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Uptake and anterograde axonal transport of wheat germ agglutinin in the chick visual system

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UPTAKE AND ANTEROGRADE AXONAL TRANSPORT OF WHEAT GERM AGGLUTININ IN THE CHICK VISUAL SYSTEM

by

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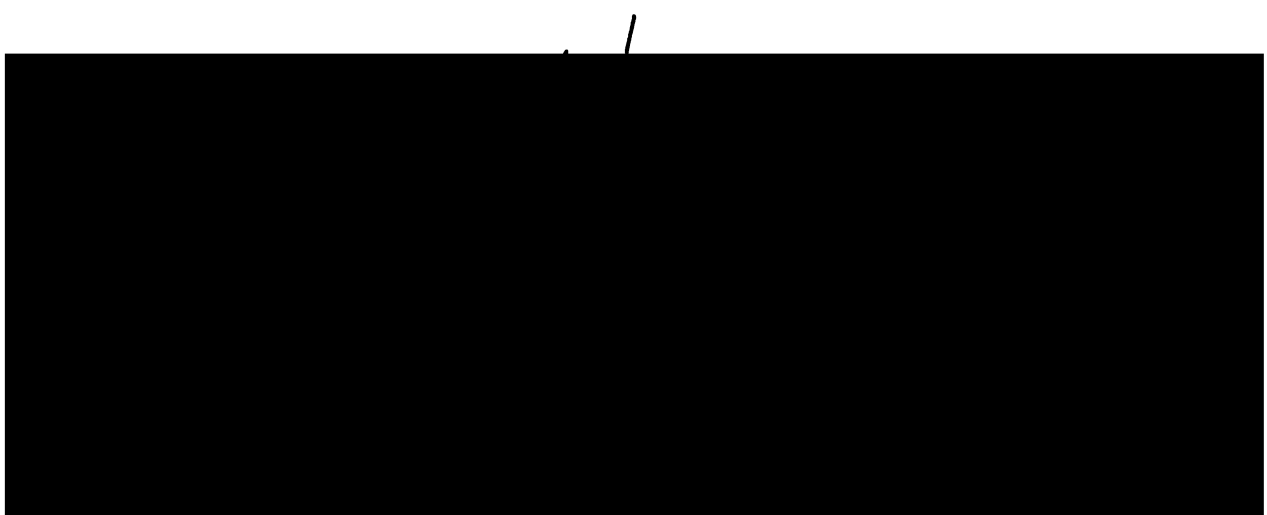

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ABSTRACT

The uptake and anterograde axonal transport of ^{125}I-wheat germ agglutinin (WGA) has been investigated in the visual system of the chick. In order to obtain a marker with specific and homogeneous binding properties, the iodinated lectin was affinity purified by passage over an N-acetylglucosamine (NAcGlu)-Sepharose column after iodination. As judged by light microscopic autoradiography, 22 hours after vitreal injection of the purified ^{125}I-WGA, radioactive label was found accumulated in the retinoreceptive layers of the contralateral optic tectum. Polyacrylamide gel electrophoresis of tectal homogenates revealed that >80% of the retrieved label ran in a band which comigrated with native WGA, and subcellular fractionation revealed that >80% of the transported radioactive label is membrane associated. In chicks injected with the fraction of the iodinated preparation that failed to bind to the affinity column, there was no evidence of tectal labeling. These findings support the hypothesis that WGA is selectively taken up by chick retinal ganglion cells and transported intact in an anterograde direction to their nerve terminals in the contralateral optic tectum.

Further experiments on the uptake and anterograde axonal transport of the WGA were carried out by dissecting the tecta of experimental chicks and assaying them 'in toto' for radioactivity in a gamma counter. As assayed in this manner, when ^{125}I-WGA was injected in the presence of 1.1 mM native WGA, the axonal transport of the labeled lectin was significantly reduced. By comparison, comparable doses of

either native soy bean agglutinin or native Ulex Europeaus-I were not very effective at reducing the uptake and transport of ^{125}I-WGA. This suggests that the uptake and subsequent transport of WGA is a saturable process which is dependent on a limited number of ganglion cell binding sites that facilitate the transport of the lectin.

The kinetics of uptake and transport of ^{125}I-WGA was also investigated, and by two lines of evidence the rate of anterograde axonal transport of ^{125}I-WGA was found to be 22-44 mm/day. This rate is slower than that reported for may other endogenously synthesized proteins or for horseradish peroxidase (HRP), the only other exogenously supplied protein studied as an anterograde transported marker. This difference in the rate of transport of WGA and HRP may reflect a difference in the intracellular pathway taken by these proteins.

In order to determine whether WGA and HRP follow different intracellular pathways following endocytosis, chicks were intravitreally injected with either HRP or a WGA-HRP conjugate and from 1 to 3 hours later the ultrastructural localization of these markers in the perikarya of retinal ganglion cells was investigated. A similar population of labeled organelles was observed following injection of these two markers. However, the relative distribution of labeled organelles in the animals injected with the WGA-HRP conjugate was different from that observed following injection of HRP. The most distinct differences were the greater relative labeling of the cisterns and vesicles of the Gogi apparatus following injection with the WGA-HRP conjugate. These findings suggest that to some extent WGA and HRP are differentially distributed following endocytosis by chick retinal

ganglion cells.

Given that the intracellular pathway followed by endocytosed WGA might reflect a pathway of membrane reutilization in the cell, the dependence of the transport of WGA on the axonal flow of newly synthesized protein was tested. Under two different sets of conditions, doses of cycloheximide which reduced the axonal flow of newly synthesized protein by up to 84% were found to have no significant effect on the transport of ^{125}I-WGA. These findings are thus consistent with the hypothesis that the axonal transport of WGA is independent of the rapid transport of newly synthesized protein, and may reflect an intracellular pathway through the cell which is important in the reutilization of endocytosed perikaryal membrane.

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CHAPTER 1

INTRODUCTION

In 1963 Aub et al. (1963) found that a wheat germ lipase preparation selectively agglutinated transformed fibroblasts and lymphocytes. Soon thereafter, Burger and Goldberg (1967) isolated this agglutinating factor, termed wheat germ agglutinin (WGA), and demonstrated that its ability to agglutinate transformed cells rested on its ability to interact with cell surface sites containing N-acetylglucosamine (NAcGlu). This property classified WGA as one of a family of plant and animal derived proteins known as lectins. Since these original observations, WGA has been highly purified and its physiochemical properties studied.

WGA is a dimeric protein composed of two identical 17,500 molecular weight subunits. Equilibrium dialysis indicates that each WGA subunit has two identical binding sites for NAcGlu each with a dissociation constant of 7.6×10^{-4}M. Oligomeric forms of NAcGlu, most notably di-N-acetylchitobiose and tri-N-acetylchitobiose, are bound more tightly with dissociation constants of 4.9×10^{-5} M and 1.2×10^{-5} M respectively. Oligomeric forms of NAcGlu greater than the triose fail to bind WGA any more tightly (Nagata and Burger, 1974; Goldstein et al., 1975). This has led to the suggestion that each WGA binding site consists of three adjacent subsites complimentary for a sequence of three linked NAcGlu units (Goldstein et al., 1975). Recent work has demonstrated that also exhibits an affinity for certain sialic acids (Bhavanandan and Katlic, 1979). Details of this interaction, however, have yet to be worked out.

Due to its ability to selectively bind to NAcGlu and sialic acid, WGA has been widely used as a probe for the recognition and purification

of membrane glycoproteins and glycolipids containing these sugar groups (Lotan and Nicolson, 1979). One of the major uses of WGA in the field of neurobiology has been to identify regional differences in cell surface carbohydrates and their changes with development. Pioneering studies in this area were conducted by Hatten and Sidman (1977), who made use of WGA induced agglutination of dissociated nerve cell populations in order to demonstrate distinct regional and developmental differences in the surface properties of mouse cerebellar cells. More recently, however, this problem has been approached through the use of WGA coupled to an electron dense marker as an ultrastructural probe. McLaughlin et al. (1980) used WGA conjugated to horseradish peroxidase (HRP) in order to identify changes in the carbohydrate composition of synaptic membrane in the chick retina during photoreceptor synaptogenesis, and Pfenninger and Maylié-Pfenninger (1975; 1981) made use of WGA coupled to ferritin in their studies of membrane biogenesis during neurite outgrowth and extension in cultured sympathetic neurons.

Techniques aimed at the selective identification of neuronal WGA receptors have also been developed. Gurd and others (Gurd and Mahler, 1974; Gurd, 1977) have used lectin affinity chromotography followed by polyacrylamide gel electrophoresis (PAGE) to isolate and identify WGA binding glycoproteins associated with rat synaptic membrane, and Mintz et al. (1981) have identified WGA binding glycoproteins in the chick neural retina by incubation of ^{125}I-WGA with solubilized neural membrane proteins previously separated by PAGE. Both techniques have proved to be sensitive enough to identify developmental changes in the composition of WGA binding proteins.

Another recent use of WGA has been as a marker for axonal transport. Thoenen and his colleagues (Stoeckel et al., 1977) originally opened this avenue by demonstrating that ^{125}I-WGA was transported in a retrograde direction (i.e. from nerve terminal to cell body) by neurons of the rat superior cervical ganglion (SCG). In further studies (Dumas et al., 1979) the nature of the binding and uptake of WGA was investigated through the use of dose response relationships; following injection of various doses of ^{125}I-WGA into the anterior eye chamber, the level of radioactivity axonally transported to the ipsilateral SCG was assayed. Analysis of the data from these experiments led Thoenen and his collaborators to the conclusion that the binding and uptake of WGA is a specific process, mediated by a single class of binding-uptake sites with a high capacity and a relatively low affinity. Similar conclusions have been drawn regarding the uptake and retrograde axonal transport of WGA coupled to HRP (WGA-HRP) (Harper et al., 1980). Whereas it is likely that the uptake of WGA is an adsorptive process similar to the uptake of tetanus toxin or cholera toxin (Stoeckel et el., 1977, Joseph et al., 1978; van Heyningen, 1974) the conclusions drawn by the above investigators are based more on speculation and analogy than on actual data.

Recently we have found that WGA is taken up and transported in an anterograde direction, i.e. from cell body to nerve terminal, by retinal ganglion cells of the chick (Margolis et al., 1981). Following intravitreal injection of ^{125}I-WGA light microscopic autoradiography revealed radioactive label in the retino-receptive layers of the contralateral optic tectum. Subcellular fractionation of tectal homogenates revealed that 81-89% of the transported radioactivity is membrane associated and

analysis of polyacrylamide gels provided evidence that greater than 80% of the labeled protein in the tectum comigrates with native WGA. Furthermore, the uptake and transport of ^{125}I-WGA appears to be a specific process dependent on the lectin's ability to bind specific carbohydrate groups associated with neuronal membrane. Whereas affinity purified ^{125}-WGA was taken up and transported, ^{125}I-WGA that had lost its ability to bind NAcGlu was not transported by these neurons.

An extensive literature exists on the subject of anterograde transport of proteins that have been newly synthesized from labeled precursors. Very little, however, is known about the anterograde transport of exogenous proteins. Prior to our findings on the uptake and anterograde transport of WGA, HRP was the only protein known to be processed by nerve cells in this manner.

The uptake and anterograde transport of HRP was first described by Hansson (1973) for rat retinal ganglion cells and has since been reported for a wide variety of neuronal cell types including chick retinal ganglion cells. Reported rates of transport range from 120 to 488 mm/day (Grafstein and Forman, 1980). Early reports on the ultrastructural localization of anterogradely transported HRP identified this protein in dense bodies, vesicles, multivesicular bodies, and smooth walled sacs and tubules which many investigators have assumed to be smooth endoplasmic reticulum (Colman et al., 1976; Turner and Harris, 1974; Sotelo and Riche, 1974; LaVail and LaVail, 1974). More recently these smooth walled profiles have been identified as part of the lysosomal system based on their positive cytochemical staining for acid phosphatase (Broadwell and Brightman, 1979). It has been suggested by Broadwell

and Brightman (1979) that these organelles mediate the anterograde transport of HRP.

WGA may be transported through the cell in a similar fashion, but two lines of evidence suggest the presence of an independent pathway for the lectin. 1) Following perikaryal endocytosis of HRP or WGA-HRP by rat sympathetic neurons Gonatas and his colleagues (Gonatas, 1979, Harper et al., 1980) found that HRP accumulated in a different set of intracellular organelles that did WGA-HRP. HRP was localized primarily in perikaryal lysosomes, whereas WGA-HRP was not only in lysosomes, but also in vesicles and saccules associated with the transforming face of the Golgi apparatus. (The ultrastructural localization of WGA-HRP in the axons and nerve terminals of these cells was not investigated). 2) Evidence from our laboratory suggests that WGA is axonally transported in an anterograde direction at 22-44 mm/day, a rate much slower than that reported for HRP. The ability of a lectin to have a different intracellular fate than HRP has been previously documented. Edelson and Cohn (1974) illustrated different intracellular fates for endocytosed HRP and Con A in macrophages. Following endocytosis HRP was rapidly degraded whereas Con A was only slowly degraded. This retarded degradation of Con A was found to be due to a failure of fusion of secondary lysosomes with Con A containing pinosomes. Rapid degradation of endocytosed Con A could be elicited by adding mannose to the culture medium. Other non-Con A-specific sugars were not effective in eliciting this response. This evidence suggests that Con A 's ability to bind specific carbohydrate containing macromolecules effects its intracellular fate in macrophages. In a similar fashion, the specific carbohydrate

binding ability of WGA may effect its intracellular fate in neurons. Specifically, it may allow WGA to follow an intracellular pathway which is different than the lysosomal pathway followed by HRP.

One might envision a number of different pathways that WGA might follow through the cell other than that followed by HRP. If WGA remains associated with components of the perikaryal membrane during its transport, the intracellular pathway followed by this lectin may reflect a pathway through the cell important in membrane recycling. Support for this concept comes from Gonatas' finding of WGA-HRP accumulation in the trans aspect of the Golgi apparatus (Gonatas, 1979; Harper et. al., 1980). Farquhar and her collaborators have implicated the Golgi apparatus in playing a major role in membrane recycling in myeloma cells (Ottosen et al., 1980, Wilson et al., 1981) and anterior pituitary mammotrophs (Farquhar, 1978). Thus, the anterograde transport of WGA might reflect the recycling of endocytosed perikaryal plasma membrane, via the Golgi appararuts for reuse at the nerve terminal. The concept of membrane recycling in nerve cells is not a new one. For years proponents of the vesicle hypothesis of neurotransmitter release have proposed schemes of vesicle membrane recycling at the nerve terminal (see Zimmerman, 1979). Although the evidence in support of membrane recycling at the nerve terminal is indirect, recycling of membrane components from plasma membrane to internal organelles and back out to plasma membrane has been directly demonstrated in a number of different cell types including macrophages (Muller et al., 1980), fibroblasts (Schneider et al., 1979), and the slime mold, Dictyostelium (Thilo and Vogel, 1980).

