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THE UPTAKE AND RELEASE OF [^{3}H]GLYCINE IN THE GOLDFISH RETINA

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SUMMARY

1. In the goldfish retina, uptake of exogenous [^{3}H]glycine follows Michaelis–Menten kinetics with increasing concentrations of glycine. This uptake can be explained kinetically by the presence of two independent affinity systems: a 'high-affinity' mechanism with an apparent K_m(H) of 8·1 μM and a V_{max}(H) of 9·12 p-moles/min.mg protein, and a 'low-affinity' mechanism with an apparent K_m(L) of 0·63 mM and a V_{max}(L) of 430 p-mole/min.mg protein.

2. The high-affinity mechanism, and probably also the low-affinity mechanism, is temperature- and Na^+-dependent.

3. The low-affinity mechanism for glycine uptake is not affected by 5 mM-isoleucine, methionine and valine in the medium. However, it is inhibited more than 90% by 5 mM-alanine, proline and serine in the medium. This result indicates that the low-affinity transport for glycine may go through system A of the neutral amino acid transport system which is present in most tissues to transport glycine and certain neutral amino acids for metabolic purposes.

4. The high-affinity mechanism for glycine uptake is, however, not affected by the presence of up to 100-fold excess of all amino acids examined.

5. Autoradiographic studies show that at least one type of amacrine cell and one type of probable interplexiform cell take up [^{3}H]glycine both in the presence and absence of 5 mM-alanine, proline and serine, indicating that these neurones possess the high-affinity mechanism for glycine uptake.

6. [^{3}H]Glycine accumulated in the retina can be released by increasing the external K^+ concentration. This release is probably Ca^{2+}-dependent since it is blocked by 10 mM-Co^{2+} in the medium. Additionally, autoradiographic studies show that [^{3}H]glycine taken up by the glycine-accumulating neurones can also be released by Ca^{2+}-dependent, K^+-depolarization of the retina.

INTRODUCTION

In the goldfish retina, autoradiographic studies have shown that at least two distinct populations of neurones possess a specific mechanism for the accumulation of exogenous glycine (Marc, Stell, Bok & Lam, 1978; Marc, Lam & Stell, 1979). These neurones have been tentatively identified by electron microscopy, autoradiography and physiological studies as (1) a type of sustained, red-sensitive, centre-hyper-

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polarizing amacrine cell with synaptic terminals predominantly in the sublamina a of the inner plexiform layer (Marc, *et al.* 1978 1979; Lam, Marc, Su, Chin, Brandon & Wu, 1980) and (2) a type of interplexiform cell with cell bodies in the middle of the inner nuclear layer and numerous processes radiating from the somas (Marc *et al.* 1979; Lam *et al.* 1980). These results suggest that glycine may be a neurotransmitter candidate for both types of neurones. This possibility has been investigated further by examining biochemically and autoradiographically the uptake and release of glycine in the goldfish retina. In this paper, the presence of two apparently independent affinity systems for glycine uptake has been demonstrated. Additionally, it is shown that both types of glycine-accumulating neurones possess the 'high-affinity' uptake mechanism for glycine and that the glycine taken up by these cells can be released by a Ca^{2+}-dependent, K^+-stimulation.

METHODS

Common goldfish (*Carassius auratus*, 5–7 in. long) were obtained from Ozark Fisheries (Stoutland, Mo.). After at least 2 hr of dark adaptation, the eyes were enucleated and the eyecups cut into halves. Retinae were isolated and all the vitreous was carefully removed using a Q-tip swab. The retinae were washed briefly with oxygenated Ringer solution (Lam, 1972; Sarthy & Lam, 1979) and preincubated with gentle shaking for 10 min in Ringer solution. Unless otherwise stated, this Ringer solution was used in all incubations and washes. The experiments were performed at 22 ± 2 °C in ambient room light. [^{3}H]glycine (specific activity: 44 c/m-mole) was purchased from Schwarz/Mann, Division of Becton, Dickinson & Company. Bovine serum albumin, used as the standard for protein determinations, and Phenol Reagent Solution for protein assays were purchased from Sigma Company. Aqueous Counting Solution (ACS) was purchased from Amersham Corporation.

