of active transport processes. Active Na extrusion in squid axons is inhibited by 2 mM-CN after a delay of more than 30 min (Hodgkin & Keynes, 1955). During this time the concentrations of ATP and arginine phosphate in the axoplasm fall to less than 10% of their

normal values (Caldwell, 1960). When 2 mM-CN was tested on glycine influx followed with an intracellular scintillator there was in three experiments a similar inhibition after 40–90 min delay (Table 6, Fig. 8). These observations are consistent with the CN inhibition of a component of glycine uptake which derives its energy from ATP break-down. In one experiment (Fig. 8), CN sea-water was replaced by ASW after

TABLE 6. The effect of CN on glycine influx in artificial sea water measured with an intracellular scintillator

	Influx (f-mole cm^{-2} sec^{-1})			
	Initial rate in CN^-	CN^- rate after 100 min	In ASW after CN^-	CN^- inhibition (%)
	163	80*	—	51*
	1123	308	—	72
	197	81	305	59
Mean ± S.E.	494 ± 314	156 ± 76		

* Later inexcitable.
Glycine concentration was 1 mM; CN^- was 2 mM, added to ASW as either NaCN or KCN.

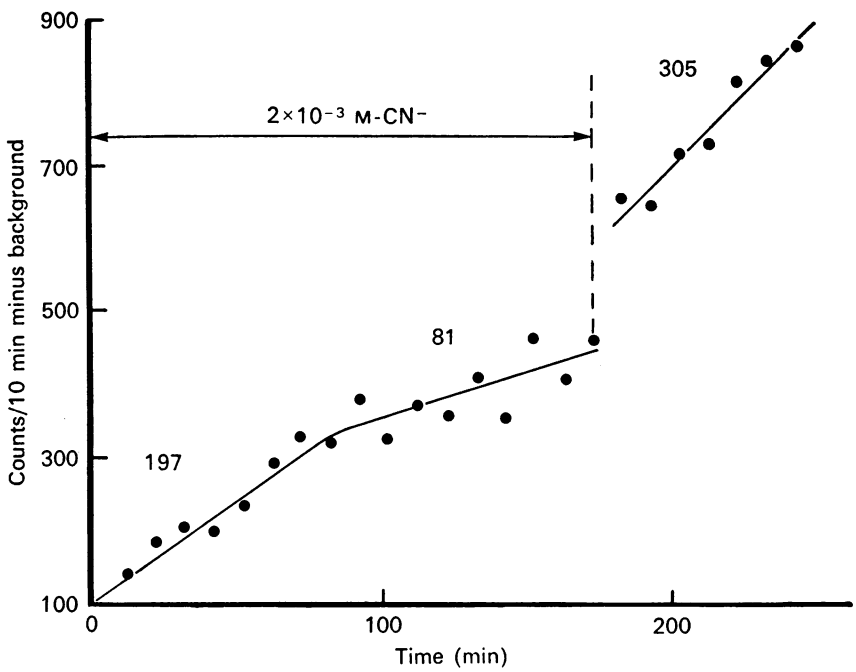

Fig. 8. The effect of 2 mM-CN on glycine influx of an axon (diameter 698 μm) in 1 mM-glycine ASW. Influx values on Figure. At vertical dashed line axon was washed with several changes of CN-free, [^{14}C]glycine-free, ASW for 15 min. It was then returned to CN-free ASW containing [^{14}C]glycine.

CN inhibition had occurred, with the result that the glycine influx rapidly recovered to a value greater than its previous initial value in CN ASW. This single result is, however, not cousidered conclusive evidence for a recovery from CN inhibition. The glycine influxes of two further axons were insensitive to CN; these axons were

dissected from squid caught in January, when the squid are spawning and dying (Holme, 1974). It is possible that if there is a glycine uptake system which is dependent on cellular metabolic energy then the activity of this system declines rapidly towards the end of the life cycle of the squid. Some evidence was also obtained that the ouabain sensitive component of glycine uptake is sometimes absent at this stage.