Alternatively, following endocytosis, WGA may dissociate from the membrane, and its transport may become associated with the flow of newly synthesized terial to the nerve terminal. WGA's affinity for specific carbohydrates make it a particularly good candidate for association with the transport of newly synthesized glycoconjugates. Several lines of evidence suggest a possible association of WGA with the axonal transport of newly synthesized glycoprotein. First, as indicated earlier, WGA-HRP has been localized to the Golgi apparatus, the presumptive site of terminal glycosylation of glycoproteins. Work by Droz (1975) suggests that glycoproteins pass through the Golgi apparatus just prior to entering on the pathway of axonal transport. Second, light microscopic autoradiographic studies of the chick optic tectum reveal similar labeling patterns following intravitreal injection of ^{125}I-WGA or ^{3}H-fucose (Margolis et al., 1981; Gremo et al., 1974). Third, Karlsson (1976) has demonstrated an affinity of WGA for 45% of the glycoprotein axonally transported in rabbit retinal ganglion cells. Lastly, in common with the transport of newly synthesized glycoproteins, WGA is rapidly transported (Margolis and LaVail, 1982).

If the axonal transport of WGA is associated with the transport of newly synthesized glycoproteins, then the pathway followed by WGA would be expected to be the same as that followed by these glycoproteins. But what is the pathway of transport of newly synthesized glycoproteins? Results of EM-ARG studies in the preganglionic axons of the chick ciliary ganglion have led Markov et al. (1976) to suggest that newly synthesized glycoproteins are rapidly transported down the axons of nerve cells through the tubules of the smooth endoplasmic reticulum from which they

are directly transfered to the axolemma, synaptic vesicles, and presynaptic plasma membrane. The smooth endoplasmic reticulum has been similarly implicated by Droz et al. (1975) in the rapid axonal transport of newly synthesized ^{3}H-leucine labeled proteins. This may not, however, be the only pathway available for the axonal transport of glycoproteins. EM-ARG and cell fractionation studies in identified neurons in the abdominal ganglion of Aplysia suggest a role for synaptic vesicles neurons in in glycoprotein transport (Thompson et al., 1976). Furthermore, based on their findings in the chick visual system Gremo and Marchisio (1975) have proposed that newly synthesized glycoproteins may be rapidly transported in the plane of the plasma membrane. They have referred to this pathway as axolemmal transport.

The axonal transport of WGA may also be associated with the transport of newly synthesized glycosaminoglycans or gangliosides. Very little is known about the transport of these carbohydrate rich macromolecules, but like WGA, they appear to be associated with the rapid phase of axonal transport (Grafstein and Forman, 1980). Furthermore, gangliosides are thought to mediate the uptake and retrograde transport of both cholera toxin and tetanus toxin (Stoeckel et al., 1977; Dumas et al., 1979; Harper et al., 1980; Joseph et al., 1977; van Heyningen, 1974).

The above pathways suggest a rather passive role for WGA, either as a marker of membrane movement through the cell or as a hitchhikker in the intracellular pathway of newly synthesized glycoconjugates. However, WGA may play a much more active role in its cellular processing. For example, WGA may induce its own endocytosis through the binding or

crosslinking of specific cell surface receptors. Uptake in this fashion has been described for a number of polypeptide hormones where it is referred to as receptor mediated endocytosis (Goldstein et al., 1979).

While this work was in progress, several investigators reported evidence for the transcellular transport of WGA in rat, chick, and monkey (Ruda and Coulter, 1982; Gerfen et al., 1982; LaVail et al., 1983; Itaya and van Hoesen, 1982). Following intravitreal injection of either WGA, ^{125}I-WGA, or WGA-HRP, the lectin or lectin-derivitive was observed not only in the axons and nerve terminals of retinal ganglion cells, but also in second order neurons of the visual system. In the chick tectum, glial cells in the vicinity of retinal ganglion cell terminals were also noted to contain the lectin. These findings suggest that subsequent to its transport to the distal regions of the retinal ganglion cell axon, a fraction of the transported WGA is released into the extracellular space and taken up by glia and post-synaptic neurons.

Although anterograde transcellular transport of exogenous protein has not been previously described in nerve cells, Schwab et al. (1979), have presented evidence for the retrograde transcellular transport of tetanus toxin in motor neurons. Furthermore, pathways for the transcellular transport of exogenous ligands have been described in a number of other cell types including endothelial cells, epithelial cells, and hepatocytes (Simionescu et al., 1975; Kraehenbuhl et al., 1979; Orlans et al., 1978). For example, in the proximal rat intestine, IgG binds Fc receptors on the apical surface of epithelial cells which appear to mediate the uptake, intracellular transport, and baso-lateral exocytosis of the ligand. Binding to this receptor is highly specific and

appears to confer on the immunoglobulin protection from lysosomal degradation, as transcellularly transported IgG is biologically active (Rodewald, 1973). In contrast, nonspecific markers of endocytosis such as horseradish peroxidase or native ferritin never appear to be transported across the epithelial cells, instead they accumulate in lysosomes and are degraded (Abrahamson and Rodewald, 1981).

Given that neurons are epithelial cell derivatives, the pathway of transcellular transport of WGA in neurons may, in many ways, resemble the pathway for the transcellular transport of IgG in epithelial cells. Furthermore, since WGA has been shown shown to bind to plasma membrane receptors for both insulin (Cuatrecasas, 1973) and NGF (Vale and Shooter, 1982), and may bind receptors for other ligands as well, the transcellular transport of WGA by retinal ganglion cells might reflect a pathway for transneuronal transport of a ligand of physiological importance to these neurons.

Alternatively, the transcellular transport of WGA might be related to the transport of newly synthesized protein destined for either secretion into the synaptic cleft, or insertion into the pre-synaptic membrane membrane. Recently, Carlson and Kelly (1981) have described a WGA-binding proteoglycan which appears to be found within purified synaptic vesicles from Torpedo. If this proteoglycan is transported within synaptic vesicles, and is subsequently released into the synaptic cleft, it would provide an ideal substrate by which the lectin might be transported transcellularly, and thus made available for endocytosis by glia and second order neurons.

CHAPTER 2

AXONAL TRANSPORT OF WHEAT GERM AGGLUTININ IN THE CHICK VISUAL SYSTEM

INTRODUCTION

Wheat germ agglutinin (WGA) belongs to a class of proteins known as lectins, that in specific carbohydrate moieties in much the same way that antibodies bind antigens. Due to its particularly high affinity for N-acetylglucosamine (NAcGlu) and sialic acid, WGA has been widely used as a probe for the recognition of membrane glycoproteins and glycolipids containing these sugar groups (Sharon and Lis, 1975). More recently, it has been useful as a marker for axonal transport. Stoeckel et al. (1977) originally opened up this avenue by their important observation that the lectin was transported in a retrograde direction, i.e., from axon endings back to neuronal cell bodies. It accumulated in rat superior cervical ganglion cells after its introduction into the anterior anterior eye chamber. Since then, they and others have extended the finding to descriptions of lectin-membrane binding characteristics (Dumas et al., 1979) and ultrastructural localization of the probe in cell bodies after retrograde axonal transport (Schwab et al., 1979; Harper et al., 1980).

In addition, other workers have recently found that the lectin may be transported in a retrograde direction by some neurons and, coincidentally but less reliably, in an anterograde direction by other neurons (Ruda and Coulter, 1980; Steindler and Deniau, 1980; Streit, 1980). In these cases of anterograde transport, relatively high concentrations of WGA have been injected, and no effort has been made to determine either the cellular mechanism of uptake or whether the lectin or breakdown

products were transported. In light of the specific binding properties of WGA, and the insight this might give us into the cellular processes of axonal transport and membrane reutilization, I have begun a systematic study of the uptake and anterograde transport of WGA. I present here evidence that, after vitreal injection, affinity purified, iodinated WGA is selectively taken up by chick retinal ganglion cells and transported intact in an anterograde direction to nerve terminals in the contralateral optic tectum. If the lectin remains associated with components of the perikaryal membrane during transport, this raises the possibility that portions of this membrane, i.e., lectin 'receptors', may be circulated within the neuron from cell body to axon terminal.

MATERIALS AND METHODS

Animals

White Leghorn chicks, 1- to 5-days-old, were used in these studies. The advantages of studying transport in the chick visual system are three-fold. First, the completely crossed projection of retinal ganglion cell axons to the opposite optic tectum enables one to study anterograde transport of radioactively labeled WGA by use of the ipsilateral optic tectum as an internal control. Second, the completely uncrossed projection of the superior cervical ganglion cell to the iris of the eye allows one to study the retrograde transport of the radioactive label in the same animal. Lastly, by vitreal injection, the retinal ganglion cell bodies can be exposed to the lectin with a minimum of pathological insult. Thus, one is able to study the uptake and transport of ^{125}I-WGA,in vivo, in a relatively undisturbed system.

Preparation of Iodinated Proteins

WGA was iodinated using a modification of the chloromine-T method of Hunter and Greenwood, as described by Burridge (1978). WGA (2.5mg/ml) and NAcGlu (5mg/ml) were combined in 200 ul of 0.2 M phosphate buffer (pH 7.4). Two mCi of carrier-free ^{125}I-WGA in 0.1N NaOH and 10 ul of chloramine-T (2.5 mg/ml) were then added. After 2-10 minutes, the reaction was stopped by the addition of 20 ul of sodium metabisulfite (12.5 mg/ml), and iodinated lectin was seperated from unreacted iodine and NAcGlu by exclusion chromotography on a Bio-Gel P-2 column. An aliquot of this preparation was reserved (fraction A). The remainder of the preparation was adjusted to pH 4.4 with 1.0 M Na acetate buffer and purified by affinity chromotography on a NAcGlu-Sepharose column (Sunberg and Porath, 1974; Vretblad, 1976). The affinity purification yielded two fractions of iodinated protein: fraction B (nonspecific) that did not bind to the column and was eluted with 50 mM sodium acetate buffer (pH 4.4), and fraction C (specific) that bound to the column. This fraction C was subsequently eluted from the affinity column with 1 M NAcGlu and dialyzed extensively against 0.1M phosphate buffer (pH 7.4) at 4°C. SDS gel electrophoresis and subsequent autoradiography of the gel served to characterize the specific and nonspecific fractions. The autoradiograms were analyzed with an E.C. Apparatus Corp. densitometer. Bovine serum albumin (BSA) was iodinated in a similar manner, but without NAcGlu in the reaction mixture. As with WGA, the reaction product was purified on a Bio-Gel P-2 column, but it was not subjected to affinity chromotography.

Axonal Transport Studies

Within 5 days of its preparation, a sample of 0.5-1.25 μCi of fraction C, the affinity purified ^{125}I-WGA (13-16 μCi/μg sp. act.), in 25 μl of 0.1 M phosphate buffer (pH 7.4), was injected into the vitreal chamber through a hole made seconds earlier in the scleral margin of one eye of each of nine chicks. Assuming a vitreal volume of 140 μl (LaVail and LaVail, 1974), this supplied about a 1 X 10^{-8}M concentration of iodinated WGA to the retina. After 21-23 hours, the animals were reanesthetized and perfused intracardially with buffer and fixative as described by LaVail et al. (1980). The dissected eyes, brains, and superior cervical ganglia from these animals were embedded in paraffin, and 8-10 um thick sections of these tissues were prepared for autoradiography. Six weeks later the emulsion-coated slides were developed and analyzed by light microscopy. As controls, 2 μCi of fraction A (13-16 μCi/μg sp. act.) was injected into the vitreal chamber of one eye of each of five chicks, and 1.0-1.8 μCi of fraction B (13-16 μCi/μg sp. act.) was similarly injected into one eye of each of five other chicks. Additionally, 9.0-19.0 μCi of ^{125}I-BSA (17.1 μCi/μg sp. act.) was injected into four chicks. Tissues from these animals were processed and analyzed in the same manner as those injected with the affinity-purified ^{125}I-WGA.

In additional studies, an aliquot of 8.0-10.0 μCi of affinity-purified ^{125}I-WGA (fraction C) in 20 μl of 0.1 M phosphate buffer (pH 7.4), was injected into the vitreal chamber of one eye of each of six chicks. After 22 hours, the animals were perfused with phosphate buffered saline for 3-5 minutes, and the tecta from four of the animals were quickly

dissected and individually homogenized in 200 µl of 5mM Tris-HCl (pH 7.4) with 0.1% SDS. The homogenates were freeze-thawed repeatedly, heated to 100 °C for 2 minutes, and centrifuged at 20,000 g for 30 minutes. The resulting supernatants were subjected to SDS discontinuous gel electrophoresis (0.1% SDS, 15% polyacrylamide), as described by Laemmli (1970). Gels were stained with 0.1% Coomassie Blue, dried onto filter paper, and exposed to Kodak X-Omat film for 10 days at -70°C, using a Dupont Image Intensification Screen. The resulting autoradiograms were analyzed with a densitometer. Cytochrome C, carbonic anhydrase, phosphorylase a, BSA, IgG heavy chain, native WGA, and ^{125}I-WGA were used as as molecular weight standards. In further studies, an aliquot of 7-14 µCi of affinity purified ^{125}I-WGA (fraction C) in 15 µl of 0.1 M phosphate buffer (pH 7.4) was injected into the right vitreal chamber of each of 10 chicks. After 25 hours, the animals were perfused through the heart with 0.1 M phosphate buffer (pH 7.4) containing 0.9% NaCl and 5% sucrose (PBSS), and the left optic tecta from all ten animals were dissected and rinsed twice in cold PBSS. The tecta were then homogenized in pairs or sets of three in 2.0 - 3.0 mls of 2.5 mM Tris-HCl buffer (pH 7.5) containing 1mM EDTA, and the tectal/Tris-HCl homogenates were then rapidly frozen and thawed repeatedly and centrifuged for 10 minutes at 800 X g. The resulting pellet was considered to consist of unruptured cells, nuclei, insoluble extracellular matrix, and large aggregrates of intracellular debris and was discarded. The supernatant, however, was collected and centrifuged for 90 minutes at 100,000 X g to seperate soluble proteins from membrane. The pellet and supernatant from this high speed centrifugation were both collected and counted in a Beckman Biogamma gamma counter with 70% efficiency.