Kinetic and uptake experiments. For kinetic experiments, retinae were transferred to Ringer solution containing [^{3}H]glycine at various concentrations ranging from 10^{-3} to 10^{-7} M – total glycine. After a 10 min incubation, the retinae were washed for 2 min in 5 ml. Ringer solution, blotted several times on weighing paper, homogenized in 1 ml. 0·4 N-perchloric acid and then centrifuged at 1000 *g* for 5 min. A 0·2 ml. of each supernatant was added to 10 ml. ACS and the mixture was counted with a Beckman Liquid Scintillation Counter. The precipitates were redissolved in 0·5 ml. each of 1 N-NaOH. Protein determinations were run in triplicate on each homogenate by Lowry's method (Lowry, Rosenbrough, Farr & Randall, 1951). The total radioactivity in each supernatant was normalized to 1 mg protein and corrected for contribution from [^{3}H]glycine present in the extracellular space of tissue, as estimated using [^{14}C]sucrose as the extracellular marker (Lam & Steinman, 1971; Sarthy & Lam, 1979). The extracellular space was found to be 35% of the total volume of the retina, a value comparable to those from other retinae (Ames & Pollen, 1969; Goodchild & Neal, 1973). The time required to remove half of the [^{14}C]sucrose from the extracellular space of the retina was found to be 1·7 min (Lam & Steinman, 1971). The velocity of glycine transport (in p-mole/min.mg protein) was calculated from the protein content and the radioactivity of each sample. The K_ms and V_{max}s for both the low- and high-affinity uptake systems were determined by means of Lineweaver–Burk and Eadie–Hofstee plots.

Retinal uptake of [^{3}H]glycine is expressed as the tissue to medium ratio (T/M) defined as c.p.m. per mg tissue/c.p.m. per μl. incubation medium. For such experiments, retinae were incubated with Ringer solution containing 1 μM-[^{3}H]glycine for 5–60 min, washed for 2 min in Ringer solution, and homogenized. The radioactivity and protein content of each sample were determined by the methods described in the preceding paragraph. The c.p.m. per mg wet weight was treated as tissue (T), and the c.p.m. per μl. of the incubation medium was treated as medium (M).

For the experiments on the Na^+ dependence of glycine uptake, $NaHCO_3$ and NaH_2PO_4 in the normal Ringer solution were substituted with $KHCO_3$ and KH_2PO_4, respectively, and NaCl was

replaced by choline chloride. In experiments using other amino acids as inhibitors of low-affinity glycine transport, both the preincubation (10 min) and the incubation media were supplemented with these amino acids. Temperature-dependent experiments were conducted at both room temperature and 2 °C.

Identification of metabolites. The radioactive products in the retina after it had been incubated for 10 min with Ringer solution containing 1 μM-[^{3}H]glycine and 5 mM-alanine, proline and serine were identified by homogenizing the retina in 1 ml. 0·1 M-HCl containing unlabelled glycine (2 mg/ml.). The homogenate was centrifuged at 1000 *g* for 5 min and the radioactive products in 50 μl. of the supernatant were separated by high voltage paper electrophoresis described earlier (6000 V, 1·5 hr, pH 1·91 Lam & Steinman, 1971; Lam, 1976). After the paper was dried in a 90 °C oven and the location of the glycine spot was determined by spraying the paper with ninhydrin (Lam & Steinman, 1971), the paper was then cut into 1 cm strips. The radioactivity in each strip was eluted with 1 ml. 0·01 M-HCl for 1 hr, mixed with 10 ml. ACS, and measured with a Beckman Liquid Scintillation Counter.

Efflux experiments. A single piece of goldfish retina was incubated for 10 min with 2 ml. Ringer solution containing 1 μM-[^{3}H]glycine, rinsed for 40 min with normal Ringer solution (4 – 10 ml.) after which the efflux of radioactivity usually stayed relatively constant. The retina was then transferred every 3 min to a petri dish containing 2 ml. of a normal or modified isotonic Ringer solution. The sequence of the Ringer solutions used was as follows: normal Ringer solution, K^+-rich Ringer solution (56 mM-NaCl in the normal Ringer solution was replaced by 56 mM-KCl), normal Ringer solution, 10 mM-Co^{2+} in K^+-rich Ringer solution, normal Ringer solution, K^+-rich Ringer solution and finally normal Ringer solution. A 1·0 ml. aliquot from each petri dish was added to 10 ml. ACS and the radioactivity was measured.

Autoradiography. Retinae from dark-adapted goldfish were isolated and incubated under red light or in darkness for 10 min at room temperature with Ringer solution containing 1 mM-[^{3}H]glycine, washed for 2 min with Ringer solution and fixed with 2% glutaraldehyde and 2% formaldehyde in 100 mM-Na phosphate buffer (pH 7·4) for 1 hr at room temperature and overnight at 4 °C. The retinae was then processed for autoradiography as described elsewhere (Lam & Steinman, 1971; Marc *et al.* 1978).