Glycine efflux and its dependence on the external glycine concentration

The rate of glycine efflux is expressed as a rate constant, given by the number of counts appearing in the outside solution per minute divided by the total number of counts in the axon. In ASW the rate constant showed an initial fall immediately

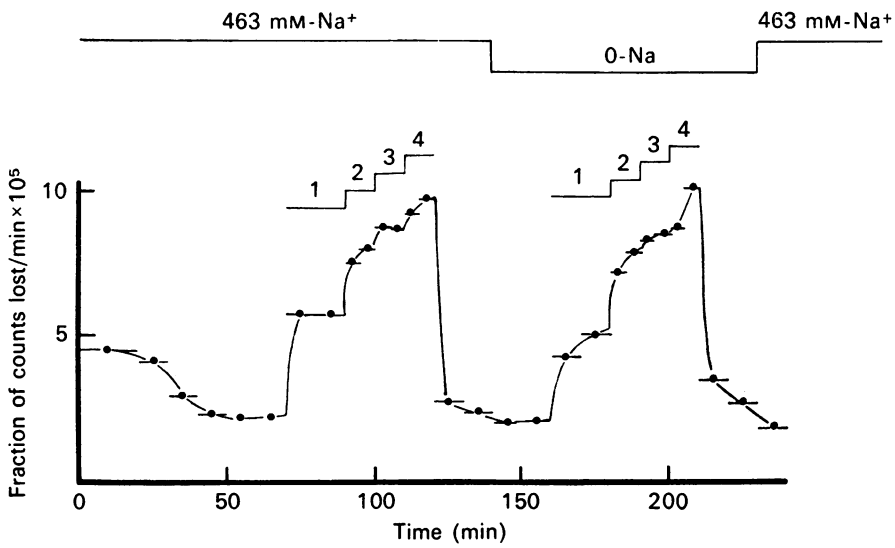

Fig. 9. The effects of different external glycine concentrations, on the glycine efflux in ASW and 0-Na^+ (choline) saline. An axon, diameter 800 μm, was injected with [^{14}C]glycine at t = 0 min. Efflux of ^{14}C-label is plotted as a rate constant (fractional loss of counts per min.). Values for external glycine concn. were 1: 0·5 mM, 2: 1 mM, 3: 5 mM, 4: 10 mM.

after injection into the axon of [^{14}C]glycine and reached a steady value of 0·3–2·7 $\times 10^{-5}$ min^{-1} (mean 1·1 $\times 10^{-5}$ min^{-1}) after about 1 hr (Fig. 9). Addition of glycine to the outside saline caused an immediate increase in the rate constant 1 mM-glycine increasing it by 3–13 times. This effect was reversible (e.g. Fig. 11). With some axons a new steady value in the rate constant was attained immediately after exposure to glycine (e.g. Fig. 9); with others the initial rate in glycine declined to a lower steady value after some time (e.g. Figs. 11, 12). It is probable that the glycine efflux in the absence of external glycine represents a passive loss of glycine from the axon while the greatly increased glycine efflux in the presence of external glycine represents the outward component of a carrier mediated exchange of the exchange diffusion type (Ussing, 1947).

Absolute values for the glycine efflux can be calculated using values for the rate constants and the concentration of free glycine in the axoplasm. This latter value

has not been measured for *Loligo forbesi*, but it has for the related *Loligo pealii*, which is caught off the coast of New England. Deffner (1961) dialysed axoplasm and obtained a figure of 11·6 mM for the concentration of glycine. Rosenberg & Khairallah (1974) found a lower value of 5·0 mM, and suggested that the higher values of Deffner for glycine and other amino acids were due to proteolytic activity in the stored axoplasm. For the purposes of this paper, a mean value of 8·3 mM for axoplasmic glycine will be used. Table 7 shows the values for glycine efflux.

