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Uptake of aspartic and glutamic acid by photoreceptors in goldfish retina

(high-affinity transport/rod and cone transmitters)

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ABSTRACT **The uptake of acidic amino acids by goldfish photoreceptors was investigated by light microscope autoradiography. Isolated retinas were incubated in media containing micromolar amounts of L-[^{3}H]aspartate, L-[^{3}H]glutamate, and D-[^{3}H]aspartate. We have four major observations. (*i*) Rods accumulate L-[^{3}H]glutamate with a high-affinity transport system; they exhibit a glutamate-to-aspartate selectivity ratio of 30:1. When incubated in 1–10 μM L-[^{3}H]glutamate, rods label more densely than cones. A unit area of rod membrane transports glutamate 30 times better than a unit area of cone membrane. (*ii*) Red-sensitive and green-sensitive cones show accumulation of L-[^{3}H]aspartate, D-[^{3}H]aspartate, and L-[^{3}H]glutamate, apparently with high affinity, but with little selectivity. Because rods have poor aspartate uptake, red-sensitive and green-sensitive cones may be preferentially labeled with L-[^{3}H]aspartate or D-[^{3}H]aspartate. (*iii*) Blue-sensitive cones show no uptake of L-[^{3}H]aspartate, D-[^{3}H]aspartate, or L-[^{3}H]glutamate other than that attributable to low-affinity transport. (*iv*) Various cell types in the goldfish retina can clearly discriminate between glutamate and aspartate, unlike acidic amino acid transport systems described in mammalian brain.**

For over two decades, the influences of aspartic and glutamic acids (aspartate and glutamate) on the vertebrate retina have been catalogued electrophysiologically. The application of sufficient quantities of aspartate or glutamate to retinas results in (*i*) suppression of nonreceptoral activity, resulting in an ostensibly pure photoreceptor mass response (1–3); (*ii*) depolarization and suppression of photoresponses in horizontal cells of turtles (4), teleost fishes (2, 5–7), and skate (8); (*iii*) hyperpolarization of on-center bipolar cells and depolarization of off-center bipolar cells in the carp retina (9). These effects are consistent with those expected of a native photoreceptor transmitter substance. The characterizations of aspartate or glutamate as retinal transmitters have been limited by the inability to identify specific enzymes or aspartate/glutamate pools uniquely associated with neurotransmission, the lack of evidence for aspartate/glutamate binding sites, and the absence of an inactivation scheme. In the mammalian central nervous system, however, sodium-dependent, high-affinity uptake has been used as a marker of possible aspartergic/glutamergic pathways, such as in perforant path terminals of area dentata and Schaffer collaterals of hippocampal pyramidal cells (10). We have therefore investigated the localization of radiolabeled aspartate and glutamate by goldfish rods and cones and conclude that: (*i*) glutamate is a plausible rod transmitter candidate; (*ii*) some acidic amino acid or an analogous substance may be the transmitter for red-sensitive and green-sensitive cones; and (*iii*) blue-sensitive cones apparently do not employ aspartate, glutamate, or any close analogue as a transmitter substance.

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METHODS AND MATERIALS

Retinal isolations, saline compositions, tissue maintenance, and histological procedures followed published methods (11). Each isolated retina was quartered and the quarters were incubated in parallel in media containing 1 μM–1.5 mM L-[^{3}H]aspartate, D-[^{3}H]aspartate, or L-[^{3}H]glutamate (New England Nuclear) for 10 or 30 min, fixed, embedded in an epoxy resin, sectioned, and coated with Kodak NTB-2 emulsion for autoradiography. Uptake was measured as total grains within each cone or rod ellipsoid by selecting only mid-sectioned, well-oriented ellipsoids and counting with a ×100 planapochromatic objective. This biases rod counts downward, because a significant fraction of grains will fall outside their small ellipsoids. Also significant is that transport is limited by surface area if the density of carriers per unit area is constant. Small cells would be less easily labeled than large cells. It is therefore possible to estimate the relative efficacies of rod and cone membrane transport by correcting label content for surface area, dilution into the cellular volume, and ligand concentration.