RESULTS

Kinetics of [^{3}H]glycine uptake

When goldfish retinae were incubated at room temperature and under ambient room light with Ringer solution containing 1 μM-[^{3}H]glycine, there was a rapid and linear accumulation of radioactivity into the retinae during the first 20 min, reaching a tissue/medium ratio of about 9 at this time (Fig. 1). Under our experimental conditions, the concentration of glycine in the incubation medium was reduced by less than 10% during a 20 min incubation period. Accordingly, the kinetic studies of glycine uptake into the retinae using 10^{-3}–10^{-7} M-exogenous [^{3}H]glycine were all performed using an incubation time of 10 min. The results were analysed by both Eadie–Hofstee and Lineweaver–Burk plots. As shown in Fig. 2, an Eadie-Hofstee plot of the kinetics of glycine uptake indicates the presence of two separate affinity systems for glycine transport. This interpretation agrees with similar analyses using Lineweaver–Burk double reciprocal plots (Fig. 3*A*, *B*). The apparent K_ms and V_{max}s for both high- and low-affinity mechanisms are shown in Table 1.

Effects of other amino acids on glycine uptake

The apparent K_m for low-affinity glycine uptake varies between 0·5 and 3 mM for different tissues (Oxender & Christensen, 1963; Sepulveda & Smith, 1978) and is therefore similar to the K_m(L) for glycine transport in the goldfish retina. Of the two

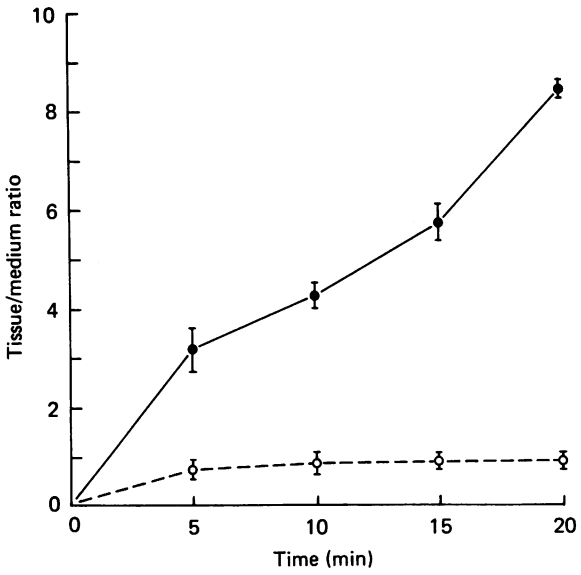

Fig. 1. Time courses of [^{3}H]glycine uptake into the goldfish retina incubated with Ringer solution containing 1 μM-[^{3}H]glycine at room temperature (filled circle) and at 2 °C (open circle). The bars are standard deviations (S.D.) from at least four experiments.

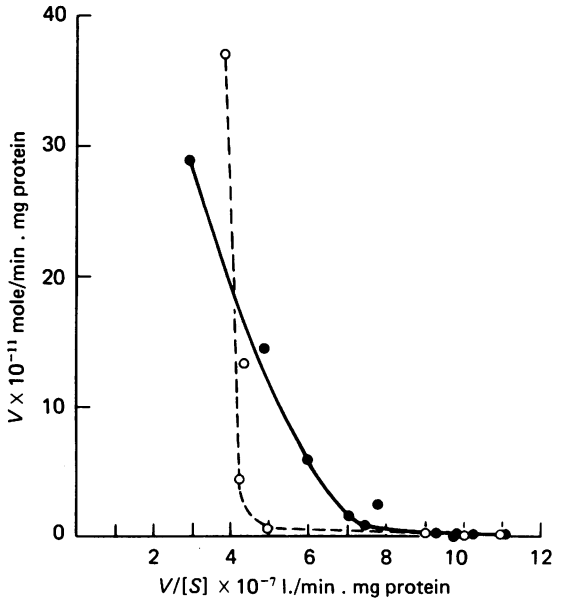

Fig. 2. Eadie–Hofstee plots of glycine uptake into the goldfish retina after a 10 min incubation with 10^{-3}–10^{-7} M-[^{3}H]glycine in the absence (filled circle) and presence (open circle) of 5 mM-alanine, proline and serine in the medium.