When the external glycine concentration was progressively increased, the glycine efflux rate constant tended to a maximum (Fig. 9). The mean results for three axons

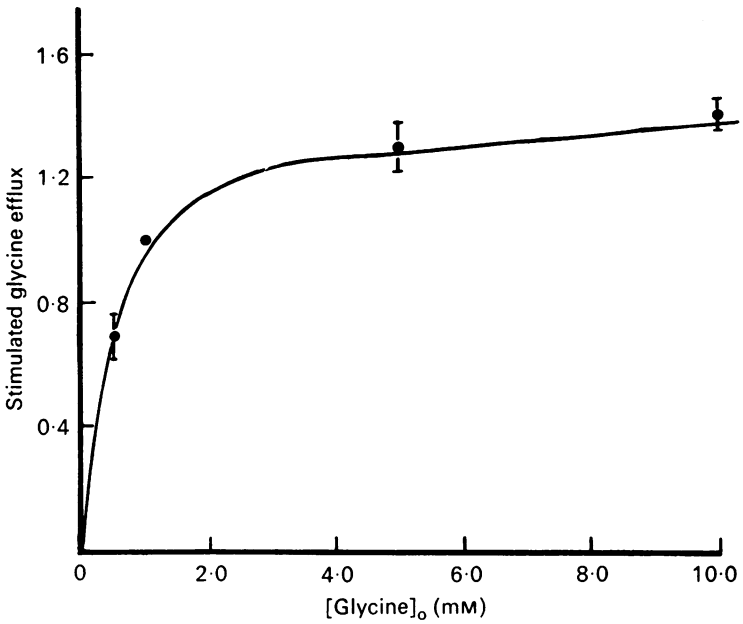

Fig. 10. Glycine stimulated efflux (efflux in glycine ASW minus efflux in ASW) for three axons (diameters 775–815 μm) injected with [^{14}C]glycine, plotted against external glycine concentration. Points are means of the stimulated efflux rate constant ($\pm$ S.E.) expressed as a fraction of that in 1 mM-glycine. The curve is drawn according to the equation

$$J = \frac{J_{(max.)}[gly]_o}{[gly]_o + K},$$

where J is the stimulated efflux, $J_{(max.)}$ is its maximum rate = 1·46, $[gly]_o$ is glycine concentration and K is the half-saturation constant = 0.51 mM.

TABLE 7. Calculation of the rates of glycine efflux

Saline	Rate constant (min^{-1})	fraction of effluxing ^{14}C, identified as glycine	efflux rate (f-mole cm^{-2} sec^{-1})
ASW	$1{\cdot}10 \pm 0{\cdot}20 \times 10^{-5}$	0·87	26·2 ± 4·8
1 mM-glycine ASW	$7{\cdot}1 \pm 1{\cdot}01 \times 10^{-3}$	0·85	160·8 ± 22·1

Values are means from thirteen or fourteen axons. ± S.E. of means.

Efflux rate was calculated for a mean axon diameter of 784 μm and an intracellular glycine concentration of 8·3 mM.

are plotted in Fig. 10; the rate constant in ASW alone was subtracted from the rate constant in glycine ASW to give the size of the stimulated component. A plot of the reciprocal of the stimulated component against the reciprocal of the glycine concentration gave a straight line, with Michaelis–Menten parameters of K_m (half-saturation constant) = 0·51 mM and J_{max} (maximum rate constant) = 1·46 × the rate constant at 1 mM-glycine outside.

Replacement of the Na in the saline by choline had little effect on the glycine efflux in either ASW or in glycine ASW (Fig. 9).

Effects of other amino acids on glycine efflux

In addition to glycine, certain other neutral amino acids also stimulated the glycine efflux to different degrees when applied externally at 1 mM, but proline and taurine, the acidic amino acids L-glutamate and L-aspartate, the basic amino acid arginine and the peptides glycylglycine and triglycine had negligible effects (Table 8 and Fig. 11). The proportion of [^{14}C]glycine in the effluxing radioactivity from axons in ASW, containing alanine, serine or leucine was the same as for axons in ASW or glycine ASW.

TABLE 8. Stimulation of glycine efflux by other amino acids

Amino acid	Stimulated efflux as % of glycine-stimulated efflux
Glycine	100·0
L-cysteine	96·6
L-α-alanine	74·4
L-serine	65·8
L-phenylalanine	52·1
L-leucine	42·0
L-isoleucine	40·7
L-tyrosine	37·3
L-threonine	31·2
L-valine	23·3
L-proline	5·4
Taurine	4·0
Glycylglycine	2·4
Glycylglycylglycine	0·2
L-glutamic acid	2·7
L-aspartic acid	5·4
L-arginine	0

The stimulated efflux is the difference between the rate constant in ASW and that in ASW containing 1 mM-amino acid. The values are single observations from six axons; each axon was tested with glycine first.