We autoradiographically estimated the K_m values of photoreceptor uptake (i.e., the concentration at which uptake velocity is half-maximal) by measuring the label content at various aspartate and glutamate total concentrations. In addition we investigated the effect of adding unlabeled ligand to each incubation. Reference concentrations of 5 μM and 15 μM were chosen for L-[^{3}H]glutamate and L-[^{3}H]aspartate, respectively. If the reference concentration was near a K_m typical of high-affinity transport (≈1–10 μM), addition of equimolar amounts of unlabeled ligand should elicit substantial label suppression. If the reference concentration was substantially less than a K_m typical of low-affinity transport (>100 μM), equimolar addition should cause less than 5% inhibition. We are therefore able to speak of "high-affinity" and "low-affinity" transport with qualitative validity.

RESULTS

Uptake by Rods. Rods selectively transported L-[^{3}H]glutamate. Incubation in 1, 5, or 10 μM L-[^{3}H]glutamate preferentially labeled the ellipsoids, nuclei, and synaptic terminal regions of rods (Fig. 1). Under conditions of identical autoradiographic responsivity [100-μCi/ml activity (1 Ci = 3.7×10^{10} becquerels); 10-min incubations; 21-day exposures; parallel processing], L-[^{3}H]glutamate was preferred over L-[^{3}H]aspartate by a factor of 30 (Table 1).

When quartered retinas were employed, synaptic terminal labeling was rare and a clear gradient of labeled rod nuclei from the outer limiting membrane inward was observed (Fig. 1*a*). Extending 10-min incubations to 30 min failed to abolish the gradient. By slicing retinal pieces into 500-μm-wide strips, it was possible to minimize the gradient effect within about 100 μm of the edge (Fig. 1 *b* and *c*); abolition of the gradient oc-