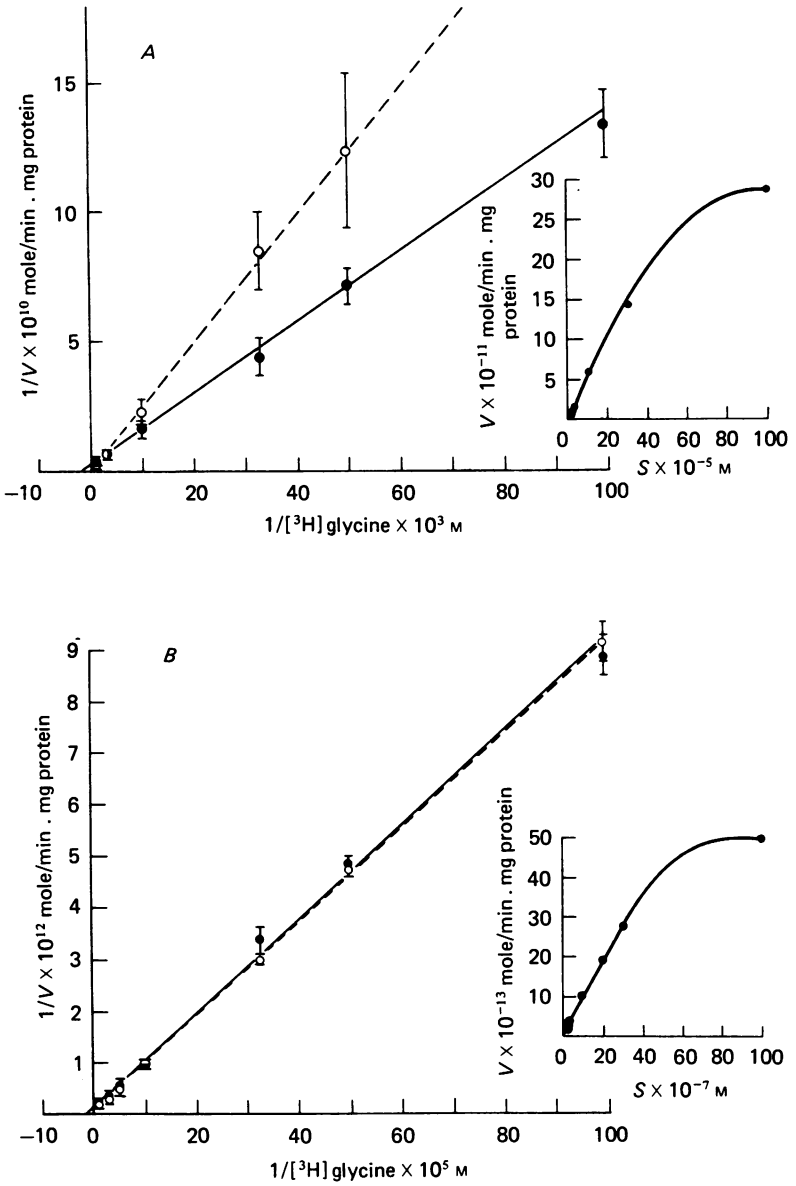

Fig. 3. Michaelis–Menten (insets) and Lineweaver–Burk plots of the rate of glycine uptake as a function of exogenous glycine concentration. The predominantly low (*A*) and high (*B*) affinity mechanisms were plotted separately to illustrate more clearly the presence of two different uptake systems. Although alanine, proline and serine inhibit the low-affinity glycine transport, they do not affect the high-affinity uptake. Each value is the mean ± S.D. for four experiments. The filled and open circles represent the absence and the presence of 5 mM-alanine, proline and serine in the incubation medium respectively.

major systems, A (alanine-preferring) and L (leucine-preferring), involved in the transport of the neutral amino acids into most tissues, glycine has been shown to enter cells predominantly through system A (Oxender & Christensen, 1963; Inui & Christensen, 1966; Sepulveda & Smith, 1978). To determine if glycine uptake in the goldfish retina also enters through these systems, retinae were incubated with [^{3}H]-

TABLE 1. Apparent K_ms and V_{max}s obtained from Lineweaver–Burk and Eadie–Hofstee plots for [^{3}H]glycine uptake into the goldfish retina