Arginine, glycylglycine and glycylglycylglycine were tested to see if they inhibited the glycine-stimulated efflux. However, at concentrations of 5 mM they showed no inhibition of the stimulated glycine efflux due to 1 mM-glycine.

The nature of the stimulated efflux and its sensitivity to CN and ouabain

It has already been shown that a component of the glycine influx is sensitive to cyanide (Table 6, Fig. 8). A component of the influx is also sensitive to ouabain (Caldwell & Lea, 1975). The sensitivity of the glycine-stimulated efflux to these

inhibitors was therefore investigated. Fig. 12 shows that the glycine-stimulated glycine efflux is unaffected by 0·1 mM-ouabain and Fig. 13 shows that it is unaffected by 2 mM-CN. An attractive explanation of these observations therefore is that the glycine-stimulated efflux is associated with a cyanide and ouabain-insensitive exchange diffusion process (Ussing, 1947), the inward component being the CN-insensitive, ouabain-insensitive component of the glycine influx. The fact that certain other amino acids can stimulate the glycine efflux suggests that this mechanism

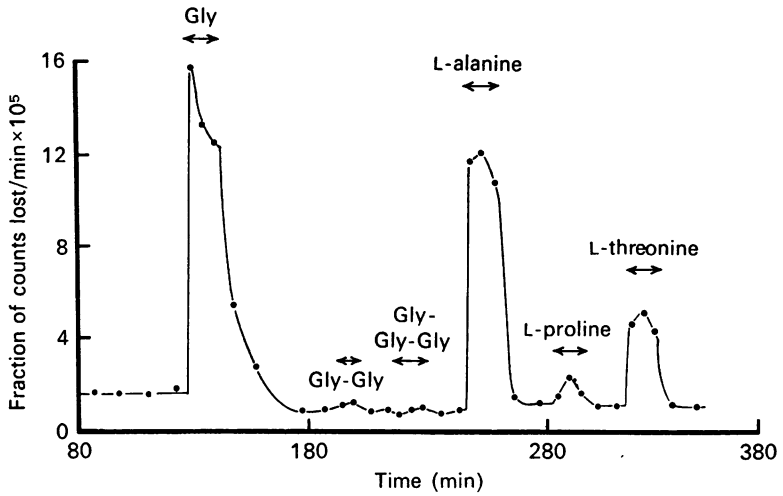

Fig. 11. The effects of some neutral amino acids and peptides (at 1 mM in ASW) on the efflux of ^{14}C label from an axon (diameter 768 μm) injected with [^{14}C]glycine at $t = 0$ min.

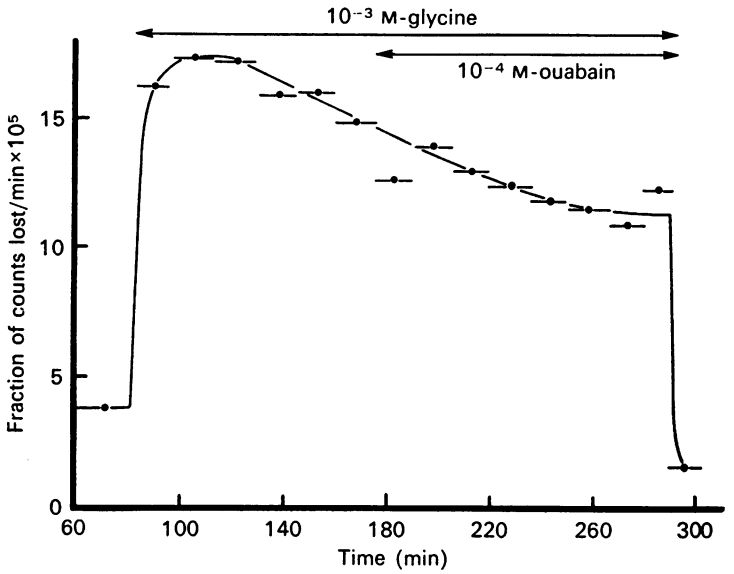

Fig. 12. The efflux of ^{14}C label from an axon injected with [^{14}C]glycine and then exposed to 1 mM-glycine ASW with and without 0·1 mM-ouabain. Axon diameter 735 μm.

may not be specific for glycine but that it is a general mechanism by means of which a group of amino acids can exchange either with themselves or with each other on a one to one basis.