RESULTS

Anterograde Transport

The ganglion cell, inner plexiform, and optic fiber layers of the retinas of all nine chicks injected with the affinity purified ^{125}I-WGA (fraction C) were heavily labeled with silver grains. In all nine animals, heavy labeling was found in the contralateral optic tectum (Fig. 2.1a) over the axons of the Stratum opticum (SO), and extending from layer a through layer f of the Stratum griseum et fibrosum superficiale (SGFS), the retino-receptive layers of the optic tectum (Crossland et al. 1973) (Fig. 2.2). Layer d appeared more heavily labeled, and layers c and f were less heavily labeled than other layers. The particularly heavy labeling of layer d was consistent with the finding of heavy labeling of this layer after vitreal injections of radioactive leucine or fucose (Hunt and Webster, 1975; Gremo et al., 1974). There was no label above background over the ipsilateral optic tecta of these animals (Fig. 1.1b).

Following intravitreal injections of the unpurified preparation of iodinated lectin (fraction A), results similar to those obtained with the affinity purified lectin were found, i.e., the contralateral optic tectum was labeled, but the ipsilateral optic tectum contained no label above background. By contrast, in five chicks that received vitreal injections of the nonspecific fraction B, neither optic tectum was labeled despite homogeneous labeling throughout the retina of the injected eye. Furthermore, the ^{125}I-BSA injected vitreally in four chicks failed to label either optic tecta.

To determine whether the radioactive labeling of the tecta seen after injections of the affinity-purified lectin was due to intact

^{125}I-WGA rather than radioactive breakdown products, the radioactive label from the optic tecta of four chicks was recovered and characterized by SDS polyacrylamide gel electrophoresis. Although multiple protein bands were retrieved from the experimental tecta of these animals, as revealed by Coomasie Blue staining, densitometer measurements (Fig. 2.3c) of the autoradiograms of these gels (Fig. 2.4, column c) revealed that >80% of the retrieved radioactive protein ran in a single band that comigrated with both native WGA and ^{125}I-WGA. In two animals, a second, fainter band at 12,000 daltons was also seen. This represented less than 15% of the recovered radioactivity and may represent a breakdown product of the iodinated lectin. Although Coomassie Blue staining of proteins from the control tecta was similar to that of the experimental tecta, no radioactive bands were seen in the autoradiograms of these gels (Fig. 2.3d; Fig 2.4, column d). Furthermore, following a crude homogenization of radioactively labeled optic tecta, >80% of the radioactivity was found to pellet at 100,000 X g (Table 1). This suggests that a large fraction of the axonally transported radioactive label is associated with membrane fractions of the cell, as would be expected for the intact lectin.

Retrograde Transport

In light of reports of the uptake and retrograde transport of labeled WGA from iris to the superior cervical ganglia in the rat (Stoeckel et al., 1977), I examined the labeling of neuronal cell bodies in the chick superior cervical ganglion after vitreal injections. Despite heavy labeling of the irises of all animals, no labeled nerve cell bodies were found in the ipsilateral superior cervical ganglion of seven of the nine chicks injected with the affinity purified ^{125}I-WGA. (In the

remaining two animals the ganglia were not examined.) Labeled pericytes and endothelial cells were, however, found in the ganglia of these animals. In contrast, in three out of five chicks injected with the nonspecific fraction (B), and in four out of five animals injected with the unpurified (fraction A) preparation, labeled nerve cell bodies were found in the ipsilateral (Fig. 2.5a), but not the contralateral (Fig. 2.5b) superior cervical ganglia.

DISCUSSION

The findings presented in this chapter support the hypothesis that ^{125}I-WGA is preferentially taken up and transported by retinal ganglion cell bodies to axon endings in the optic tectum. Although other investigators have reported a retrograde (Schwab et al., 1979; Harper et al., 1980) or bidirectional transport (Ruda and Coulter, 1980; Steindler and Deniau, 1980; Streit, 1980) of this lectin by neurons, this is the first evidence that the selective affinity of WGA for specific carbohydrate moities may play a role in its uptake and anterograde transport. Furthermore, this is the first evidence that a fraction of the lectin avoids lysosomal degradation and is transported intact down the axon.

Despite equivalent or greater concentrations of the nonspecific fraction B (the one that failed to bind to the affinity column), or of ^{125}I-BSA, only the fraction of WGA that retained its affinity for NAcGlu following iodination (fraction C) was taken up and transported in an anterograde direction by chick retinal ganglion cells. This observation supports the hypothesis of selective uptake and transport.

The affinity of ^{125}I-WGA for carbohydrate moities of glycoproteins and glycolipids may thus play a role in the selective uptake of the

lectin and its subsequent transport. In a fashion analogous to concanavalin A's induced patching and capping in lymphocytes (Yahara and Edelman, 1973), WGA may crosslink plasmalemmal glycoproteins or glycolipids and induce its endocytosis. Once inside the cell, a fraction of the ^{125}I-WGA is transported rapidly toward axon terminals.

Although I have not systematically studied the rate of transport, assuming a minimum retinal ganglion cell axon length of 15 mm to the posterior pole of the tectum (Crossland et al., 1973) and a transit time of 22 hours, it must be at least 16mm/day. Given this rate this transport and the affinity of the lectin for glycoproteins, it is conceivable that the transport of the lectin is linked to the transport of endogenous glycoproteins. The anterograde transport of glycoproteins in retinal ganglion cells of developing chicks has been well documented (Marchisio et al. 1973)

After injection of affinity-purified ^{125}I-WGA into four chicks, greater than 80% of the radioactive protein that was recovered from the experimental optic tecta appeared as a single radioactive band that comigrated with both native WGA and ^{125}I-WGA on SDS polyacrylamide gels gels. Because cells do not appear to reincorporate iodinated tyrosine into new protein (Ryser, 1963), this protein band was considered to represent axonally transported ^{125}I-WGA. This observation extends the findings of Ruda and Coulter (1980) concerning an immunologically identifiable fragment of the lectin in the optic pathway of rats following vitreal injections. If the WGA is not only intact, but remains associated with perikaryal membrane components after endocytosis and during transport, it raises the possibility that portions of this membrane may be circulated within the neuron, as has been recently proposed by Muller

et al. (1980) for plasma membranes of cultured macrophages.

Given earlier reports of the preferential uptake and retrograde axonal transport of WGA (Schwab et al., 1979; Harper et al. 1980), the labeling of neuronal cell bodies in the superior cervical ganglia after vitreal injections of the lectin was also examined. In contrast to these findings, I found no labeled neurons in the ipsilateral superior cervical ganglia in any of the animals injected with the affinity-purified ^{125}I-WGA. The differences between these results and those of others may be due to differences in species, methods of preparation, or concentrations of the WGA, survival times, or sites of injection (anterior chamber vs. vitreal chamber). However, it is relevant to note that I succeeded in labeling nerve cell bodies of the superior cervical ganglia in three out of five animals in which the nonspecific fraction (B) was injected. Thus, it seems likely that inactivated WGA, or some iodinated breakdown product, is responsible for the retrograde labeling reported here and may, at least in part, be responsible for the retrograde transport seen by others.

Not suprisingly, when aliquots of the iodinated lectin that had not been passed over the affinity column (fraction A) were injected, both the anterograde transport of label to the contralateral optic tectum and the the retrograde transport of label to superior cervical ganglion cell bodies was observed. Thus, this data suggests that the heterogeneity of tagged WGA molecules is responsible for the bidirectional transport observed by others (Ruda and Coulter, 1980; Steindler and Deniau, 1980; Streit, 1980).

This problem of heterogeneity was addressed by Dumas et al. (1979) who depended on hemagglutination to assay for lectin binding activity

after chloramine-T iodination. Such an assay, however, is relatively insensitive to slight changes in lectin binding activity. Because the nonspecific fraction (B) always represented <20% of the radioactive yield, the associated decrease in binding activity might easily have been undetected with a hemagglutination assay.

In summary, I have demonstrated the selective uptake and anterograde transport of the membrane marker WGA. I believe that this extends the usefulness of WGA as a probe for the study of neuronal cell development, endocytosis, and membrane recycling. In addition, this study highlights the need for affinity purification of tagged lectins, as has already been emphasized in other systems by Sharon and Lis (1975) and Maylié-Pfenninger and Jamieson (1979).

TABLE 1

CRUDE FRACTIONATION OF HOMOGENIZED TECTA FOLLOWING TRANSPORT OF ^{125}I-WGA

Number of combined tecta	Pelletable radio-activity at 100K X g	Soluble radio-activity at 100K X g
2	83%	17%
2	81%	19%
3	85%	15%
3	89%	11%

CHAPTER 3

FURTHER STUDIES ON THE SPECIFICITY OF UPTAKE AND ANTEROGRADE AXONAL TRANSPORT OF WGA

INTRODUCTION

In the previous chapter I have suggested that affinity purified ^{125}I-WGA is taken up by chick retinal ganglion cells and axonally transported to their nerve terminals in the contralateral optic tectum. Furthermore, I presented evidence which suggests that the uptake and subsequent transport of ^{125}I-WGA is a specific process, dependent on the lectin's ability to bind NAcGlu. Subsequent to the publication of these findings other investigators have described the anterograde transport of native WGA (Ruda and Coulter,1982; Lechan et al., 1981), ^{3}H-acetyl-WGA (Steindler, 1982, Steindler et al. 1982), and WGA conjugated to HRP (WGA-HRP) (Robertson and Aldskogius, 1982; Gerfen et al., 1982; Pugh and Kalia, 1982; Trojanowski et al., 1981; Wiley et al., 1982; Itaya and van Hoesen, 1982). The emphasis of these studies, however, has been to describe the utility of WGA and WGA derivitives as markers for tracing neuronal connections. As such, the data presented in these studies is largely morphological, i.e., derived from autoradiographic, histochemical, and immunocytochemical localization of the lectin or lectin derivitive. Whereas these techniques are appropriate for use in localization of the transported lectin, they are handicapped by the limits of reproducibility and only crude quantification. Since an in depth study of the transport of WGA might require a more quantitative approach, an alternative method for analyzing the transport of this lectin was sought. In this chapter I describe a relatively convenient and

reliable method for quntitatively assessing the amount of ^{125}I-WGA transported to the chick optic tecta. This method of assessing transported lectin is then used to provide data which confirm and extend the findings presented in chapter 2 regarding the specificity of uptake and transport of ^{125}I-WGA.

MATERIALS AND METHODS

Animals used in this study were 1 to 3-day old White Leghorn cockerels. WGA, Soy Bean Agglutinin (SBA) and Ulex Europeanus-I (UEA-I) were purchased from Vector Labs. Bovine serum albumin (BSA) and N,N-diacetylchitobiose were purchased from Sigma Chemical Co.. ^{125}Iodine was purchased from New England Nuclear and ^{3}H-proline (sp. act. 100 Ci/mmole) was purchased from Amersham. The ^{3}H-proline was stored in 2% ethanol. Prior to its use, it was dessicated under nitrogen and redissolved in phosphate buffered saline (PBS), pH 7.4. ^{125}IBSA (sp. act. 20 μCi/μg), affinity purified ^{125}I-WGA (sp. act. 14 - 19 μCi/μg), and that fraction of ^{125}I-WGA which failed to bind to an NAcGlu affinity column (sp. act. 16 -18 μCi/μg) were all prepared as described in chapter 2. Unless otherwise stated, '^{125}I-WGA' will refer to affinity purified ^{125}I-WGA, and 'inactive ^{125}I-WGA' will refer to that fraction of ^{125}I-WGA with a decreased affinity for NAcGlu.

All animals were injected with 15 ul of solution while under ether anesthesia. Unless specified otherwise, all radioactive material was injected in a solution of 0.1M phosphate buffer, pH 7.4. Injections were made into the right vitreal chamber through a hole made a few seconds earlier at the scleral margin. To minimize damage to the neural retina and maintain consistency from injection to injection, the tip of

the syringe was placed in the middle of the vitreous humour, directly behind the lens. Placement was directed using a Zeiss dissecting microscope with a 10X objective and 20X occulars. In a small number of animals, reflux of injected material out of the eye was observed. These animals were excluded from further analysis.

To obtain a quantitative assessment of the transported lectin, ten chicks were each injected with 15.0 μCi of ^{125}I-WGA, and twenty-five hours later these animals were perfused through the heart with 0.1 M phosphate buffer, pH 7.4, containing 0.9% NaCl and 5% sucrose (PBSS). The optic tecta were carefully dissected, weighed, rinsed in two changes of cold PBSS, and counted in a Beckman Biogamma gamma counter with a 70% counting efficiency. Since dissected optic tecta were very similar in size (61.7 $\pm$ 0.5 mgs (SEM)), radioactivity was expressed as dpm/tectum. In order to determine the relative contributions of acid precipitable and acid soluble radioactivity in the tectum, an additional 9 chicks were each injected with 15 μCi of ^{125}I-WGA. Twenty-five hours later these animals were perfused with PBSS and their optic tecta were carefully dissected. Each optic tectum was subsequently rinsed in cold PBSS and homogenized in 0.5 ml of distilled water at 4°C. The homogenizer was washed with 0.3 ml of distilled water, and the wash was pooled with the initial homogenate. Aliquots of 0.2 ml of cold 60% trichloroacetic acid (TCA) were added to the homogenates. Following a 40 minute incubation at 4°C, the TCA-homogenates were centrifuged, and the pellets were washed twice with 10% TCA at 4°C. The supernatants from the centrifugations were combined and assayed for gamma radioactivity as described above. The washed TCA-insoluble pellets were similarly assayed for

gamma radioactivity. In order to examine the relationship between the dose of injected ^{125}I-WGA and the amount of radioactive label transported to the tectum, 49 animals were intravitreally injected with varying doses (1 - 52 μCi) of ^{125}I-WGA. Twenty-five hours later these animals were perfused with PBSS, and the optic tecta were dissected, rinsed in cold PBSS, and assayed for gamma radioactivity. As controls, an additional 50 animals were injected with similar doses of either ^{125}I-BSA or the ^{125}I-WGA which failed to bind to the NAcGlu affinity column.

In order to determine whether the uptake and transport of WGA by retinal ganglion cells is a saturable process, each of 44 chicks was injected intravitreally with a solution of 0.1 M phosphate buffer containing 15 uCi of ^{125}IWGA and excess native WGA. The concentration of native WGA ranged from 15 um to 1.1 mM (a 10- to 750-fold excess). Twenty-five hours later the animals were killed by cardiac perfusion with PBSS, and the optic tecta were dissected, rinsed, and assayed for radioactivity as described above. As controls, additional chicks were each injected with a solution containing ^{125}-WGA (15 μCi) and either SBA (1.1 mM), UEA-I (1.1 mM), or BSA (15 μM - 1.1 mM).