	Lineweaver–Burk plot	Eadie–Hofstee plot	Remarks*
K_m (L) $\times 10^{-4}$ (M)	6·33 ± 2·2	6·77	(−)
	no		(+)
V_{max} (L) $\times 10^{-10}$ (mole/min.mg protein)	4·30 ± 1·51	4·57	(−)
	no		(+)
K_m (H) $\times 10^{-6}$ (M)	8·08 ± 0·70	8·04	(−)
	8·17 ± 0·23		(+)
V_{max} (H) $\times 10^{-12}$ (mole/min.mg protein)	9·12 ± 0·24	9·00	(−)
	8·71 ± 0·54		(+)

* (−) and (+) representing without and with alanine, proline and serine.

glycine in the presence of 5 mM each alanine, proline and serine, or isoleucine, methionine and valine to inhibit system A or L respectively. As shown in Table 1, Figs. 2 and 3*A* low-affinity glycine transport is inhibited by the addition of alanine, proline and serine to the incubation medium. These amino acids, however, did not affect the high-affinity uptake system (Fig. 3*B*). In addition, isoleucine, methionine and valine, inhibitors of system L, had no effect on either the high- or low-affinity mechanism (not shown in the Figures).

Furthermore, our autoradiographic studies of goldfish retinae incubated with Ringer solution containing 1 μM-[^{3}H]glycine and 5 mM-unlabelled alanine, proline and serine show that the glycine-accumulating neurones described by Marc *et al.* (1978, 1979; R. E. Marc & D. M. K. Lam, in preparation) are still heavily labelled (Pl. 1*A*). The silver grains over the entire retina, especially those in photoreceptor cells, are however greatly reduced presumably due to the absence of low-affinity uptake. This result indicates that the glycine-accumulating neurones take up glycine selectively and predominantly through the high-affinity uptake mechanism.

Effects of temperature and external Na^+ concentrations

Compared to glycine uptake at room temperature, uptake of [^{3}H]glycine into the retina at 2 °C is reduced by over 80% (Fig. 1). The effect of external Na^+ concentrations on glycine transport was examined by measuring the uptake of [^{3}H]glycine in the presence of different Na^+ concentrations in the incubatiou medium. Fig. 4 shows that glycine uptake increases almost linearly with increasing extracellular Na^+ concentration. These results indicate that glycine uptake in the goldfish retina is a Na^+-dependent and temperature-dependent process. Furthermore, the uptake of [^{3}H]glycine into the retina in the presence of 5 mM-alanine, serine and proline in the incubation medium was also found to be Na^+- and temperature-dependent. Thus, the high-affinity mechanism for glycine transport in the goldfish retina is a Na^+- and temperature-dependent process.

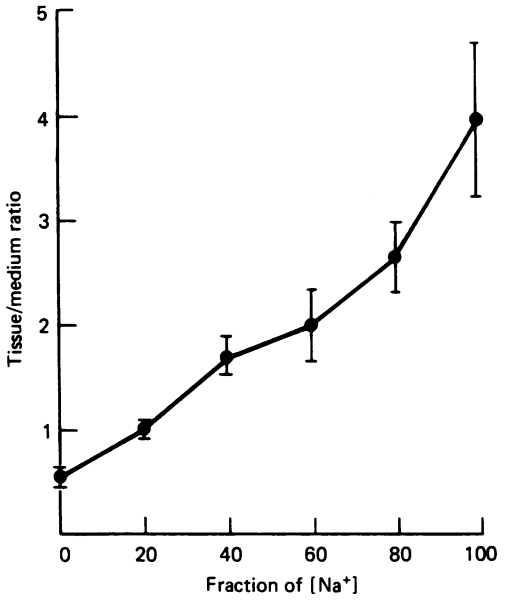

Fig. 4. The effect of Na^+ concentration on [^{3}H]glycine (1 μM) uptake in goldfish retinae incubated for 10 min with isotonic Na^+-free Ringer solution supplemented with varying proportions of Na^+ and choline. Each value is the mean ± S.D. of at least three experiments.