Evaluation of the methods used for the study of glycine efflux

Axons injected with [^{14}C]glycine were immersed in sea water containing glycine or another amino acid for 5–6 hr, and then the sea water and extruded axoplasm were analysed by high voltage paper electrophoresis. Table 9 and Fig. 14 show the results. Three peaks of radioactivity were usually recognized, a large glycine fraction, a neutral fraction which did not migrate from the origin (NF) and a fraction X

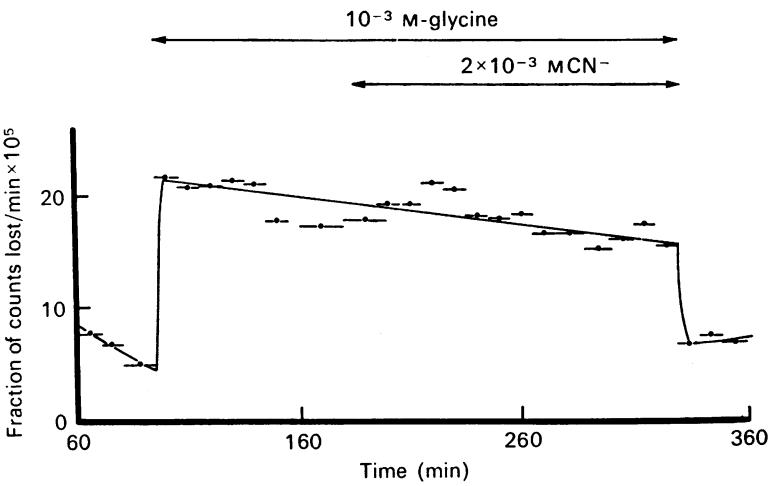

Fig. 13. Efflux of ^{14}C label from an axon injected with [^{14}C]glycine and then exposed to 1 mM-glycine ASW with and without 2 mM-CN$^-$. Axon diameter, 503 μm.

which migrated more slowly than aspartic acid. The counts in each fraction are expressed as a percentage of the total counts in an aliquot of the sample put on the paper after electrophoresis. For seven axons, immersed in glycine, alanine, leucine and serine-containing sea waters, 61·2–83·4% (mean 74·1) of the effluxing counts were in the glycine fraction on the ninhydrin-treated paper. The percentage of counts recoverable as glycine in a sample untreated with ninhydrin will be greater than this value, since reaction of [^{14}C]glycine with ninhydrin caused a loss of up to half the activity of glycine whereas the substances in fractions X and NF were apparently ninhydrin-negative and suffered no loss of radioactivity during ninhydrin staining. Taking this into consideration, a corrected value for the counts in the glycine fraction is 85·2%.

The extruded axoplasm was dispersed in water for 12 hr, and samples of the dispersate were then analysed. Of the counts in these samples, 89·2% were recoverable as glycine on the ninhydrin treated paper. If allowance is made for a halving of the counts from [^{14}C]glycine by ninhydrin the actual contribution of [^{14}C]glycine to the total axoplasm radioactivity will be 94·3%.