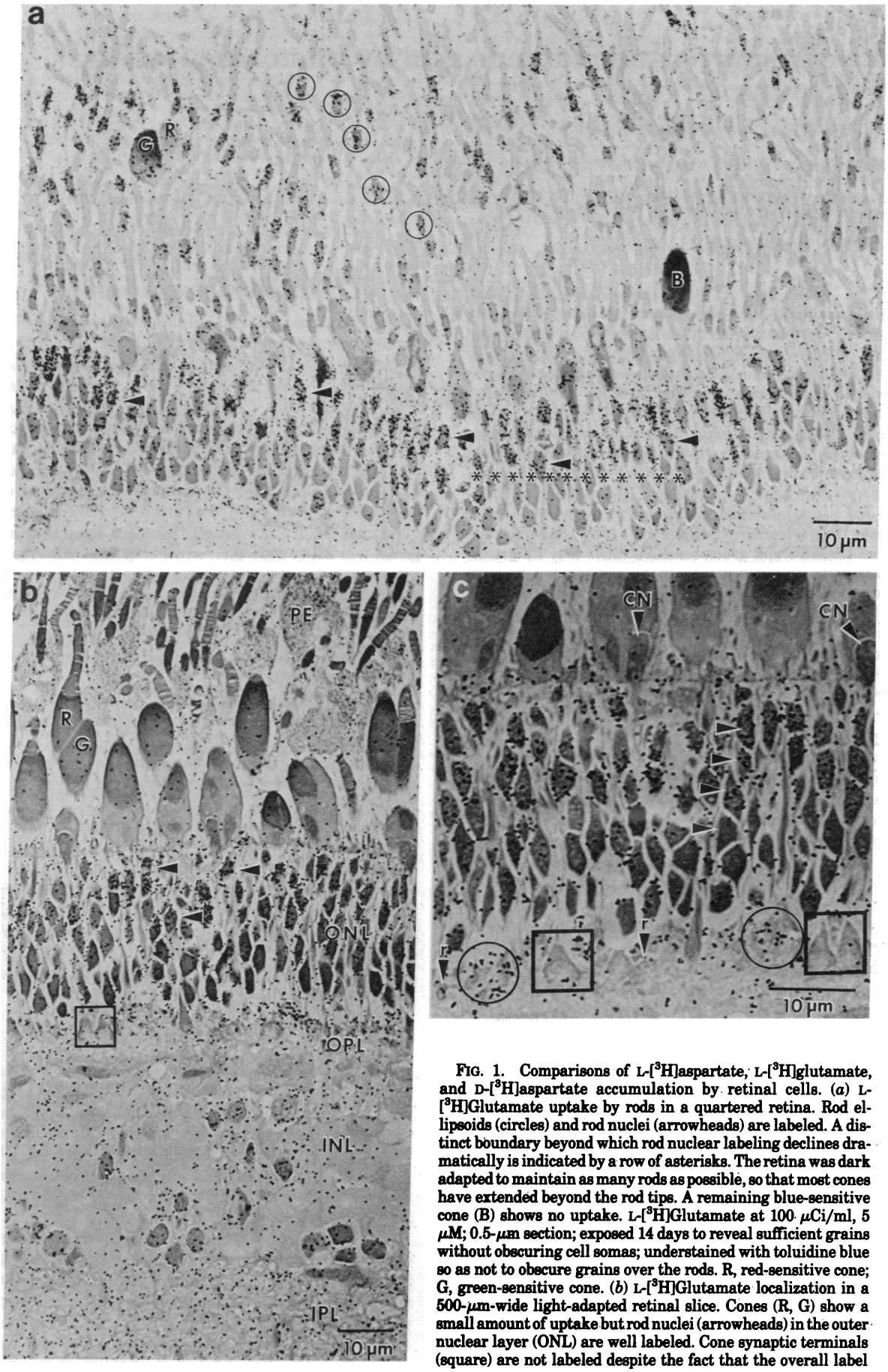

FIG. 1. Comparisons of L-[^{3}H]aspartate, L-[^{3}H]glutamate, and D-[^{3}H]aspartate accumulation by retinal cells. (*a*) L-[^{3}H]Glutamate uptake by rods in a quartered retina. Rod ellipsoids (circles) and rod nuclei (arrowheads) are labeled. A distinct boundary beyond which rod nuclear labeling declines dramatically is indicated by a row of asterisks. The retina was dark adapted to maintain as many rods as possible, so that most cones have extended beyond the rod tips. A remaining blue-sensitive cone (B) shows no uptake. L-[^{3}H]Glutamate at 100 μCi/ml, 5 μM; 0.5-μm section; exposed 14 days to reveal sufficient grains without obscuring cell somas; understained with toluidine blue so as not to obscure grains over the rods. R, red-sensitive cone; G, green-sensitive cone. (*b*) L-[^{3}H]Glutamate localization in a 500-μm-wide light-adapted retinal slice. Cones (R, G) show a small amount of uptake but rod nuclei (arrowheads) in the outer nuclear layer (ONL) are well labeled. Cone synaptic terminals (square) are not labeled despite the fact that the overall label

Table 1. Raw scores and specific uptake numbers

Cell type	^{3}H-Labeled ligand	Conc., μM	Incubation time, min	Exposure time, days	Raw score, grains/cell	Specific uptake, grains/μm^2 per mM
Rods	L-Aspartate	15	10	21	2 ± 2*	130
	L-Glutamate	5	10	21	21 ± 6*	3830
Red/green cones	L-Aspartate	15	10	21	30 ± 8*	60
	L-Glutamate	5	10	21	20 ± 5*	120
Blue cones	L-Aspartate	15	10	21	3 ± 2*	11
	L-Glutamate	5	10	21	5 ± 3*	50
Rods	D-Aspartate	10	30	24	27 ± 9†	
Red/green cones	D-Aspartate	10	30	24	122 ± 18†	
Blue cones	D-Aspartate	10	30	24	26 ± 6†	

Ligand activity was 100 μCi/ml in all cases.
* Mean ± 1 SD for a total of 300 cells summed from three experiments.
† Mean ± 1 SD for a total of 100 cells from a single experiment.

curred only in 40-μm-wide strips at the edges of slices. Three points are clear: (*i*) because terminal labeling was obtained in slices under conditions identical to those used with retinal quarters, the terminals themselves must have transport capacities—i.e., intracellular redistribution cannot account for terminal labeling; (*ii*) the somas of rods have transport capacities; and (*iii*) the layer of rod nuclei constitutes a significant barrier to free intraretinal diffusion of exogenously applied L-glutamate. Rod glutamate uptake appears to be quite strong and supports the view that glutamate could be a rod transmitter, consistent with the strong effects of acidic amino acids on mixed rod–cone bipolar cells (9).

Incubations in 50–500 μM L-[^{3}H]glutamate did not lead to substantial increases in labeling over 50 μM glutamate, suggesting uptake saturation at ligand concentrations less than typical for low-affinity transport. Moreover, addition of equimolar unlabeled glutamate to a reference medium containing 5 μM L-[^{3}H]glutamate led to substantial inhibition, suggesting that rod glutamate transport is mediated by a high-affinity system (Table 2). Unlabeled L-aspartate was not a particularly effective inhibitor at low concentrations but became effective at 500 μM; 1 mM D-aspartate also suppressed uptake.