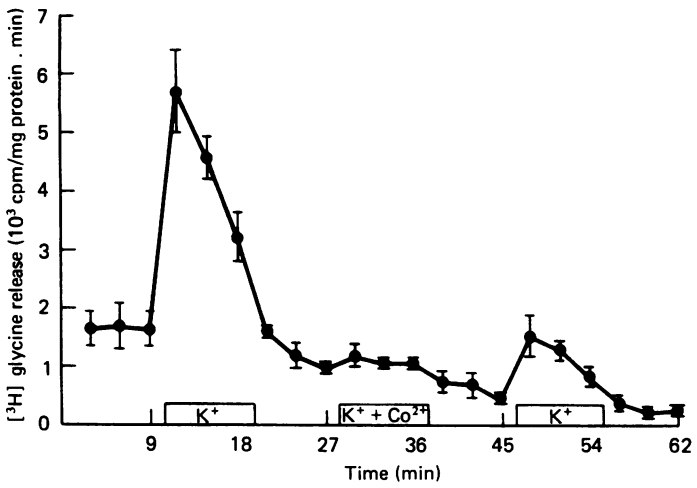

Fig. 5. The efflux [^{3}H]glycine from the goldfish retina in normal and K^+-rich Ringer solution. A piece of retina incubated for 10 min with Ringer solution containing 1 μM-[^{3}H]glycine was washed for 40 min with Ringer solution and transferred to 2 ml. each of a series of modified isotonic Ringer solutions at 3 min intervals. The sequence of the isotonic Ringer solutions was: normal Ringer solution, K^+-rich Ringer solution (K^+), normal Ringer solution, 10 mM-Co^{2+} in K^+-rich Ringer solution ($K^+ + Co^{2+}$) normal Ringer solution, K^+-rich Ringer solution (K^+) and normal Ringer solution.

K^+-stimulated release of [^3H]glycine

The efflux of [^{3}H]glycine was examined by incubating a piece of retina with 1 μM-[^{3}H]glycine for 10 min and rinsing the retina for 40 min with several changes of Ringer solution. When a steady efflux of radioactivity was reached, the retina was placed in Ringer solution containing 56 mM-K^+ and the efflux of [^{3}H]glycine was measured. As shown in Fig. 5, K^+-rich Ringer solution caused a transient increase in the efflux of radioactivity into the medium (37 % of the total radioactivity). The efflux rates returned to basal levels when the retina was transferred to normal Ringer solution. The K^+-stimulated increase in [^{3}H]glycine release was inhibited by the presence of 10 mM-Co^{2+} in the K^+-rich Ringer solution. Upon removal of Co^{2+} from the medium, K^+-rich Ringer solution again caused a transient increase in [^{3}H]-glycine efflux (10 % of the total radioactivity).

Additionally, our autoradiographic studies show that much of the [^{3}H]glycine taken up by the glycine-accumulating neurones is lost in response to K^+-depolarization of the retina (Pl. 1 *B*). This result suggests that the [^{3}H]glycine released into the medium by K^+ depolarization originates from the glycine-accumulating neurones.

Identification of metabolites

Since glycine is readily taken up by all retinal cells for metabolic purposes, it was necessary to estimate the percentage of the total radioactivity that was associated with free [^{3}H]glycine. Using the procedure described in the methods section, over 90 % of the radioactivity in retinae incubated for 10 min with Ringer solution containing 1 μM-[^{3}H]glycine and 5 mM-alanine, proline and serine was found to be associated with [^{3}H]glycine.

DISCUSSION

Biochemical, electrophysiological and autoradiographic studies indicate that at least some interneurones of the mammalian spinal cord use glycine as the neurotransmitter (Aprison, Davidoff & Werman, 1970; Iversen & Bloom, 1972; Price, Stocks, Griffin, Young & Peak, 1976; Davidson, 1976). Recent studies have also suggested that the neurones R3-R14 in the *Aplysia* parieotoviceral ganglion might be glycinergic (Price *et al.* 1978; McAdoo, Iliffe, Price & Novak, 1978). In the rabbit retina, strychnine, an antagonist of glycine receptors, has specific effects on the firing patterns and receptive field properties of certain ganglion cells (Ames & Pollen, 1969; Caldwell, Daw & Wyatt, 1978; Caldwell & Daw, 1978), while [^{3}H]glycine is taken up by some amacrine cells and can be released by appropriate light stimulation (Brunn & Ehinger, 1972; Ehinger & Lindberg-Bauer, 1976). In the rat retina, kinetic analysis indicates the presence of both high- and low-affinity mechanisms for glycine uptake (Neal, Peacock & White, 1973). Similarly, specific uptake and release of [^{3}H]glycine has been observed in the frog retina (Voaden, Marshall & Murani, 1974; Voaden, 1974) and neurophysiological studies indicate the presence of glycinergic amacrine cells in the *Necturus* retina (Miller, Dacheux & Frumkes, 1977). These results suggest that glycine may be a neurotransmitter in the vertebrate retina.