The identities of the substances in fractions X and NF are not known. Radioactive substances in NF may have been introduced into the axons as impurities of the

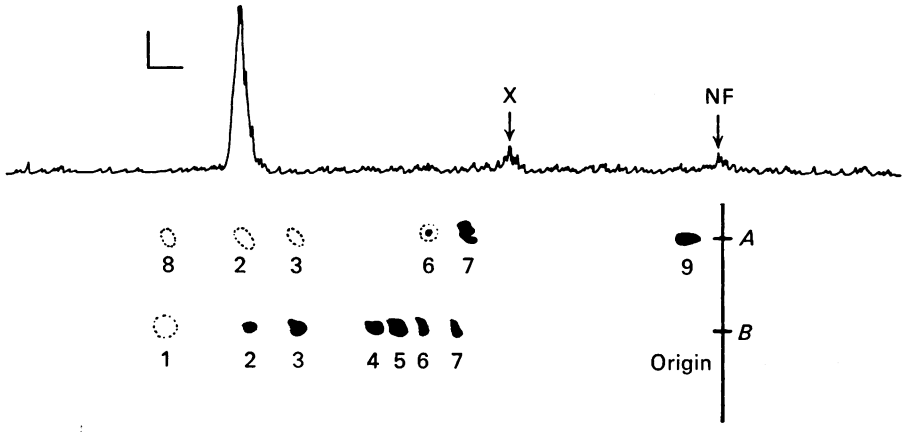

Fig. 14. Analysis, by high-voltage paper electrophoresis, of the ^{14}C-labelled compounds effluxing from an axon which was injected with [^{14}C]glycine and then soaked in saline for several hours. Effluent sample (*A*) and standard amino acid solution (*B*) were applied at the origin and electrophoresis was carried out at pH 2·1 using 5 kV for 30 min. The amino acids moved towards the cathode. Identifiable ninhydrin-positive spots are 1, histidine; 2, glycine; 3, alanine; 4, leucine; 5, threonine; 6, glutamate; 7, aspartate; 8, arginine and 9, taurine. Top trace shows ^{14}C activity of sample *A*, measured by a Berthold scanner. Calibrations: vertical, ^{14}C activity in arbitrary units; horizontal, 2 cm. Apart from the glycine peak, two small ^{14}C peaks, referred to as X and NF in the text can be recognized.

TABLE 9. Distribution of effluxing ^{14}C compounds in various salines after [^{14}C]glycine injection

Saline and amino acid (1 mM)	% of counts in various fractions of the effluxing ^{14}C			% of counts in various fractions of the axoplasmic ^{14}C			Counts after removal of acid labile $^{14}CO_2$ as a % of the counts before removal	
	Glycine	X	NF	Glycine	X	NF	Effluxing ^{14}C	Axoplasmic ^{14}C
Glycine ASW	61·2	—	—	74·8	—	—	—	—
Glycine ASW*	83·4	0·6	0·4	77·5	1·3	2·8	97·1	95·2
Alanine ASW	68·2	4·8	8·3	74·7	4·3	2·8	110·1	92·4
Alanine ASW*	82·0	1·8	0·8	105·6	5·6	6·9	84·3	104·2
Leucine ASW	73·4	8·0	5·2	91·3	3·8	2·2	91·4	96·7
Serine ASW	73·1	4·3	3·1	—	—	—	96·0	—
ASW	77·2	2·7	5·6	111·3	10·0	4·0	91·4	101·0

98·5 % of the counts in the [^{14}C]glycine used for the injections were found to be present as glycine, 1·0 % as X and 3·6 % as NF.

* The counts in the fractions of the effluxing ^{14}C are expressed here as a % of the counts in the efflux sample before passage through an ion exchange column. When the column was not used these counts are expressed as a % of the counts in a standard efflux sample which was applied to the paper after electrophoresis and before staining. All axons were excitable at the end of the efflux period. Calculated rates of ^{14}C efflux were in agreement with those in other experiments.

[^{14}C]glycine, which when analysed contained 3·6% of the total activity as NF. The proportion of the counts in the X fraction of the injected [^{14}C]glycine was significantly lower than those in the axoplasm and effluxing samples. Either [^{14}C]-glycine was metabolized to X in the axoplasm and this left the axon, or [^{14}C]glycine in the sea water and extruded axoplasm samples was broken down by bacterial or enzymic action during storage. Analysis of the effluent samples showed significant amounts of glutamate, aspartate and taurine (Fig. 14). These are the three most abundant amino acids in squid axoplasm (Deffner, 1961).