The viability of retinal ganglion cells exposed to 1.1 mM WGA was assessed by the ability of these cells to synthesize and transport protein to the optic tectum. Each of 18 chicks was injected with either 15 μCi of ^{3}H-proline in PBS or a mixture of 15 μCi of ^{3}H-proline and native WGA (1.1 mM) in PBS. Twenty-five hours later the animals were perfused with PBSS, and each optic tectum was carefully dissected, rinsed in two changes of cold PBSS, and stored in 1 ml of 1 M NaOH at

4° C. The following day the tecta were homogenized in NaOH, and aliquots of the homogenate were mixed with toluene based scintillation fluid containing 25% (v/v) Triton X100, 0.5% (w/v) phenyloxazole, and 0.01% (w/v) 1,4 bis-2-(4-methylphenoxazolyl)benzene. The samples were then counted for 10 minutes in a Beckman LS 8000 liquid scintillation counter (at 59% effieciency). Disintegrations per minute (dpm) were calculated using a quench curve and an unquenched external standard, from which total tectal radioactivity was calculated. Tectal radioactivity was expressed as dpm/tectum, and the total transported radioactivity was calculated as the dpm from the left side minus the value from the right side (background). Since 98% of the label that is transported to the tectum following intravitreal injection of ^{3}H-proline is thought to be transported as protein (see chapter 5), the amount of radioactive label transported to the tecta of these animals was used as a measure of the amount of transported protein.

The effect of the disaccharide, N,N-diacetylchitobiose, on the uptake and subsequent transport of affinity purified ^{125}I-WGA was also examined. To accomplish this, each of 36 chicks was injected with a solution of 0.1 M phosphate buffer containing a mixture of 13 uCi of ^{125}I-WGA and N,N-diacetylchitobiose. The iodinated lectin was incubated in this sugar solution for at least 30 minutes at room temperature prior to its injection. The concentration of N,N-diacetylchitobiose in the injected solution ranged from 0.15 M to 0.77 M. Twenty-five hours after intravitreal injection, the animals were killed by cardiac perfusion with PBSS and the optic tecta were dissected, rinsed, and assayed for radioactivity as described above.

RESULTS

Quantitative Assessment of Transported ^{125}I-WGA

Twenty-five hours after intravitreal injection of 15 μCi of affinity purified ^{125}I-WGA, radioactive label was found in both the ipsilateral and contralateral optic tecta of all ten animals investigated (Table 2). The amount of of tectal labeling varied from animal to animal, but the average amount of radioactive label in the the contralateral tecta of these animals, 4984 dpm, was more than ten times the amount of label found in the ipsilateral tecta (467 dpm). Furthermore, since the visual pathway of the chick is completely crossed, the level of radioactive label in the ipsilateral tecta of these animals serves as a measure of background labeling. The difference between the amount of label in the contralateral and ipsilateral optic tectum of each animal, may therefore be used as a measure of the amount of radioactive label that had accumulated in the contralateral tectum by axonal transport. Thus, an average of 4508 dpm of radioactive label had accumulated in the contralateral optic tecta of these animals by axonal transport. Since 94% of the transported label is TCA precipitable (Table 3), this value represents the transport of approximately 3 fmoles of radioactive protein, or one ten thousandth of the injected ^{125}I-WGA.

Tectal Labeling as a Function of the Dose of Injected ^{125}I-WGA

As is illustrated in Figure 3.1, the amount of axonally transported label found in the tecta of animals after intravitreal injection of ^{125}I-WGA varied with the amount of labeled lectin which was injected. Detectable levels of transported label were found following injection of

as little as 1 μCi of ^{125}I-WGA (2 pmoles), and progressively increasing levels of tectal label were observed following injection of up to 52 μCi of the labeled lectin.

Also illustrated in Figure 3.1 are the results of a study in which axonally transported label was measured as a function of the intravitreal dose of either ^{125}I-BSA or inactive ^{125}-WGA. In contrast to the light microscopic autoradiographic findings presented in chapter 2, axonally transported label was found in the contralateral tecta of these animals. The amount of transported label, however, was always less than the amount of background labeling (see Table 4). Furthermore, the amount of label transported to the tecta of these animals was significantly less than the amount of label transported to the tecta of animals injected with similar doses of affinity purified ^{125}I-WGA.

These findings suggest that the difference in the amount of radioactive label found in the contralateral and ipsilateral optic tectum of each animal may be used to establish quantitative differences in the amount of lectin transported by retinal ganglion cells under different experimental conditions.

Saturation of the Uptake and Transport of ^{125}I-WGA

As presented above, doses of up to 52 μCi of injected ^{125}I-WGA (3 μg) failed to saturate the system responsible for the uptake and subsequent axonal transport of this lectin. In order to determine whether this transport system is saturable, (i.e. a limited number of receptors are available for uptake and transport of the lectin) chicks were injected with 15 μCi of ^{125}I-WGA in a 1.1 mM solution of native WGA. Twenty-five

hours after intravitreal injection, the tecta from these animals were assayed for axonally transported label. As summarized in Figure 3.2, there was a 75% reduction in the amount of label transported to the contralateral tecta of these animals as compared to animals injected with ^{125}I-WGA in buffer containing no excess lectin. Comparable doses of either SBA or UEA-I, lectins with specificities for N-acetylgalactosamine and fucose respectively (Goldstein and Hayes, 1978), also reduced the amount of axonally transported label, but were not as effective as native WGA. Co-injection of SBA reduced the amount of transported label by only 5%, and co-injection of UEA-I reduced the amount of transported label by 27%.

When ^{125}I-WGA was injected in the presence of lower concentrations of native WGA (15 μM to 286 μM), a paradoxical effect was seen. The amount of label transported to the contralateral tecta of these animals was greater than the amount of label transported to the tecta of animals injected with only ^{125}I-WGA (see Fig. 3.3). When comparable concentrations of BSA were co-injected with ^{125}I-WGA, similar findings were observed (see Fig. 3.3). Increased tectal labeling was even seen following co-injection of BSA at a concentration of 1.1 mM. As noted above, when ^{125}I-WGA was co-injected with native WGA at this concentration, there was a significant reduction in the amount of label transported to the tectum.

The above findings suggest that the total concentration of protein in the injected solution may play some non-specific role in increasing the amount of ^{125}I-WGA taken up by retinal ganglion cells and subsequently

transported to their axons and nerve terminals in the contralateral tectum.

Competition with Diacetylchitobiose

Earlier in this chapter, as well as in the previous chapter, I presented evidence which suggests that the selective uptake and transport of ^{125}I-WGA by chick retinal ganglion cells is dependent on the lectin's ability to bind NAcGlu. This evidence was based on the differential uptake and transport of affinity purified ^{125}I-WGA and a fraction of ^{125}I-WGA which had lost its ability to bind NAcGlu. Further evidence for this conclusion might be obtained by effectively competing for the uptake and transport of the lectin with N,N,-diacetylchitobiose, a dimeric form of NAcGlu which binds to WGA with a greater affinity than does NAcGlu (Nagata and Burger, 1974). As illustrated in Table 5, concentrations of diacetylchitobiose as high as 0.77 M failed to reduce the amount of label subsequently transported to the optic tecta of these animals. Concentrations of 0.77 M had little effect on the amount of transported label, whereas lower concentrations of diacetylchitobiose led to a pronounced increase in tectal labeling.

Viability of Retinal Ganglion Cells Exposed to WGA

To determine whether native WGA was toxic to retinal ganglion cells at the concentrations used in this study, chicks were each injected with with either 15 μCi of ^{3}H-proline, or a mixture of WGA (1.1 mM) and 15 μCi of ^{3}H-proline. Twenty-five hours later the tecta from these animals were analyzed for transported radioactive label. As analyzed by Student t test there was no significant difference between the amount of label

transported to the tecta of these two groups of animals ($p = 0.183$; $df = 15$). Thus, as assayed in this manner, intravitreal injection of WGA at a concentration of 1.1 mM, does not appear to be toxic to chick retinal ganglion cells.

DISCUSSION

In the experiments presented in this chapter, the tecta from experimental animals were dissected 'in toto' and directly assayed for gamma radioactivity. The difference between the amount of radioactivity in the ipsilateral and contralateral tectum of each animal was then used as a measure of axonally transported label. Since 94% of this label is TCA precipitable, and cells do not reutilize iodotyrosine (Ryser, 1963), axonally transported label was thought to reflect reliably the amount of ^{125}I-WGA that had accumulated in the contralateral tecta by axonal axonal transport. Assessing transported ^{125}I-WGA in this manner allows one to appreciate quantitative differences in the amount of lectin transported to the tectum under different experimental conditions (see Fig 3.1). Furthermore, since tectal radioactivity is measured directly and background levels of labeling are taken into account for each individual animal, this method of assaying transported ^{125}I-WGA appears to be both more accurate and more reliable than morphological methods such as autoradiography.

When this assay was used to study the amount of label that was axonally transported to the contralateral tectum of animals previously injected with either affinity purified ^{125}-WGA, inactive ^{125}I-WGA, or ^{125}I-BSA, results similar to those obtained obtained by light microscopic

autoradiography were observed: The affinity purified ^{125}I-WGA was taken up and transported selectively as compared to either ^{125}I-BSA or inactive ^{125}I-WGA. However, in contrast to the light microscopic autoradiographic findings of the previous previous chapter, in which no evidence for the transport of either ^{125}I-BSA or inactive ^{125}I-WGA was found, a small amount of transported label was detected in the contralateral tecta of animals injected with these labeled proteins. This suggests that within the range of doses of ^{125}I-WGA that were tested, a small percent of the transported label may be taken up and transported nonselectively, i.e., fluid phase, in a manner similar to the uptake and transport of either inactive ^{125}I-WGA or ^{125}I-BSA. More specifically, with an injected dose of 15 μCi of ^{125}I-WGA, up to 9% of the transported label may have reached the tectum via such a pathway. Labeled material which has been transported in this fashion might account for the TCA soluble fraction of axonally transported label in the tectum or for a fraction of the transported protein which does not migrate with WGA on SDS polyacrylamide gels.

If, in fact, the uptake and transport of WGA is facilitated by the lectin's ability to bind a limited number of lectin 'receptors', then at high enough concentrations of WGA, saturation of these binding sites should occur. Although this was not observed when ^{125}I-WGA was injected at concentrations up to 4.2 uM (52 μCi), when ^{125}I-WGA was co-injected with native WGA at a concentration of 1.1 mM, the amount of axonally transported label was reduced by 75%. Since injection of native WGA at this concentration does not appear to be toxic to retinal ganglion cells, nor do comparable levels of other lectins reduce tectal labeling to a

similar extent, this reduction presumably reflects competition between WGA and ^{125}I-WGA for a limited number of intra- or extracellular binding sites which facilitate transport of the lectin. Other investigators claim to have demonstrated competition between WGA-HRP and WGA for uptake and transport by neurons of the rat and cat CNS (Pugh and Kalia, 1982; Trojanowski et al., 1982; Wiley et al., 1982), however, the lack of appropriate controls, and inadequately described methods, i.e., the number of animals used, and the techniques used for quantifying HRP reaction product in neuronal terminal fields, have made the findings of these investigators difficult to interpret.

Assuming that native WGA and ^{125}I-WGA compete equally well for retinal ganglion cell binding sites, then it may be estimated that at least 550 fmoles of WGA may be axonally transported to the optic tectum over a twenty-five hour period. Since there are approximately 2.6 million ganglion cells in a chick retina (Rager, 1980), it may be further estimated that at least 5,000 molecules of WGA may be transported to the tectum per retinal ganglion cell per hour.*

While neither 1.1 mM SBA or UEA-I were as effective at competing for ^{125}I-WGA receptors as was native WGA, co-injection of these lectins did lead to a small reduction in the amount of transported ^{125}I-WGA. This reduction is likely to be due to stereological inhibition of unoccupied WGA binding sites by adjacently bound SBA or UEA-I. A similar mechanism has been proposed by Gurd (1977) to explain reciprocal inhibition between WGA and Lens culinaris phytohemagglutinin for binding to a synaptic

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* Assuming a delay of 8.5 hours (see chapter 4), tectal accumulation is only for 16.5 hours. This works out to 7,600 molecules/cell/hour.

membrane fraction isoloated from rat cerebral cortex.

Furthermore, competition between labeled and native WGA for uptake and transport by retinal ganglion cells might occur at concentrations of injected lectin much lower than 1.1 mM. This was, however, difficult to assess since lower concentrations of native WGA (15 μm to 286 μM) co-injected with ^{125}I-WGA lead to a paradoxical increase in the amount of label axonally transported to the tectum was observed. This enhancement in tectal labeling is likely to be a non-specific effect, due to the presence of additional protein in the injected volume, since co-injection of ^{125}I-WGA with comparable amounts of BSA produced similar results. The most likely explanation for this finding is that the additional protein decreases the availabilty of a large number of non-specific binding sites in the retina. A high level of nonspecific background labeling has been observed in studies of lectin binding to the cell surfaces of cultured neurons (Pfenninger and Maylié-Pfenninger, 1981; Gonatas et al. 1980), and pre-washing the cells in a BSA solution has, in part, been effective at reducing this labeling.

A number of other possible mechanisms, however, might also account for this increase in the amount of axonally transported label. Higher concentrations of protein in the vitreous might have a direct effect on enhancing retinal ganglion cell endocytosis, as has been shown for poly-l-ornithine (Itaya et al., 1978). Alternatively, higher concentrations of protein in the vitreous might indirectly increase availability of WGA to these cells by saturating mechanisms responsible for the clearance of WGA from the vitreous. These mechanisms might be either proteolytic or endocytic in nature.

To further test the hypothesis that the uptake and subsequent transport of WGA is dependent on the lectin's ability to bind specific carbohydrate residues, ^{125}I-WGA was co-injected with N,N diacetylchitobiose. Despite the greater affinity of WGA for this sugar (K_d = 4.9 X 10^{-5} M) than for NAcGlu (K_d = 7.6 X 10^{-4} M) (Nagata and Burger, 1974), concentrations of N,N diacetylchitobiose as high as 0.77 M failed to reduce the amount of label axonally transported to the tecta of these animals. (Due to the solubility characteristics of N,N-diacetylchitobiose in 0.1M phosphate buffer, higher concentrations were not attainable.) Furthermore, co-injection of N,N diacetylchitobiose at concentrations less than 0.77 M led to an increase in the amount of axonally transported label. Other investigators have reported a similar difficulty in blocking the anterograde transport of WGA-HRP by co-injection with either NAcGlu or N,N,N-triacetylchtobiose (Wiley et al. 1982; Pugh and Kahlia, 1982). Furthermore, Borges and Sidman (1982), reported an enhancement of the retrograde transport of WGA when this lectin was co-injected with a chitin hydrolysate.