Uptake by Cones. Cones did not discriminate strongly between L-[^{3}H]aspartate (Fig. 2*a*), D-[^{3}H]aspartate (Fig. 2*b*), or L-[^{3}H]glutamate (Fig. 1*b*; see also Table 1). Red-sensitive and green-sensitive cones (12–14) exhibited localization of label in ellipsoid, myoid, and nuclear regions; synaptic terminal labeling was equivocal. Regardless of the ligand employed, blue-sensitive cones (12–14) failed to show uptake beyond diffuse labeling attributable to low-affinity transport (Figs. 1*a* and 2). In view of the fact that high-affinity uptake can sometimes be influenced by stimulus conditions (11, 15), we incubated retinas in darkness, and under red, green, and blue lights of various fluxes and temporal patterns. In no case did blue-sensitive cones show significant uptake. Blue-sensitive cone uptake did not saturate at 100 μM and continued to increase even at 500 μM L-[^{3}H]glutamate and 1.5 mM L-[^{3}H]aspartate; the uptake thus appears to be low affinity. Clear localization of L-[^{3}H]aspartate and D-[^{3}H]aspartate occurred in red-sensitive and green-sensitive cones, but the specific uptake number was rather low: 60 grains per μm^2 of surface per mM for 15 μM L-[^{3}H]aspartate. The uptake of acidic amino acids by these cones was definitely high affinity, because labeling saturated at or below 100 μM and because significant inhibition with unlabeled ligands occurred at low concentrations (Table 2). It appears that there may be two transport sites on cones: one selective for aspartate and one for glutamate. We conclude this because homologous inhibition [e.g., L-[^{3}H]aspartate + unlabeled L-aspartate) was always more effective than heterologous inhibition (Table 2)].

Uptake by Nonreceptoral Elements. Aspartate and glutamate were clearly distinguished by retinal glia, the Müller's cells, and by horizontal, bipolar, and amacrine cells. Both D- and L-[^{3}H]aspartate were taken up quite avidly by Müller's cells (Figs. 2*b* and 3) and strongly excluded by horizontal cells, bi-

Table 2. Uptake inhibition

Ligand, activity, and conc.	Inhibitor	Conc., μM	No. of exps.	Label,* grains/cell: Red/green cones	Blue cones	Rods
L-[^{3}H]Asp,	—	—	3	30 ± 8	3 ± 2	2 ± 2
100 μCi/ml,	L-Asp	15	3	5 ± 3	2 ± 2	0
15 μM	L-Asp	150	2	0	0	0
	L-Asp	1500	2	0	0	0
	D-Asp	1000	2	0	0	0
	L-Glu	15	3	18 ± 8	0	1 ± 1
	L-Glu	150	3	17 ± 6	0	0
	L-Glu	1500	2	6 ± 4	0	0
L-[^{3}H]Glu,	—	—	3	20 ± 5	5 ± 3	21 ± 6
100 μCi/ml,	L-Asp	5	3	16 ± 5	2 ± 2	17 ± 6
5 μM	L-Asp	50	2	18 ± 10	2 ± 2	13 ± 4
	L-Asp	500	2	13 ± 4	0	7 ± 4
	D-Asp	1000	2	2 ± 1	0	3 ± 2
	L-Glu	5	3	16 ± 6	2 ± 1	9 ± 3
	L-Glu	50	2	10 ± 3	0	4 ± 3
	L-Glu	500	2	5 ± 3	0	0

* Data are presented as mean ± 1 SD; 100 cells per experiment.

density in the outer plexiform layer (OPL) has increased over that seen in larger retinal pieces. The remainder of the retina is diffusely labeled with grain accumulations over bipolar cell nuclei in the inner nuclear layer (INL) and over the inner plexiform layer (IPL) in general. PE, pigment epithelium. L-[^{3}H]Glutamate at 100 μCi/ml, 5 μM; 0.5-μm section; exposed 8 days; stained with toluidine blue to reveal cone terminals and proximal retinal cells. (*c*) Higher magnification view of a 0.5-μm section immediately adjacent to that shown in *b*. Here the localization of label to rod nuclei (arrowheads) is clear, while the labeling of cone nuclei (CN) and their surrounding cytoplasm is low to moderate. The distal-to-proximal label gradient observed in rod nuclei shown in *a* is dramatically decreased in the slice preparation but not abolished. Cone terminals (squares) show no labeling, while clusters of rod terminals (circles) between cone endings are heavily labeled. The spaces between rod nuclei, occupied primarily by Müller's cell processes, are quite free of label. r, Individual rod terminals.