For the past several years, neurotransmitters have been used as physiological and morphological probes to identify and map the different neuronal pathways in the

goldfish retina (Marc *et al.* 1978, 1979; Lam, 1976; Sarthy & Lam, 1979; Lam *et al.* 1978, 1979). Because of the possibility that glycine may be a neurotransmitter in the goldfish retina, autoradiographic, biochemical and physiological studies have been instigated to characterize the glycinergic pathways in the goldfish retina. In studies reported elsewhere, light and electron microscope autoradiography (Marc *et al.* 1978, 1979; R. E. Marc & D. M. K. Lam, in preparation) has shown that a type of sustained, red-sensitive, centre-hyperpolarizing amacrine cell with synaptic terminals predominantly in sublamina a of the inner plexiform layer and a type of interplexiform cell with somas in the middle of the inner nuclear layer selectively accumulate [^{3}H]glycine. It is of interest that these glycine-accumulating cells are functionally and morphologically different from the probable GABA-ergic and dopaminergic neurones of the goldfish retina (Marc *et al.* 1978; Dowling & Ehinger, 1978; Lam *et al.* 1979; Sarthy & Lam, 1979). Electron microscope autoradiography is in progress using high-affinity glycine uptake as a marker for mapping the putative glycinergic pathways in the retinae (Marc *et al.* 1979; R. E. Marc & D. M. K. Lam, in preparation).

Unlike dopamine uptake, which in the goldfish retina is mediated by a single high-affinity system (Sarthy & Lam, 1979), glycine appears to be taken up by both low- and high-affinity transport systems similar to choline uptake reported for other retina (Neal & Gilroy, 1975; Baughmann & Bader, 1977). Autoradiographic studies (Sarthy & Lam, 1979) have shown that when goldfish retinae are incubated with [^{3}H]dopamine for various time intervals, synaptic terminals are labelled much more easily than the corresponding somas, suggesting that the channels for dopamine uptake may be predominantly localized in presynaptic dopaminergic terminals. In contrast, under similar experimental conditions, goldfish retinae incubated with [^{3}H]glycine for only a few minutes show heavy labelling patterns over both the somas and some terminals of glycine-accumulating neurones, suggesting that channels for high-affinity glycine uptake may be present in both the somas and presynaptic terminals of these neurones. This conjecture is in agreement with our recent observation that high-affinity glycine uptake by certain differentiating neurones occurs much earlier than the onset of synaptic formation during development of the *Xenopus* retina (Rayborn, Sarthy, Hollyfield & Lam, 1980).

As with other transport processes, glycine uptake is strongly dependent on both the temperature and the Na^+ concentration in the medium. In particular, results presented here show that the high-affinity mechanism of glycine uptake possessed by the glycine-accumulating neurones is a Na^+- and temperature-dependent process.

The funcational significance of the high-affinity uptake is not known. In analogy with the role of norepinephrine uptake in adrenergic transmission (Iversen, 1976), high-affinity glycine uptake may be involved in the removal and therefore inactivation of glycine released by presynaptic glycinergic terminals. In addition, an efficient mechanism of glycine uptake into glycinergic neurones may decrease the need for continual glycine synthesis and degradation during prolonged neuronal stimulation. In this regard, it is of interest that both GABA and dopamine have been shown to be selectively accumulated by probable GABA-ergic and dopaminergic cells of the goldfish retina by high-affinity uptake systems (Lam & Steinman, 1971; Marc *et al.* 1978; Sarthy & Lam, 1979; Lam *et al.* 1980).

Our autoradiographic and biochemical studies indicate that the glycine-accumu-

lating neurones in the goldfish retina release [^{3}H]glycine by K^+-depolarization. This release is likely to be Ca^{2+}-dependent because it is inhibited by the presence of Co^{2+} in the incubation medium. It is, however, not known whether this K^+-induced release is truly synaptic and whether endogenous glycine is also released.

In summary, the findings presented in this paper show that certain neurones in the goldfish retina possess a specific, high-affinity system for glycine uptake. The morphologies of these glycine-accumulating neurones are different from those of putative GABA-ergic and dopaminergic neurones of this retina (Lam *et al.* 1980). Furthermore, the glycine taken up into the glycine-accumulating neurones can be released by K^+-depolarization of the retina. This release is probably Ca^{2+}-dependent because it is reversibly inhibited by the presence of Co^{2+} in the K^+-rich Ringer solution. Taken together, our results point to glycine as the probable neurotransmitter used by these neurones.