Although these findings are in apparent conflict with the rest of the data presented in this chapter, for a number of reasons it is likely that the injected N,N,-diacetylchitobiose was simply not effective at competing with the retinal ganglion cells for WGA's specific carbohydrate binding sites. First, since the free volume of the vitreal chamber is about 140 µl (LaVail and LaVail, 1974), the effective concentration of the injected sugar was reduced almost ten-fold, to a final concentration of approximately 0.015 - 0.077 M. To inhibit specific lectin binding to cultured neurons, tissue sections, or synaptic membrane fractions, con-

centration of the appropriate hapten sugar ranging from 0.1 to 0.5 M are generally used (Pfenninger and Maylié-Pfenninger, 1981a; Gonatas and Avrameas, 1977; McLaughlin et. al., 1980, Gurd, 1977). Second, the excess injected sugar might be rapidly cleared from the vitreous by capillary endothelial cells of the pecten occularis, whereas bound WGA would not. Thus, over the twenty-five hour test period, the effective concentration of the competing sugar would drop relative to the concentration of WGA available to retinal ganglion cells. Lastly, since WGA has four carbohydrate binding sites, it may be stereologically possible for one WGA molecule to be bound to several different retinal ganglion cell glycoconjugates at the same time. This would lead to a exponential decrease in the effective K_d between WGA and the plasma membrane of retinal ganglion cells, thus making any competition with N,N,-diacetylchitobiose essentially impossible to detect with the assay which was used. (For example, as described by Adair and Kornfeld (1974) the isolated erythrocyte receptor for WGA appears to bind the lectin 15,000 times more effectively than does the haptene sugar NAcGlu.)

The observed increase in tectal labeling is more difficult to explain. Borges and Sidman (1982) have suggested that this may be due to enhanced uptake of the lectin-sugar complex as a result of binding a carbohydrate selective receptor in the neuronal plama membrane. Alternatively, N,N, diacetylchitobiose may directly induce an increased level of endocytosis in retinal ganglion cells as well as in other neurons.

TABLE 2

TECTAL LABELING 25 HOURS AFTER INTRAVITREAL INJECTION OF 15 μCi ^{125}I-WGA

	N	DPM *
Ipsilateral Tectal Radioactivity	10	467 ± 66
Contralateral Tectal Radioactivity	10	4984 ± 650
Transported Radioactivity $\frac{\Sigma(C - I)}{N}$	10	4508 ± 626

* $\bar{X}$ and SEM

TABLE 3

TCA PRECIPITABLE AND TCA SOLUBLE FRACTIONS OF TRANSPORTED LABEL 25 HOURS TER INTRAVITREAL INJECTION OF 15 μCi ^{125}I-WGA

	N	% TCA PPT*	% TCA SOL*
Ipsilateral Tectal Radioactivity	9	33 ± 2	67 ± 2
Contralateral Tectal Radioactivity	9	77 ± 4	23 ± 4
Transported Radioactivity $\frac{\Sigma(C - I)}{N}$	9	94 ± 1	6 ± 1

* $\bar{X}$ and SEM

TABLE 4

TECTAL LABELING 25 HOURS AFTER INTRAVITREAL INJECTION OF ^{125}I-BSA OR INACTIVE ^{125}I-WGA

Sample	N	Dose	Ipsilateral (DPM)*	Contralateral (DPM)*	Transported (DPM)*
^{125}I-BSA	6	4 µCi	339 ± 39	377 ± 54	41 ± 61
"	6	7 µCi	227 ± 21	271 ± 31	44 ± 26
"	6	13 µCi	429 ± 46	613 ± 44	77 ± 29
"	3	17 µCi	419 ± 76	489 ± 103	100 ± 46
"	7	26 µCi	530 ± 56	647 ± 264	107 ± 56
"	4	32 µCi	584 ± 130	857 ± 173	144 ± 34
"	6	59 µCi	1474 ± 53	1606 ± 46	133 ± 33
inactive ^{125}I-WGA	3	4 µCi	290 ± 31	389 ± 53	97 ± 27
"	2	10 µCi	259 ± 3	416 ± 3	150 ± 10
"	2	12 µCi	263 ± 40	589 ± 109	387 ± 79
"	7	17 µCi	501 ± 20	881 ± 91	381 ± 93
"	4	25 µCi	393 ± 80	616 ± 83	226 ± 63

* $\bar{X}$ and SEM

TABLE 5

TECTAL LABELING 25 HOURS AFTER INTRAVITREAL CO-INJECTION OF 13 μCi OF ^{125}I-WGA AND VARYING AMOUNTS OF N,N, DIACETYLCHITOBIOSE

Conc. of N,N Diacetyl-chitobiose (M)	N	Transported DPM * (% of control)
0	6	3889 ± 601 (100 %)
0.15	9	14,570 ± 2270 (375 %)
0.30	9	13,344 ± 1387 (343 %)
0.55	9	10,199 ± 1257 (262 %)
0.77	9	5174 ± 811 (133 %)

* $\bar{X}$ and SEM

CHAPTER 4

RATE OF ANTEROGRADE TRANSPORT OF WHEAT GERM AGGLUTININ FROM RETINA TO OPTIC TECTUM IN THE CHICK

INTRODUCTION

Due to its particularly high affinity for N-acetylglucosamine (NAcGlu) and sialic acid, wheat germ agglutinin (WGA) has been widely used as a probe for the recognition of specific membrane glycoconjugates during neuronal development (DeSilva et al., 1979; McLaughlin et al., 1980, Mintz et al., 1981; Pfenninger and Maylié-Pfenninger, 1981). Recently, its use has been extended to the study of axonal transport. Thoenen and his colleagues (Dumas et al., 1980; Stoeckel et al., 1977) and Gonatas and co-workers (Gonatas, 1979; Gonatas et al., 1979) have studied the retrograde axonal transport of this lectin by rat superior cervical ganglion cells, and we (Margolis et al., 1981) have examined the selective uptake and anterogrde transport of WGA by retinal ganglion cells of the chick. Following intravitreal injection of ^{125}I-WGA, radioactive label was found by light microscopic autoradiography over the retino-receptive layers of the contralateral optic tectum. Polyacrylamide gel electrophoresis of this transported label revealed a single major radioactive band which comigrated with native WGA. Whereas affinity purified ^{125}I-WGA was taken up and transported, ^{125}I-WGA that had lost its ability to to bind NAcGlu was not transported by these neurons.

Although an extensive literature exists on the subject of anterograde transport of proteins that have been newly synthesized from labeled precursors, very little is known about the anterograde transport of

exogenous proteins introduced to neuronal cell bodies. Prior to our findings, horseradish peroxidase (HRP) was the only protein known to be processed by nerve cells in this manner (Hansson, 1973; LaVail and LaVail, 1974; Mesulam and Mufson, 1980; Sotelo and Riche, 1974). Since it seems possible that the intracellular pathway taken by such proteins would differ from that taken by a newly synthesized one, and in the case of WGA may parallel the path of selectively endocytosed and recycled neuronal membrane, we have begun a systematic investigation of the uptake and anterograde transport of ^{125}I-WGA. In this paper we report rates of anterograde axonal transport of ^{125}I-WGA in retinal ganglion cells of the chick visual system that are based on two lines of evidence.

MATERIAL AND METHODS

Affinity purified ^{125}I-WGA was prepared as described in chapter 2. Various concentrations of the labeled lectin in 10 µl of 0.1 M phosphate buffer (pH 7.4) were injected into the vitreal chamber of 1-day-old chicks through a hole made a few seconds earlier in the scleral margin.

For the initial study of accumulation of ^{125}I-WGA in the tectum, each of 58 chicks was injected with 8 µCi of ^{125}I-WGA (sp. act. 13-16 µCi/µg). These chicks were then killed by perfusion through the heart with 0.1 M phosphate buffer (pH 7.4) containing 5% sucrose and 0.9% NaCl (PBSS) at times ranging from 4 to 48 hours later. The optic tecta of these animals were rapidly dissected, rinsed in 2 changes of cold PBSS and counted in a Beckman Biogamma gamma Counter (70% efficiency).

Since retinal ganglion cell axons of one eye innervate only the contralateral and not the ipsilateral optic tectum, the amount of radioactivity in the ipsilateral optic tecta of these animals serves as a

measure of nonspecific tissue labeling. The difference between the amount of radioactivity in the contralateral and ipsilateral optic tecta of each animal may, therefore, be used as a measure of the amount of label that was axonally transported to the contralateral tectum. We shall refer to this value as axonally transported label (ATL).

RESULTS

As seen in Fig. 4.1, axonally transported label was first detected in the contralateral optic tectum at 12 hours after injection. Thereafter, it accumulated linearly in the tectum for up to 48 hours as determined by analysis of variance for significant slope ($P = 0.48$; $df = 7,8$) (Zar, 1974).

In an effort to determine whether our method of detection was simply not sensitive enough to detect lectin that might have arrived in the tectum before 12 hours, additional chicks were injected with higher concentrations of ^{125}I-WGA (up to 52 μCi) and sacrificed from 6 to 10 hours later. In these chicks, the earliest time at which axonally transported label was detected in the contralateral optic tectum was 8.5 hours after injection.

Assuming a transit time of 8.5 hours and a minimal retinal ganglion cell axon length of 8.0 mm from the retina to the anterior pole of the tectum (LaVail and LaVail, 1974), an estimate of 22 mm/day is obtained for the rate of axonal transport of ^{125}I-WGA. Since even greater concentrations of injected ^{125}I-WGA might reveal shorter times of transit, this value might be an underestimation of the most rapid rate of transport of this lectin.

A more direct and possibly more accurate measure of the rate of

axonal transport was suggested to us by the linear accumulation of label in the optic tectum. This pattern of accumulation suggests that the rate of clearance of degraded ^{125}I-WGA from the optic tectectum is nominal in the first 48 hours. Given this assumption (discussed below), the rate of accumulation of label during this time period reflects the rate of arrival of ^{125}I-WGA to the tectum from the optic optic tract. If we also know how much label is passing through a known length of optic tract en route to the tectum at a given time, we can calculate how many times this section of nerve must be cleared of its label in order to account for the observed rate of arrival. The net result of such an analysis is an estimate of the rate of axonal transport as expressed by the following equation:

$$R_{(mm/day)} = \frac{ATL_{(optic\ tectum)} \ / \ t_{(h)}}{ATL_{(optic\ tract)} \ / \ L_{(mm)}} \times 24\ h/day$$

where R is rate of transport, t is the number of hours of accumulation of label in the optic tectum (calculated by subtracting the time of first arrival of axonally transported label to the tectum from the time of survival) and L is the length of optic tract sampled.

To accomplish such an analysis 12 chicks were injected intravitreally with 12-52 μCi of ^{125}I-WGA (18 μCi/μg spec. act.). Twenty-five hours later they were killed by cardiac perfusion with PBSS. The brains were quickly removed from the skull, and the dura was cut and stripped from the base of the brain. The optic tecta as well as sections of the optic tract were then dissected out, rinsed and assayed for radioactivity

(Table 1). The excised portions of optic tract measured 2.2 $\pm$ 0.08 mm in length. Assuming that axonally transported ^{125}I-WGA first arrives in the optic tectum 8.5 hours after injection, the rate of transport was then calculated for each of these animals using the above equation. The data from these calculations yield an average rate of axonal transport of ^{125}I-WGA of 34.5 $\pm$ 10 mm/ day (Table 6), and analysis of variance indicates no significant differences between the rates of axonal transport determined with different doses of injected lectin ($P = 0.10$; $df = 2,9$).

There are several advantages of using this method to determine the rate of transport. First, it is independent of estimates of axon length. Second, it selectively distinguishes rate of axonal transport from rates of endocytosis or transport through the cell body. Lastly, it is independent of the concentration of injected lectin.

However, it must also be recognized that an accurate determination of the rate of axonal transport by this equation depends on at least 4 assumptions: (1) the rate of accumulation of the label in the optic tectum must be constant. This condition is satisfied, as is illustrated by the linearity of the data presented in Fig. 6; (2) the amount of labeled protein in the optic tract that is not being transported must represent only a small percentage of the total radioactivity that is present in the tract. The work of Cuénod and Schobach (1971) as well as others (Ochs, 1975), demonstrates that at least for rapidly transported, endogenously synthesized proteins, this assumption is valid; (3) the proportion of label in the optic tract destined for transport to a site other than the optic tectum must be minimal. Based on the work of

Crossland et al. (1973) and our own observations, the major site of termination of retinal fibers in the avian brain is the optic tectum; (4) lastly, the clearance of degraded label from the optic tectum must be relatively small as compared to the rate of arrival of labeled protein to the tectum. We have assumed this to be the case in the present study because of the linear accumulation of label in the tectum with time. A progressively decreasing rate of accumulation would otherwise be expected.

Regardless of hypothetical arguements, the true test of this method for dedetermining rates of axonal transport rests on its accuracy and reliability. In this study, rates of axonal transport calculated by this method were reproducible within the range of doses injected and they were in reasonable close agreement with estimates of rate obtained by the method of time of first arrival.

DISCUSSION

Newly synthesized proteins are transported down the axons of nerve cells at rates varying from 0.2 to 500 mm/day (Grafstein and Forman, 1980), and proteins characteristic of specific transport rates have been hypothesized to move as both structural and functional complexes (Black and Lasek, 1980). The rate of transport of ^{125}I-WGA is determined in this study is much slower than that found for many endogenous proteins. It is also slower than that found for the rate of anterograde axonal transport of HRP, the only other exogenous protein known thus far to be processed by neurons in this manner. HRP has been described as moving at a range of rates the least of which is 120 mm/day (Harper et al., 1980; Mesulam and Mufson, 1980; Sotelo and Riche, 1974). This difference

in the rate of transport of WGA from that of HRP may reflect a difference in the intracellular pathway taken by these proteins. If so, it extends the findings of Gonatas (1979) and Harper et al. (1980) who found that following endocytosis by rat superior cervical ganglion neurons, HRP had a different ultrastructural localization than did HRP conjugated to WGA. HRP was localized primarily in lysosomes in the cell body, whereas HRP conjugated to WGA was not only in lysosomes but also in elements of the Golgi apparatus.