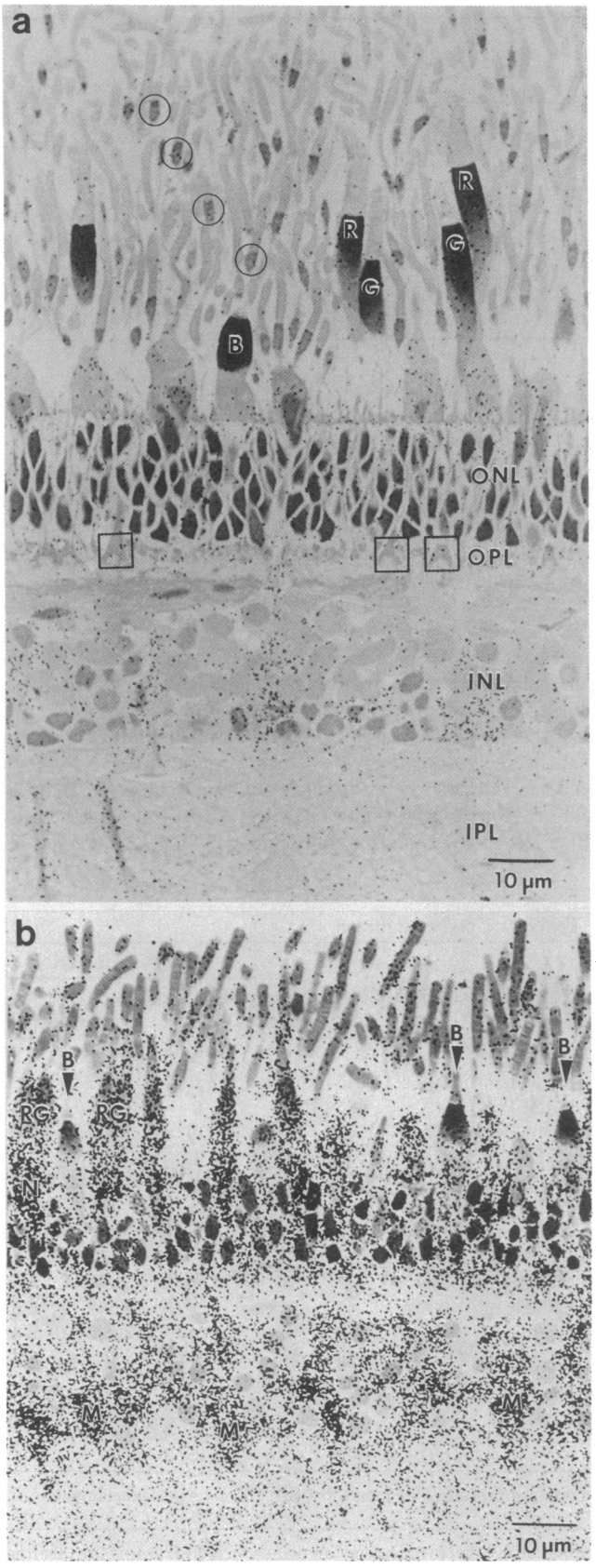

FIG. 2. L-[^{3}H]Aspartate accumulation by cones. Cones identifiable as red-sensitive (R) or green-sensitive (G) show light but distinct localization of L-[^{3}H]aspartate. Blue-sensitive cones (B) and rods (circles) do not. No selective labeling of cone terminals (squares) is observed. Neural elements in the remainder of the retina do not accumulate L-[^{3}H]aspartate. L-Aspartate at 100 μCi/ml, 15 μM; 0.5-μm section; exposed 14 days for direct comparison with Fig. 1*a*. (*b*) D-[^{3}H]Aspartate uptake by cones and glial cells. This preparation was incubated three times as long as all other experiments (30 min versus 10 min) to assess how selectively red-sensitive and green-sensitive cones (RG) could be labeled. Despite the long incubation, rods and blue-sensitive (B) cones labeled lightly. Also heavily labeled are Müller's cells (M), which showed little uptake of L-[^{3}H]glutamate. N, cone nucleus. D-[^{3}H]Aspartate at 100 μCi/ml, 10 μM; 0.5-μm sections; 24-day exposure.

polar cells, bipolar cell axon terminals, and what are likely to be amacrine cell somas (Fig. 3). In contrast, L-[^{3}H]glutamate is diffusely localized in retinal neurons (Fig. 1*b*) and appears not to label Müller's cells well. The spaces between rod nuclei are composed largely of Müller's cell processes, yet they are quite unlabeled (Fig. 1*c*).