The possibility that ^{14}C was leaving the axon as $^{14}CO_2$ arising from metabolism of the glycine was tested by the addition of excess HCl to sea water and axoplasm samples and the removal of dissolved CO_2 by evaporation. Table 9 shows that there was virtually no $^{14}CO_2$ in either the effluxing material or the axoplasm.

The rate of incorporation of [^{14}C]glycine into axon proteins was not measured. However, Fischer & Litvak (1967) have measured the rate of incorporation of ^{14}C-labelled serine, valine and leucine into axon proteins of the squid *Dosidicus gigas*. They found that 100 min after injection of the [^{14}C]amino acids, 2·8–4·0% of the counts had been incorporated into a TCA insoluble fraction, most of which originated from the axon sheath.

It can be concluded that usually [U-^{14}C]glycine is not metabolized to any great extent during the course of a typical efflux experiment and that ^{14}C-labelled glycine gives a reliable estimate of glycine efflux.

DISCUSSION

The results which have been obtained seem to validate the use of the intracellular glass scintillator for the measurement of the influx of ^{14}C-labelled amino acids into squid giant axons. The close agreement between the influx rates obtained with the internal scintillator and those obtained by counting the activity of extruded axoplasm indicates that the distribution of [^{14}C]amino acids in the axoplasm is relatively uniform and that if appreciable amounts are taken up into surrounding Schwann cells or elsewhere then these are beyond the range of detection of the scintillator. The case for this is strengthened by the failure of the internal scintillator in most cases to detect much of the [^{14}C]amino acid activity in the saline outside the axon plasma membrane and by the calibration curve for the scintillator which shows that ^{14}C activity at a distance greater than 150 μm from the scintillator surface is virtually undetected. The relatively simple internal structure of the axon is probably responsible for the apparent lack of absorption of the scintillation light output of a glass scintillator inside an axon. The [^{14}C]amino acids which enter the axoplasm across the axolemma must diffuse easily and reach the area of detection of the scintillator within the counting period of 5 min.

A simple interpretation of the results obtained for glycine uptake by squid giant axons is that this involves two main mechanisms one of which is sensitive to CN and ouabain while the other is not. The former probably derives energy from ATP break-down. Both mechanisms appear to be insensitive to sodium removal but a third Na-sensitive component may appear under certain circumstances. The glycine efflux which is also insensitive to Na removal, CN and ouabain is probably involved

in a mechanism in which it and the CN-insensitive, ouabain-insensitive influx may form two of the components of a generalized ATP-independent amino acid exchange system of the exchange diffusion type. This latter possibility is made more likely by the fact that the CN-insensitive, ouabain-insensitive glycine fluxes are of similar size. When the external concentration for glycine is 1 mM the mean glycine efflux is 166 f-mole cm^{-2} sec^{-1}. The mean glycine influx at this external concentration is 498 f-mole cm^{-2} sec^{-1}. About 30% of this influx, equivalent to 149 f-mole cm^{-2} sec^{-1}, is ouabain-insensitive (P. C. Caldwell & T. J. Lea, unpublished) while the mean CN-insensitive influx at this concentration is 181 f-mole cm^{-2} sec^{-1}. Both these influxes are of similar size to the CN-insensitive, ouabain-insensitive glycine efflux. An exact 1:1 exchange of glycine for glycine by this exchange diffusion mechanism is not to be expected, however, since the stimulation of the glycine efflux by certain other amino acids indicates that they too can enter the axon in exchange for glycine by this mechanism. Table 8 shows that these amino acids include cysteine, alanine, serine, phenylalanine, leucine, isoleucine and tyrosine. When present in the artificial sea waters at 1 mM these amino acids stimulate the glycine efflux to 37–97% of its value in 1 mM-glycine. Influx values for cysteine and isoleucine are not available but the influxes of the others are all at least as large as the increased glycine effluxes which they bring about. The influxes of alanine, leucine and serine are similar to the total glycine influx. Data for cysteine uptake by giant axons of *Loligo pealii* (Hoskin & Brande, 1973) suggest that the influx is similar to or greater than the glycine influx in axons of *Loligo forbesi*. It seems likely therefore from the data in Tables 2 and 8 that the entry into squid axons of cysteine, alanine, serine, phenylalanine, leucine, tyrosine and probably isoleucine, threonine and valine, partly takes place by a process which involves some exchange with glycine. It is likely that both glycine and this group of amino acids can also enter in exchange for other amino acids of the group as well as glycine but there is as yet no evidence available for this. The data in Tables 2 and 8 indicate that the amino acids most actively involved with this exchange diffusion system are glycine, alanine, serine and cysteine and it is of interest that the data in Table 1 of Deffner (1961) indicate that the concentration gradients across the axolemma of *Loligo* axons of glycine, alanine and serine are similar. This would be expected if an amino acid exchange system of the type discussed contributed a major component to the influx and efflux of these amino acids and played a major role in determining their concentration gradients.