Although apparently different than the rate of axonal transport of HRP, the rate of transport of WGA is within the range of transport rates of the group II proteins described by Lorenz and Willard (1978). These newly synthesized proteins were found to move down the axons of rabbit retinal ganglion cells at a rate of 34-68mm/day. Thus, similar mechanisms might be involved in the the axonal transport of WGA and the group II proteins. Alternatively, this similarity in rates may be merely coincidental, and WGA may be transported through the cell by a different mechanism than group II proteins. Since WGA is known to have a high affinity for specific glycoproteins associated with the neuronal plasma membrane (Gurd, 1977), the intracellular route followed by this lectin may reflect a novel pathway important in membrane recycling or receptor mediated endocytosis.

TABLE 6

SUMMARY OF ESTIMATED RATES OF ANTEROGRADE AXONAL TRANSPORT OF ^{125}I-WGA BY CHICK RETINAL GANGLION CELLS (Number of animals is shown in parenthesis. Radioactivity in optic tract and optic tectum is the radioactivity in the contralateral side minus the radioactivity in the ipsilateral side ± S.E.M.)

Dose (μCi)	Radioactivity in optic tract (dpm)	Radioactivity in optic tectum (dpm)	Estimated rate (mm/day)
12 (5)	116.6 ± 47	1150.0 ± 351	35 ± 4.3
23 (4)	200.0 ± 35	2275.0 ± 701	41 ± 5.2
52 (3)	1046.7 ± 134	7900.0 ± 989	25 ± 5.8

CHAPTER 5

INDEPENDENCE OF ^{125}I-WGA UPTAKE AND TRANSPORT ON AXONAL TRANSPORT OF NEWLY SYNTHESIZED PROTEINS

INTRODUCTION

In previous chapters I have suggested that WGA is taken up and axonally transported in an anterograde direction by chick retinal ganglion cells in a manner that differs from that of HRP, a marker of fluid phase endocytosis. The uptake and anterograde transport of ^{125}I-WGA is a saturable process which correlates with the lectin's ability to bind NAcGlu. Furthermore, the rate of anterograde axonal transport of ^{125}I-WGA (22-44mm/day) (Margolis and LaVail, 1982), is significantly slower than that reported for HRP. How, then, is WGA taken up and transported through the cell?

One might envision a number of different pathways that WGA could follow through the cell other than that followed by HRP. One possibility is that WGA enters the cell by adsorptive endocytosis, remains bound to glycoconjugates in the membrane, and subsequently moves with intracellular membrane. Thus, the anterograde transport of the lectin might reflect a pathway for the reuse of endocytosed perikaryal membrane at the nerve terminal. Alternatively, following endocytosis, WGA may dissociate from the membrane, and its transport may become associated with the flow of newly synthesized material destined for the axon and nerve terminal. WGA's affinity for specific carbohydrates makes it a candidate for an association with the transport of newly synthesized glycoconjugates through direct linkages to the sugar groups of these

macromolecules. Several lines of indirect evidence suggest the possible association of WGA with the transport of newly synthesized protein, specifically glycoprotein. First, light microscopic autoradiographic studies of the chick optic tectum reveal similar labeling patterns following intravitreal injection of either ^{125}I-WGA (see chapter 2), ^{3}H-leucine (Crossland et al., 1973), or ^{3}H-fucose (Gremo et al., 1974). Second, the rate of transport of ^{125}I-WGA, 22-44 mm/day, is similar to the rate of transport of the group II proteins described by Willard et al. (1973) in rabbit retinal ganglion cells as well as a distinct group of glycoproteins described by Levin (1977) in rat locus coeruleus neurons. Third, Karlsson (1979) has demonstrated an affinity of WGA for 45% of the glycoprotein rapidly transported in rabbit retinal ganglion cell bodies. Fourth, following endocytosis by rat superior cervical ganglion cells, WGA-HRP has been localized to the Golgi apparatus (Harper et al., 1980), the presumptive site of terminal glycosylation of glycoprotein. Work by Droz (1967) and Rambourg and Droz (1969), as well as more recent work by Ellisman and coworkers (Lindsey et al., 1981; Hammerschlag et al., 1982) suggest that rapidly transported proteins and glycoproteins pass through the Golgi apparatus prior to their axonal transport. Lastly, electron microscopic autoradiographic analysis of axonally transported ^{125}I-WGA in chick retinal ganglion cells suggests that the lectin accumulates in subaxolemmal regions of axons (LaVail et al., 1983). A similar but less restricted distribution has been described for rapidly transported protein in pigeon retinal ganglion cells (Markov et al., 1976) and rapidly transported glycoprotein in chick parasympathetic neurons (Schonbach et al.,

al., 1972).

In this chapter I present evidence which supports the hypothesis that the axonal transport of ^{125}I-WGA is not associated with the flow of newly synthesized protein to the nerve terminal. Other possible pathways for the transport of WGA are discussed.

MATERIALS AND METHODS

Animals used in this study were 1 to 3-day-old White Leghorn chicks. Cycloheximide (CHX) was obtained from Sigma Chemical Co., and ^{3}H-proline (sp. act. 100 Ci/mmole) from Amersham Inc. Prior to its use, the tritiated proline was dessicated under nitrogen and redissolved in phosphate-buffered saline (PBS) (pH 7.4) to a final concentration of 1 μCi/μl. Affinity purified ^{125}I-WGA (sp act. 15-18 μCi/μg) was prepared as described in chapter 2 and injected in 0.1 M phosphate buffer (pH 7.4) at a final concentration of 1 μCi/μl, i.e., approximately 1.4 μM.

All animals were injected with 15 μl of solution while under ether anesthesia. Injections were made into the right vitreal chamber through a hole made a few seconds earlier in the scleral margin. In a small number of animals reflux of injected material out of the eye was observed. These animals were excluded from further analysis.

In order to determine the amount of radioactive label transported to the chick optic tectum 25 hours after intravitreal injection of ^{3}H- proline, each of sixteen chicks was injected with 15 μCi of ^{3}H-proline. Twenty-five hours later these animals were killed by perfusion through the heart with phosphate-buffered saline (pH 7.4) containing 5% sucrose (PBSS). Each optic tectum of these animals was rapidly dissected,

rinsed in two changes of cold PBSS, and stored in 1 ml of 1M NaOH overnight at 4°C. The following day the tecta were homogenized in NaOH and 50 ul of the resulting homogenates were mixed with 12 mls of toluene based scintillation fluid containing 25% (v/v) Triton X 100, 0.5% (w/v) diphenyloxazole, and 0.01% (w/v) 1,4 bis-2(4-methylphenoxazolyl)-benzene. The samples were then counted for 10 minutes in a Beckman Liquid Scintillation counter. Disintegrations per minute were calculated using a quench curve and an external standard, from which total tectal radioactivity was calculated. Since all dissected tecta are very similar in size (see chapter 3) total tectal radioactivity was expressed as dpm/tectum.

In order to determine the relative contributions of acid precipitable and acid soluble radioactivity in the tectum, an additional twelve chicks were injected with 15 μCi ^{3}H-proline, and twenty-five hours later perfused as described above. Each optic tectum from these animals was subsequently dissected, rinsed twice in cold PBSS, and homogenized in 0.5 ml of distilled water at 4°C. An additional 0.3 ml of distilled water used to rinse the homogenizer was combined with the homogenate. Aliquots of 0.2 ml of 60% trichloroacetic acid (TCA) were then added to the homogenates. Following a 40 minute incubation at 4°C, the TCA homogenates were centrifuged and the pellets were washed twice with 10% TCA. The supernatants from the centrifugations were combined, and aliquots from these supernatants were mixed with scintillation fluid and assayed for radioactivity as described above. The washed TCA insoluble pellets were dissolved in 1 N NaOH and aliquots from these solutions were similarly assayed for radioactivity.

In order to determine the relative size of the free proline pool available to the retinal ganglion cells for new protein synthesis, chicks were intravitreally injected with 15 uCi of ^{3}H-proline at five different specific activities. Eight chicks were injected with ^{3}H-proline at a specific activity of 100 Ci/mmole, and an additional 36 chicks were injected with ^{3}H-proline ranging in specific activity from 10 mCi/mmole to 10 Ci/mmole. Twenty-five hours after intravitreal injection the animals were perfused with PBSS, and their tecta were dissected and assayed for radioactivity as described above, with the exception that Hydroflour (National Diagnostics) replaced the toluene based scintillant used earlier.

In order to determine the effect of CHX on the axonal transport of newly synthesized proteins, each of 56 chicks was injected intravitreally with either 15 µl of PBS, or 15 µl of PBS containing CHX (1-80 µg/animal). One hour later, these same chicks had the same eye injected with PBS containing 15 µCi of ^{3}Hproline. Twenty-five hours after the ^{3}H-proline injection the chicks were perfused through the heart with PBSS, and the optic tecta were dissected, incubated in NaOH, mixed with toluene based scintillation fluid, and assayed for radioactivity as described above.

In order to determine the effect of CHX on the uptake and anterograde axonal transport of ^{125}I-WGA, each of 30 chicks were injected intravitreally with either PBS or CHX (1-20 µg) in PBS. One hour later, the same eye of each chick was injected with 15 µCi of ^{125}I-WGA in 15 µl of 0.1 M phosphate buffer (pH 7.4). Twenty-five hours after this second

injection, the chicks were perfused with PBSS and the optic tecta were dissected, rinsed twice in cold PBSS, and assayed for radioactivity in a Beckman Biogamma gamma counter with a 70% counting efficiency. The amount of radioactive label which was axonally transported to the contra lateral tecta of these animals was calculated as described in chapter 3.

In order to determine the effect of CHX on the uptake and transport of WGA during saturating conditions, an additional sixteen chicks were each injected intravitreally with either PBS or CHX (20 μg) in PBS. One hour later these same animals were each injected with a mixture of 15 μCi ^{125}I-WGA and excess native WGA (286 μM) in 0.1 M phosphate buffer. Twenty-five hours after this second injection these animals were perfused with PBSS, and their tecta were assayed for radioactivity as described above.

RESULTS

Axonal Transport of Protein to the Chick Optic Tectum

Twenty-five hours after intravitreal injection of ^{3}H-proline, radioactive label was found in both the ipsilateral and contralateral optic tecta of all ten chicks investigated (see Table 7). Since the visual pathway of the chick is completely crossed, the level of radioactivity in the ipsilateral tectum of each animal was considered to be a measure of background labeling, and was subtracted from the level of radioactivity in the contralateral tectum of each animal in order to obtain a measure of axonally transported label. As analyzed in this fashion, the average amount of background labeling in these animals was 10,160 $\pm$ 493 (SEM) dpm/tectum, and the average amount of label transported to the contralateral tecta of these animals was 42,956 $\pm$ 4711 (SEM) dpm.

In order to determine what fraction of this tectal label was due to the transport of radioactive protein, twelve additional animals were injected with ^{3}H-proline, and twenty-five hours later their tecta were assayed for TCA precipitable radioactivity. As summarized in Table 8, 98% of the transported radioactive label was TCA precipitable. This suggests that protein accounts for at least 98% of the radioactive label axonally transported to the contralateral optic tectum in these animals.

Concentration of Free Proline Available to Chick Retinal Ganglion Cells

The amount of native proline which is available to chick retinal ganglion cells for incorporation into rapidly transported protein was determined by isotope dilution. The experimental procedure was similar to that used by Neale et al. (1974) in determining the size of the free proline pool available for protein synthesis by goldfish retinal ganglion cells. In my experiments, chicks were each injected with either 15 μCi of ^{3}H-proline at a specific activity of 100 Ci/mmole or 15 μCi of ^{3}H-proline which had been diluted with native proline to a lower specific activity. Twenty-five hours later the optic tecta from these animals were assayed for transported radioactive label. As summarized in Table 9, an average of 46,486 dpm of transported label had accumulated in the contralateral tecta of those animals injected with ^{3}H-proline at a specific activity of 100 Ci/mmole, whereas an average of 26,160 dpm of transported label had accumulated in the contralateral tecta of those animals injected with ^{3}H-proline at a specific activity of 10 mCi/mmole. Thus, a ten thousand-fold dilution in specific activity resulted in a 1.8 fold difference in the amount of label transported to the tectum. In those animals injected with ^{3}H-proline where the specific activity was

diluted only ten, one hundred, or one thousand-fold, no significant differences in the amount of transported label were observed as assessed by the Students t test ($p > 0.1$).

This data has been used to estimate the size of the native proline pool available to ganglion cells in the retina, as well as the total amount of protein which is synthesized and subsequently transported to the tectum within a twenty-five hour period. The derivations of the equations necessary for these calculations are presented in the Appendix, as are several underlying assumptions. By applying these equations to the data presented above, one may calculate that the steady state size of the freely interchangeable native proline pool in the chick retina is 0.77 μmoles, and that during the 25 hour experimental period, approximately 8.85 nmoles of proline are transported to the tectum by retinal ganglion cells.

Effect of CHX on the Transport of Protein By Retinal Ganglion Cells

CHX is a potent inhibitor of protein synthesis and has been shown to reduce dramatically the synthesis and axonal transport of ^{3}H-proline containing proteins in both rabbit and goldfish retinal ganglion cells (Sturman, 1979; Ingoglia et. al., 1978; Heacock and Agranoff, 1977). To test the effect of CHX on the ability of retinal ganglion cells to synthesize and transport protein, chicks were intravitreally injected with CHX, and one hour later these same animals were injected with ^{3}H-proline. Twenty-five hours after the injection of ^{3}H-proline the tecta of these animals were assayed for transported radioactive label. Since 98% of transported label is TCA precipitable (see above), the amount of transported label served as a measure of the amount of transported protein.

Animals which had been pre-injected with PBS instead of CHX served as controls. As summarized in Figure 5.1, there was a dose dependent reduction in the amount of radioactive label transported to the optic tecta of chicks pre-injected with CHX as compared to those pre-injected with PBS. This reduction in tectal labeling was statistically significant (Student t test, $p > 0.1$) for all doses of CHX used, and ranged from a 60% reduction in tectal labeling for a 1 μg dose of CHX, to a 92% reduction for a 60 μg dose of CHX. Doses of 40 μg and higher, however, produced signs of systemic toxicity. One out of 7 animals injected with 40 μg of CHX exhibited marked lethargy, ascites and intestinal enlargement, 6 out of 8 animals injected with 60 μg of CHX died within twenty-hours of injection, and all 8 animals injected with doses of 80 μg of CHX died within 18 hours of injection. These findings indicate that dosages of 20-30 μg of CHX per animal produce the maximal reduction in transport of radioactive label (and thus protein) to the tectum (84-85%) without producing obvious systemic effects.