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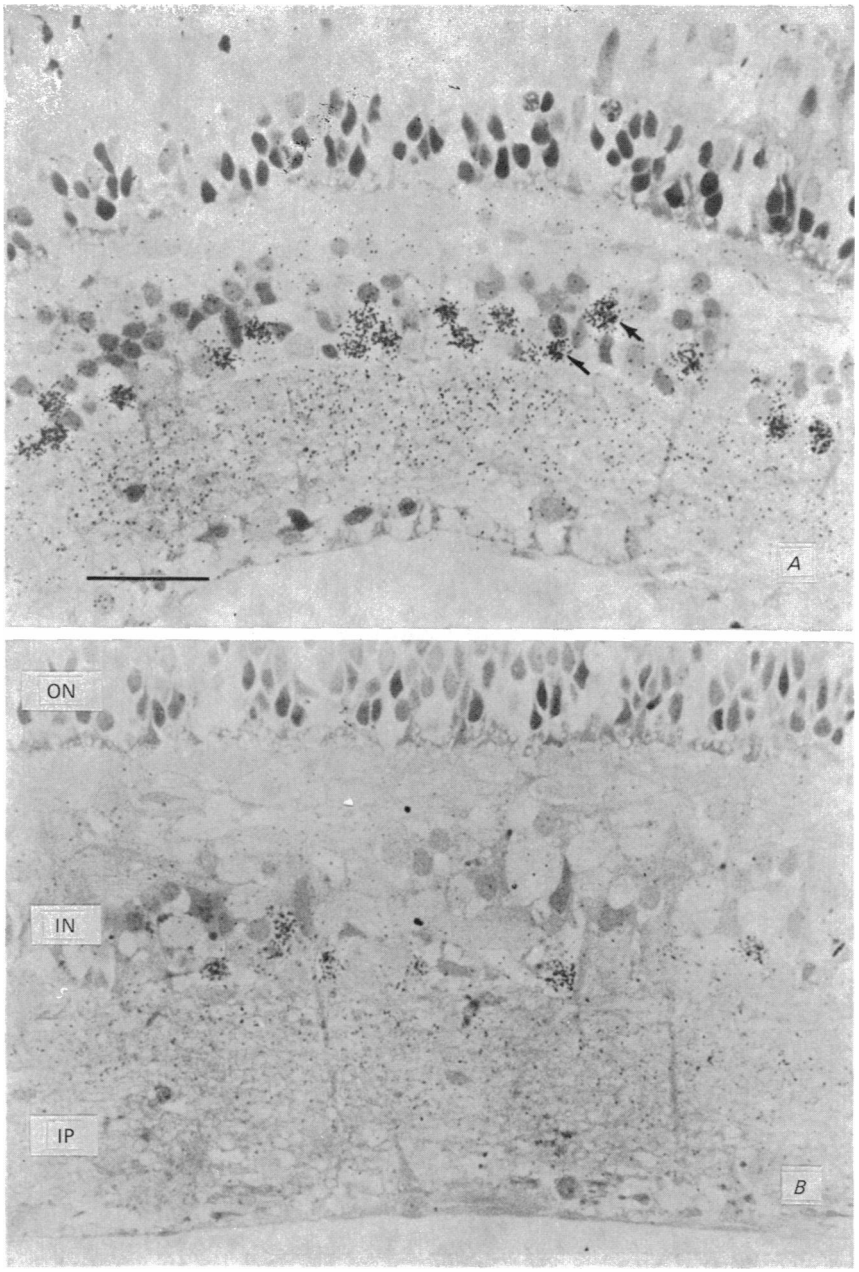
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EXPLANATION OF PLATE

Light microscope autoradiography of [^{3}H]glycine uptake and release in the goldfish retina. A piece of retina was incubated with Ringer solution containing 1 μM-[^{3}H]glycine and 5 mM-alanine, proline and serine for 10 min at room temperature and rinsed with Ringer solution for 20 min. The retina was then cut into two pieces. One piece was placed in Ringer solution while the other was placed in an isotonic K^+-rich Ringer solution (56 mM-K^+) for 5 min. Each piece was then fixed with glutaldehyde-formaldehyde and processed for autoradiography. *A*, retina incubated with normal Ringer solution. *B*, retina incubated with K^+-rich Ringer solution. Note fewer silver grains over glycine-accumulating cells in *B*. Arrow, probable glycine-accumulating interplexiform cell; half-arrow, probable glycine accumulating amacrine cell; ON, outer nuclear layer; IN, inner nuclear layer; IP, inner plexiform layer; Bar, 20 μm.