The data for glutamate, aspartate and arginine in Tables 2 and 8 indicate that these amino acids do not exchange with glycine, since their influx values are low and the glycine efflux is not stimulated by them. The data of Deffner (1961) also indicates that the energy involved in the transport of these amino acids differs considerably from that for glycine. It also seems likely that proline is not transported in exchange for glycine since it does not affect the glycine efflux even though its influx approaches that of glycine. The amino acid exchange system just discussed probably enables certain relationships between the concentration ratios of the amino acids involved to be maintained. It also enables certain amino acid gradients set up by metabolically linked active transport and possibly by other means to provide the energy needed to drive the transport of other amino acids. It seems in some ways similar to the system which has been characterized as a result of studies on the

exchange of amino acids in the Ehrlich tumour cell (Heinz, 1954; Oxender & Christensen, 1963; Gillespie, 1967) although the latter shows Na dependence. It is difficult to identify the amino acid exchange system with either the high affinity system (J_1 and K_1) or low affinity system (J_2 and K_2) for glycine uptake discussed in connexion with Fig. 4 and eqn. (5) since the K_1 = 0·16 mM and K_2 = ~ 2·7 mM while the Michaelis–Menten half-saturation constant for the activation of glycine efflux by extracellular glycine = 0·51 mM.

Glutamate uptake is Na-dependent and almost completely inhibited by both CN (Baker & Potashner, 1973) and ouabain (Caldwell & Lea, 1975). The uptake of this amino acid seems therefore to take place by a metabolically dependent active process similar to the CN-dependent, ouabain-sensitive component of glycine uptake. The need for such active uptake processes is made evident by the data of Deffner (1961) for *Loligo pealii* which indicate axoplasm to blood ratios of 14·5 for glycine and 30 for the glutamate ion which also enters against an adverse electrical gradient. Although Rosenberg & Khairallah (1974) have argued that Deffner's values for axoplasmic amino acid concentrations were too high on account of protein breakdown by proteolytic enzymes, it is difficult to see why evidence for metabolically linked amino acid transport should be found if energy was not needed to move amino acids into the axons against substantial electrochemical gradients.

Many of the known amino acid transport systems in other cells and tissues are Na-dependent (Schultz & Curran, 1970), so that the metabolically dependent glycine active transport system of the squid axon may prove to be an important exception. Since there has been controversy regarding the individual contributions of the Na gradient and metabolic energy to these systems, knowledge of the mechanism of a Na-independent glycine system driven by metabolism could prove valuable. A parallel can be drawn between the two glycine systems in the squid axon and the A and L systems for neutral amino acids in the Ehrlich tumour cell (Christensen, 1970). Thus the CN-sensitive active transport of glycine and possibly other amino acids may serve as primary concentrators, while the exchange diffusion system may act as a secondary concentrator by 'counter-transporting' neutral amino acids using the glycine and other amino acid gradients as an energy source. A further possibility is that the exchange diffusion system uses the alanine gradient rather than the glycine gradient as a primary energy source, the alanine gradient being mainly maintained not by active transport, but by the formation of alanine from pyruvate by transamination. It is still uncertain whether the A and L systems are independent systems or just different modes of a single carrier (Jacquez, 1973). The apparent ability of ouabain to separate the two glycine systems in the squid axon suggests that in this cell they are independent.

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