Effect of CHX on the Uptake and Transport of ^{125}I-WGA

Next, to test the dependence of WGA uptake and transport on the continued synthesis and transport of newly synthesized protein, 23 chicks were injected with CHX, and the effect of this drug on the uptake and subsequent anterograde transport of ^{125}I-WGA by retinal ganglion cells was assessed. As seen in Figure 5.1, pre-injection of chicks with CHX (1-20 μg) failed to produce any significant effect on the amount of label transported to the optic tectum following injection with ^{125}I-WGA. This was true for all doses of cycloheximide tested.

The above findings suggest that either the transport of WGA is independent of the transport of newly synthesized protein e.g.,the transport of WGA is not due to a direct linkage between the lectin and a carbohydrate side chain of a glycoprotein, or that the reduced amount of protein which continues to be synthesized and transported in the presence of CHX is sufficient for the transport of approximately 16,000 dpm (1.35×10^{-14} moles) of ^{125}I-WGA.

In order to differentiate between these possibilites an additional experiment was carried out. Each of five chicks was pre-injected with 20 µg of CHX, and one hour later injected with 15 µCi of ^{125}I-WGA in a 286 µM solution of native WGA. At these levels of injected lectin the pathway for the specific uptake and transport of WGA is limiting, as evidenced by competition between labeled and native WGA (see Table 10), and any effects that CHX has on on this pathway will be more likely apparent. The amount of radioactive label transported to the tecta of these animals (6498 dpm) was somewhat less than the amount of label transported to the tecta of control animals (8250 dpm) which were pre-injected with PBS. This apparent reduction in tectal labeling, however, was not statistically significant ($t = 1.5976$; $p = 0.13$; $df = 12$).

DISCUSSION

A major function of axonal transport is to provide newly synthesized proteins to axons and nerve terminals which lack protein synthetic machinery. Thus, newly synthesized proteins are continuously being shipped out of the neuron cell body by axonal transport for their use in the axon and nerve terminal. One way in which investigators have studied this flow of newly synthesized proteins is by labeling them

isotopically through the use of radioactive amino acids. In this study I have made use of ^{3}H-proline to label proteins which are synthesized by chick retinal ganglion cells and subsequently transported to their nerve terminals in the optic tectum. Chicks were injected with ^{3}H-proline, and twenty-five hours later the tecta from these animals were assayed for radioactivity by liquid scintillation spectrophotometry. The difference between the amount of radioactivity in the ipsilateral and contralateral tectum of each animal was then used as a measure of axonally transported label. Since 98% of this label is TCA precipitable, this was also used as a measure of the amount of ^{3}H-labeled protein that had accumulated in the contralateral tecta of these animals by axonal transport.

Twenty-five hours after chicks were intravitreally injected with 15 μCi of ^{3}H-proline, axonally transported label accounted for approximately 43,000 dpm of the total radioactive label that had accumulated in the contralateral tecta of these animals. This represents the axonal transport of 194 fmoles of ^{3}H-proline. Since over the time course of this experiment ^{3}H-proline accounts for 0.0022% of the total proline pool (^{3}H-proline + native proline) available to retinal ganglion cells for synthesis into rapidly transported protein (see Appendix), then the total amount of proline (^{3}H-proline + native proline) which was rapidly transported to the tecta of these animals may be estimated as 8.85 nmoles. Assuming that that rapidly transported protein has a proline content of 4% (Lehninger, 1970) and an average molecular weight of 100,000 daltons (Lorenz and Willard, 1978), then this represents the rapid axonal transport of approximately 295 pmoles of newly synthesized

protein to the chick optic tectum over a 25 hour period.*

By comparison, in the course of these experiments the maximum amount of WGA which was observed to be transported to the contralateral optic tectum by retinal ganglion cells wass 550 fmoles (see chapter 3). Thus, if WGA is transported to the distal axon and nerve terminal by piggy-backing with newly synthesized glycoprotein, then 0.09% of the newly synthesized protein destined for the nerve terminal must exhibit a stereologically accessible WGA binding site. Since 45% of rapidly transported glycoprotein in rabbit retinal ganglion cells has an affinity for WGA as assayed by affinity chromotography (Karlsson, 1979), and a large fraction of rapidly transported protein is likely to be glycoprotein (Grafstein and Forman, 1980), from theoretical arguments it would seem possible for such an association to exist. Experimental data presented in this chapter, however, suggests otherwise.

When chick retinal ganglion cells were incubated with CHX by means of intravitreal injection, a dose dependent reduction in the axonal transport of newly synthesized protein was observed. Similar concentrations of CHX, however, were not effective at reducing the axonal transport of ^{125}I-WGA, even under experimental conditions where the pathway for the uptake and and transport of the lectin approached saturation. These findings thus support the hypothesis that the axonal transport of ^{125}I-WGA is independent of the rapid transport of newly synthesized protein. However, before discussing alternative pathways for the

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* If one assumes a delay of two hours before any radioactive protein reaches the tectum, then the test period is really twenty-three hours long. This was taken into account in the Appendix when proline pool sizes were determined using isotope dilution data.

transport of ^{125}I-WGA, two limitations of the present findings should be discussed.

First, it should be emphasized that through the use of CHX the axonal transport of newly synthesized protein could only be reliably inhibited by up to 84%. Since a reduced amount of protein continues to be synthesized and transported under these conditions, the successful transport of ^{125}I-WGA in CHX treated animals may be due to an association between the lectin and this remaining fraction of transported protein. The possibility of an unrecognized association between the transport of ^{125}I-WGA and the transport of newly synthesized protein should even be considered in those experiments that were carried out at saturation, since the limiting step in such a pathway may not be the availability of stereologically accessible lectin binding sites on newly synthesized protein, but rather some aspect of endocytosis or perikaryal sorting.

Second, the experimental sample size in the CHX/WGA experiment conducted under limiting conditions should be considered. This experimental group consisted of only 5 animals. Given the relatively large variances in both the experimental and control group of animals, the lack of a statistically significant difference between the amount of ^{125}I-WGA transported in these groups of animals may have been due to the small sample size.

Although the transport of WGA may not be dependent on the transport of newly synthesized protein, the possibility of an association with the transport of newly synthesized lipid, particularly glycolipids such as gangliosides, should be considered. One possible problem with such a proposed pathway, is that in addition to blocking protein synthesis,

CHX appears to inhibit the synthesis and/or transport of lipids (McNamara et al., 1969; Edstrom and Mattsson, 1972; Grafstein et al., 1975). Thus, treatment with CHX would be expected to reduce the transport of ^{125}I-WGA by such a pathway; which is contrary to the findings presented in this chapter. A similar problem arises in proposing an association between WGA and newly synthesized proteoglycans.

Thus, I conclude that the transport of ^{125}I-WGA is likely to follow a pathway that is independent of all newly synthesized material. One possiblility is that ^{125}I-WGA enters the cell by adsorptive endocytosis and remains bound to internalized plasma membrane lectin receptors. The linkage between ^{125}I-WGA and its receptors may even be covalent in nature, as has been described for the receptor binding of both ^{125}I-insulin and ^{125}I-EGF prepared by the chloramine-T method (Saviolakis et al., 1981; Comens et al., 1982). The subsequent transport of ^{125}I-WGA may then reflect a pathway for the reutilization of endocytosed perikaryal membrane at the nerve terminal. Although direct evidence for an intracellular pathway of this sort has yet to be reported in neurons, pathways for the transcellular transport and/or re-use of plasma membrane have been described for a number of cell types including macrophages (Muller et. al., 1980), fibroblasts (Schneider et al., 1979), hepatocytes (Fehlman et al., 1982; Fisher et al., 1979) and epithelial cells (Abrahamson and Rodewald, 1981; Herzog, 1981).

Furthermore, the existence of such a pathway would explain some peculiar findings about the axonal transport of glycoprotein and glycolipid. For example, in their study of axonal transport in mouse retinal ganglion cells, Specht and Grafstein (1977) described evidence for a

delayed release of rapidly transported ^{3}H-labeled glycoprotein 6 days after intravitreal injection of ^{3}H-fucose. Similarly, in rabbit retinal ganglion cells, Haley et al. (1979) have described a 17 day delay in the release of a fraction of rapidly transported ^{3}H-glycerol labeled ganglioside. Although neither group of investigators elaborated on the meaning of these findings, this delayed release of glycoprotein and glycolipid from the neuronal cell body most likely reflects perikaryal use of these membrane precursors prior to their axonal transport. If these glycoconjugates were to be used in the perikaryal membrane prior to their axonal transport, they then could serve as the hypothetical plasma membrane lectin receptors discussed above. Furthermore, the delayed transport of these glycoconjugates would provide a means of axonal transport for the lectin which is independent of the transport of newly synthesized protein or lipid.

TABLE 7

TECTAL LABELING 25 HOURS AFTER INTRAVITREAL INJECTION OF 15 μCi OF ^{3}H-PROLINE

	N	DPM *
Ipsilateral Tectal Radioactivity	10	10,160 ± 493
Contralateral Tectal Radioactivity	10	51,104 ± 4561
Transported Radioactivity $\frac{\Sigma(C-I)}{N}$	10	42,956 ± 4711

* $\bar{X}$ and SEM

TABLE 8

TCA PRECIPITABLE AND TCA SOLUBLE FRACTIONS OF TRANSPORTED LABEL 25 HOURS AFTER INTRAVITREAL INJECTION OF 15 μCi OF ^{3}H-PROLINE

	N	% TCA PPT *	% TCA SOL *
Ipsilateral Tectal Radioactivity	12	73 ± 1.6	27 ± 1.6
Contralateral Tectal Radioactivity	12	91 ± 1	9 ± 1
Transported Radioactivity $\frac{\Sigma(C-I)}{N}$	12	98 ± 0.4	2 ± 0.4

* $\bar{X}$ and SEM

TABLE 9

TECTAL LABELING 25 HOURS AFTER INTRAVITREAL INJECTION OF 15 μCi OF ^{3}H-PROLINE

Specific Activity	N	Background DPM * (Ipsilateral label)	Transported DPM * (Contra. minus Ipsi. label)
100 Ci/mmole	8	7020 ± 380	46,480 ± 3328
10 Ci/mmole	8	6160 ± 240	46,940 ± 5894
1 Ci/mmole	10	6620 ± 420	39,940 ± 2220
100 mCi/mmole	8	7160 ± 880	48,400 ± 7740
10 mCi/mmole	10	6160 ± 280	26,160 ± 1620

* $\bar{X}$ and SEM

TABLE 10

EFFECT OF PRE-INJECTION OF CHX ON TECTAL LABELING

Dose of ^{125}I-WGA (μCi)	N	Conc. of native WGA (μM)	Dose of CHX (μg)	Transported DPM *
15	7	0	0 (PBS)	15,539 ± 2957
15	9	286	0 (PBS)	8250 ± 831
15	5	286	20	6498 ± 364

* $\bar{X}$ and SEM

CHAPTER 6

PRELIMINARY ULTRASTRUCTURAL FINDINGS ON THE UPTAKE OF WGA-HRP BY CHICK RETINAL GANGLION CELLS

INTRODUCTION

In previous chapters I have suggested that the uptake and transport of WGA by chick retinal ganglion cells might reflect a pathway of perikaryal membrane reuse at the nerve terminal. This hypothesis was based on a number of observations, most notably the affinity of WGA for neuneuronal plasma membrane glycoconjugates (Mintz et al., 1981; Pfenninger and Maylie-Pfenninger, 1981; Gurd, 1977; Huck and Hattan, 1981), the specificity of uptake and anterograde transport of WGA (chapter 2 and 3), the independence of the uptake and transport of WGA on the transport of newly synthesized protein (chapter 5), and the accumulation of WGA-HRP in cisterns of the Golgi apparatus following endocytosis by sympathetic neurons of the rat superior cervical ganglion (Harper et al., 1980). The observation that WGA may accumulate in cisterns of the Golgi apparatus following endocytosis is a particularly significant piece of evidence in support of this hypothesis, since Farquhar and co-workers (Farquhar, 1978; Ottosen et al., 1980; Wilson et al., 1981) have presented evidence which suggests that the Golgi apparatus plays a pivotal role in the reutilization of plasma membrane by myeloma cells, plasma cells and anterior pituitary mammotrophs.

To examine whether WGA-HRP also accumulates in the Golgi cisterns of chick retinal ganglion cells, as well as to begin to study other aspects of the intracellular transport of WGA, a preliminary study on

the ultrastructural localization of WGA-HRP was conducted. Furthermore, since WGA is a multivalent ligand which may alter membrane distribution in these cells and thus influence its intracellular fate, a preliminary stereological study of the intracellular distribution of membrane following injection of either WGA-HRP or HRP was conducted.

In this chapter I provide ultrastructural evidence for differences in the intracellular pathways of WGA-HRP and HRP in chick retinal ganglion cells. Furthermore, I provide evidence that HRP may not be acting as a passive marker of fluid phase endocytosis in these cells.

MATERIALS AND METHODS

Animals used in this study were 1 to 2 day old White Leghorn cockerels. HRP (type VI) as well as WGA which had been conjugated to HRP (WGA-HRP) were purchased from Sigma Chemical Co. Prior to their use, WGA-HRP and HRP were dissolved in 0.1 M phosphate buffer (pH 7.4) containing 0.9% NaCl (PBS). HRP was injected at a final concentration of 130 µg/µl and WGA-HRP was injected at a final concentration of 33 µg/µl.

A total of 23 chicks were used in this study. Six of the animals composed an experimental group which received intravitreal injection of WGA-HRP. These animals were sacrificed at either 1, 2, or 3 hours after injection of the lecconjugate. Twelve additional control animals were injected with either HRP or PBS and sacrificed at similar time points.

All animals were injected with 15 µl of solution while they were under ether anesthesia. Injections were made into the right vitreal chamber with the aid of a dissecting microscope as described in chapter 2. One,

2, or 3 hours after injection, the chickens were re-anesthetized and sacrificed by decapitation. The corneas were then slit, and the retinas were fixed by intraoccular perfusion with an ice cold solution of 1% formaldehyde, 1.25% glutaraldehyde and 0.12% CaCl in 0.1 M cacodylate buffer (pH 7.4) followed by immersion in this same solution for 4 hours at 4°C. After fixation the retinas were dissected into strips approximately 0.5 mm wide, and stored overnight at 4°C in 0.1 M tris maleate buffer (pH 7.4) containing 5% sucrose. The following day the tissues were rinsed in two changes of 0.1 M tris maleate buffer (pH 5.5) containing 5% sucrose and then incubated for 3 hours at 4°C in a solution of 0.05% diaminobenzidine (DAB), 0.002% hydrogen peroxide, and 5% sucrose in this same buffer. The tissue was then rinsed in 0.1 M phosphate buffer (pH 7.4) containing 5% sucrose, and postfixed for 3 hours at 4°C in a solution of 1% osmium tetroxide and 1.5% potassium ferricyanide in the same buffer. It was rapidly dehydrated in a graded series of ethanol and embedded in an Epon-Araldite mixture.