DISCUSSION

For most amino acid transmitters, high-affinity uptake is thought to represent a neurotransmitter inactivation mechanism. Incubations of goldfish retinas in 50–500 μM L-[^{3}H]glutamate and 150–1500 μM L-[^{3}H]aspartate do not lead to major increases in rod or cone uptake, suggesting that the K_m values are probably in the 1–10 μM range. The K_m for high-affinity aspartate uptake in whole goldfish retina is 6 μM (unpublished data). This was also approximately indicated by the inhibition data.

Our results do not allow us to assign a plausible cone transmitter; L-glutamate seems as good a candidate as L-aspartate (Table 2). On the other hand, dose–response curves for depolarization of goldfish L-type horizontal cells by application of nebulized aspartate or glutamate show aspartate to be more effective (6). While the specificities of uptake need not reflect the specificities of postsynaptic receptor molecules, the difference in horizontal cell sensitivity to aspartate and glutamate may have been inflated by rapid rod clearance of glutamate from the extracellular space after brief nebulizer applications. Recently, Ishida and Fain (16) used the isolated, perfused goldfish retina to show that D-aspartate, applied at concentrations we have shown will suppress rod L-glutamate uptake (Table 2), potentiates the depolarization evoked by L-glutamate in L-type horizontal cells but does not potentiate responses to L-aspartate. In these experiments, L-glutamate appears to be a more effective ligand. This is consistent with our view that buffering of extracellular L-glutamate levels by rods is an important factor in pharmacological studies of the retina. We must stress, however, that the identity of a transmitter for cones is, in our view, totally unresolved.

It has often been reported that aspartate and glutamate share the same high-affinity uptake systems in the mammalian central nervous system (17–19). Contrary to that view, goldfish rod photoreceptors selectively transport glutamate with a high-affinity carrier; aspartate is not a good ligand for that system. Red-sensitive and green-sensitive cones are not as discriminatory. Even so, we are not certain that the same carrier is involved in cone transport of both aspartate and glutamate, especially because homologous inhibition of uptake is always superior to heterologous inhibition (Table 2). Beyond photoreceptor selectivity, there is again no doubt that retinal glia, the Müller's cells, are aspartate selective and that the remaining retinal cells exclude aspartate while showing some uptake of glutamate. These findings emphasize the importance of autoradiographic characterizations of uptake sites in systems that have been well studied structurally. The goldfish retina provides strong evidence that spatially and chemically discrete aspartate and glutamate transporters exist in ectotherm vertebrate nervous tissue.

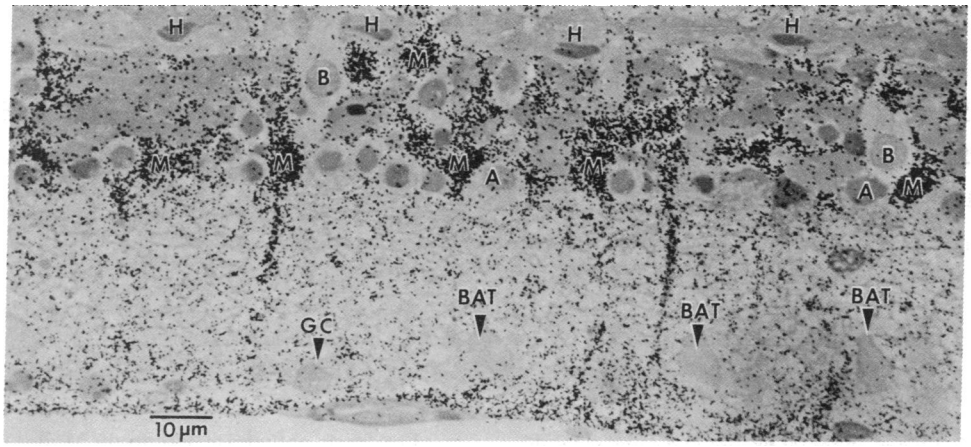

FIG. 3. L-[^{3}H]Aspartate accumulation by Müller's cells (M) and exclusion by horizontal cells (H), bipolar cells (B), amacrine cells (A), ganglion cells (G), and bipolar cell axon terminals (BAT). L-[^{3}H]Aspartate 150 μM; 0.5-μm section; 21-day exposure.

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