Plastic sections cut 1-2 μm thick and stained with toluidine blue were used for light microscopic examination as well as to orient the tissue for thin sectioning. Thin sections, estimated to be about 50-60 nm thick, were cut on an LKB 8800 ultramicrotome and collected on formvar coated copper grids. After staining for 15 seconds with lead citrate thin sections were examined on a Zeiss EM-10 electron microscope. Some sections were examined without heavy metal staining.

As has been reported previously (LaVail and LaVail, 1974), the retinal injection sites failed to react evenly for HRP reaction product when the tissue was prepared in this manner. Extracellular reaction product

was only observed along the edges of the blocks of embedded tissue, extending in about 10–15 μm from either edge in a decreasing gradient of intensity. The observations in this study were therefore restricted to the outer surfaces of the cut retinal slices.

Sterological proceedures were carried out using a coherent multipurpose test lattice (spacing = 5mm) (Weibel et al., 1966) superimposed on randomly selected micrographs of retinal ganglion cells (total magnification = 25,000). The surface density of a given organelle within a set of test micrographs was calculated from the number of intersections of the test lines of the lattice with the membrane of that organelle by using the formula $S_{vi} = 4N_{Li}/P_tZ$ where S_{Vi} is surface density, N_{Li} is the number of times a test line intersects a membrane of interest, Z is the calibrated length of the test line, and P_t is the number of test line end points falling within the cytoplasm of the cells of interest (Weibel et. al., 1966). As determined in this manner, surface density is expressed as membrane area per unit volume of cytoplasm, eg., mm^2/mm^3 (Williams, 1977). The total cytoplasmic area of ganglion cells within a given set of test micrographs was calculated by using the formula $A = \sqrt{3}/2\ P_t\ Z^2$, where P_t and Z are defined as described above (Weibel et al., 1966).

To study the intracellular distribution of WGA-HRP and HRP in a semi-quantitative manner, over 400 ganglion cells which contained intracellular reaction product were photographed, and then printed at a final magnification of 25,000 X. One hundred and twenty of these micrographs, covering a total cytoplasmic area of 3100 μm^2, were then randomly selected, and the labeled organelles within these micrographs were

systemtically categorized according to their morphological structure. Categories included small vesicles (diameter less than 160 nm), SER (smooth walled tubular structures), lysosomes (dense bodies, multivesicular bodies and large vesicles), Golgi associated vesicles (small vesicles within 200 nm of a Golgi cistern) and Golgi cisterns. Multivesicular bodies, dense bodies and large vesicles (diameter > 160 nm) were all included in a single category due to the difficulty in differentiating between these organelles when they were filled with reaction product. Since the absolute number of labeled organelles within a given test area of cytoplasm varied for differing experimental conditions, the data from this study were normalized, and expressed as the percent of total labeled organelles which were associated with each of the organelle categories.

These same micrographs were used to study the membrane surface density of organelles in the ganglion cells of WGA-HRP and HRP injected animals. Randomly selected micrographs of retinal ganglion cells from PBS injected animals served as controls. The surface densities of all cytoplasmic organelles were included in this study. Categories of organelles included those mentioned above as well as mitochondria (outer membrane only) and the rough endoplasmic reticulum. Measurements of the plasma membrane and nuclear membrane were not included in this study. A total cytoplasmic area of over 4600 μm^2 was examined in the course of this analysis. By sampling this amount of cytoplasmic area, enough organelle membrane was analyzed so that the confidence level of all surface density measurements could be set at $\pm$ 10%. This was determined by using the method of Williams (1977) for setting confidence limits by

calculation of a progressive mean.

RESULTS

Light Microscopic Observations

At 1, 2 and 3 hours after intravitreal injection of WGA-HRP, brown reaction product was selectively distributed in the retina. The inner limiting membrane, optic nerve fiber layer, and inner plexiform layer were all heavily labeled with reaction product. In addition, a heavy layer of reaction product surrounded the cell bodies of the ganglion cell layer. Small granules of reaction product were also observed within the perkarya of the ganglion cells and were rarely noted within the cell bodies of the photoreceptors. Reaction product was either scant or not present in the inner nuclear layer, outer nuclear layer, or outer plexiform layer of the retina.

In contrast, following intravitreal injection of HRP a more uniform distribution of enzymatic reaction product was noted. All layers of the neural retina were labeled although the fiber layers were more heavily labeled than the cellular layers Furthermore, cell bodies in all layers of the retina appeared to have endocytosed the HRP as evidenced by the presence of small granules of reaction product within the perikarya of these cells. Moreover, at all three time points the retinas of animals that had been injected with HRP appeared more heavily stained with reaction product than did the retinas of animals that had been injected with WGA-HRP. The one area of the retina which was more heavily stained following injection with WGA-HRP was the inner limiting membrane. No evidence of reaction product was observed in the retinas of those animals that had been injected with PBS.

Ultrastructural Observations

The extracellular distribution of reaction product was similar in specimens fixed at 1, 2, and 3 hours after intravitreal injection of WGA-HRP. Reaction product was evenly distributed throughout the extracellular space of the optic fiber layer, ganglion cell layer, and inner plexiform layer of the retina. A heavy accumulation of dense reaction product was noted on the inner limiting membrane (Fig 6.1). Other layers of the retina were not examined for the presence of reaction product. A similar extracellular distribution of label was observed following intravitreal injection of HRP. No extracellular labeling was noted following intravitreal injection of PBS.

At 1, 2, and 3 hours following intravitreal injection of WGA-HRP, reaction product was also observed intracellularly, within membrane bound organelles of Mueller cells and ganglion cells (Fig. 6.1). In the Mueller cells reaction product was found almost exclusively in multivesicular bodies (MVBs). In the ganglion cells, however, reaction product was noted in vesicles ranging in diameter from 30 nm to 360 nm, dense bodies, MVBs, elements of the smooth endoplasmic reticulum (SER), and Golgi associated vesicles (Fig. 6.2 a,b,c). Reaction product was also occaisionally observed in 1 or 2 of the outermost cisternae of the Golgi apparatus (Fig. 6.2 a,c), but due to the heterogeneous appearence of the Golgi complex in these cells, it was impossible to determine whether the labeled cisternae were present only at the transforming face of the organelle as has been suggested by Harper et al. (1980). Reaction product was never observed in association with mitochondria, nuclei, or elements of the rough endoplasmic reticulum.

A similar population of labeled organelles was observed following intravitreal injection of HRP (Fig. 6.3 a,b). However, the relative distribution of reaction product in these organelles differed from that which was seen in the ganglion cells of animals which had been injected with WGA-HRP (see Table 11). The most distinct difference was the greater relative labeling of the Golgi cisterns and Golgi associated vesicles in the WGA-HRP injected animals (see Table 11). This difference was most dramatic at 1 hour after intravitreal injection when the relative labeling of the Golgi cisterns in WGA-HRP injected animals was six times as great as the relative labeling which was observed in HRP injected animals.

The data presented in Table 11 also indicates that following both WGA-HRP and HRP injections there is a shift in the relative labeling of organelles with time. The most distinctive changes which are noted in both sets of animals are a decrease in the relative labeling of the Golgi associated vesicles and an increase in the relative labeling of lysosomal structures. In those animals injected with PBS, no intracellular reaction product was observed.

Stereological Assessment of Cytoplasmic Membrane Following Intravitreal Injection of WGA, WGA-HRP, or Saline

Since WGA might disrupt the normal intracellular distribution of membrane in retinal ganglion cells in a manner similar to the way Concanavalin A disrupts the normal distribution of membrane in macrophages (Goldman, 1974; Edelson and Cohn, 1974) a study of the surface density of cytoplasmic organelles in the ganglion cells of animals injected with WGA-HRP was carried out. The surface density of cytoplasmic organelles

from the retinal ganglion cells of animals injected with either HRP or PBS served as controls. As summarized in Table 12, there are some distinct differences in the cytoplasmic distribution of membrane in the three sets of animals. Only those differences which are greater than the $\pm$ 10% confidence limits of these measurements, however, were considered significant and thus will be discussed. Some of these differences were noted at both 1 hour and 3 hours after intravitreal injection.

At 1 hour after injection, the surface density (and thus membrane surface area) of small vesicles and Golgi associated vesicles in ganglion cells exposed to both HRP and WGA-HRP was greater than the surface density of these organelles in ganglion cells exposed to PBS. In WGA-HRP injected animals this amounted to a 54% greater surface density of small vesicles and a 36% greater surface density of Golgi associated vesicles. In HRP injected animals this amounted to a 59% greater surface density of small vesicles and a 48% greater surface density of Golgi associated vesicles. In addition, at 1 hour after intravitreal injection, the surface density of lysosomal organelles in ganglion cells exposed to HRP had a greater surface density than was observed in either PBS or WGA-HRP injected animals. This amounted to a 46% greater surface density than was observed in PBS injected animals and a 34% greater surface density than was observed in WGA-HRP injected animals.

At 3 hours after intravitreal injection, the surface density of small vesicles in ganglion cells exposed to either HRP or WGA-HRP was still greater than than the surface density of small vesicles in PBS injected animals. In WGA-HRP injected animals this amounted to a 118% greater

surface density, and in HRP injected animals this amounted to a 75% greater surface density. In addition, in both WGA-HRP and HRP injected animals, the surface density of lysosomal organelles and SER was greater than the surface density of the corresponding organelles in PBS injected animals. In WGA-HRP injected animals this amounted to a 56% greater surface density of lysosomal organelles and a 112% greater surface density of SER. In HRP injected animals this amounted to a 29% greater surface density of lysosomal organelles and a 62% greater surface density of SER. Furthermore, at this survival time the surface density of Golgi associated vesicles in HRP injected animls was 29% less than the surface density of Golgi associated vesicles in PBS injected animals.

At neither 1 hour nor 3 hours after intravitreal injection of either WGA-HRP, HRP, or PBS were any significant differences observed in the surface density of mitochondria or the rough endoplasmic reticulum.

It would thus appear that relative to PBS injected animals, injection of WGA-HRP and HRP lead to distinct changes in the intracellular distribution of membrane in retinal ganglion cells. Furthermore, the changes induced by WGA-HRP appear to be more dramatic than those induced by HRP.

A second finding that may be observed from the data presented in Table 12 is that the intracellular distribution of membrane in ganglion cells that have been exposed to either WGA-HRP, HRP, or PBS changes with time. In PBS injected animals the only significant change was a decrease in the surface density of small vesicles. In HRP injected animals there was a decrease in in the surface density of small vesicles and Golgi associated vesicles, and an increase in the surface density of SER. In

WGA-HRP injected animals there was an increase in the surface density of SER and lysosomal organelles, and no significant decreases.

DISCUSSION

Light Microscopy

Following intravitreal injection of WGA-HRP the distribution of peroxidase reaction product in the chick retina was different than that which was observed following intravitreal injection of HRP. At the light microscopic level, the most distinct difference was the selective labeling of the inner plexiform, ganglion cell, and optic fiber layers following intravitreal injection of the lectin conjugate. This restricted distribution of WGA-HRP probably reflects the presence of macromolecules which are rich in NAcGlu or sialic acid in the inner layers of the retina. Moreover, since plasma membrane receptors for WGA have been reported in a number of different neuronal populations (Pfenninger and Maylié-Pfenninger, 1981; Gurd, 1977; Huck and Hattan, 1981), including chick retinal neurons (Mintz et al., 1981; McLaughlin et al., 1980), it is likely that this restricted region of retinal labeling may, in part, be due to the presence of WGA-specific glycoconjugates on the axons, dendrites, and perikarya of the retinal ganglion cells.

Electron microscopy

At the ultrastructural level, further differences in the intracellular distribution of WGA-HRP and HRP in retinal ganglion cells were observed. In the retinal ganglion cells of chick, injection of either WGA-HRP or HRP led to peroxidase labeling of morphologically similar classes of organelles. The relative frequency with which these

classes of organelles were labeled, however, depended on the nature of the injected marker. The most distinct example of this different intracellular distribution of WGA-HRP and HRP, was the greater relative frequency of labeling of Golgi cisterns and Golgi associated vesicles in the retinal ganglion cells of animals which had been injected with WGA-HRP. It is unlikely that this differential distribution of reaction product may be accounted for by differences in the amount of WGA-HRP endocytosed by these cells, since this would be expected to increase the labeling of all intracellular organelles, not just one select compartment. Thus, there appears to be some difference in the intracellular sorting of WGA-HRP and HRP in these cells. This finding is consistent with the hypothesis that WGA-HRP and HRP may be transported to the nerve terminals of retinal ganglion cells by two distinct intracellular pathways. Furthermore, since it has been suggested that the Golgi apparatus plays a central role in the reutilization of endocytosed perikaryal membrane (Farquhar and Palade, 1981; Orci et al., 1978) the relatively high frequency with which WGA-HRP was found to be associated with the Golgi apparatus (at 1 hour after injection 53% of all labeled organelles were either Golgi cisterns or Golgi associated vesicles) suggests that the lectin conjugate may be associated with endocytosed perikaryal membrane which is destined for reutilization either at the nerve terminal or elsewhere in the cell.

The differences in the intracellular distribution of HRP and WGA-HRP which were observed in this study, however, were not as distinct as those observed by Harper et. al. (1980) in rat sympathetic neurons. These investigators reported that endocytosed HRP was never observed in

cisterns of the Golgi apparatus, whereas in this study HRP labeled cis-cisterns were occaisionally observed. Furthermore, both Broadwell and Brightman (1979) and Sherlock et al. (1975) have reported infrequent labeling of the Golgi cisterns of rat pituitary neurons following extracellular exposure to HRP, and Holtzmann and colleagues (1973) have described the accumulation of HRP in Golgi cisterns of cultured chick sympathetic neurons. Reports such as these are rare, but suggest that in certain neuronal cell types and/or with the appropriate conditions, accumulation of HRP in cisterns of the Golgi apparatus may occur.

One possible explanation for the accumulation of HRP in the cisterns of the Golgi of these cells may be that some populations of neurons, such as chick retinal ganglion cells, are less efficient at sorting the content of pinocytic vesicles than other neurons. Thus, rather than delivering the entire fluid phase content of pinocytic vesicles to lysosomes, a portion of the solute (including the HRP), may be inappropriately delivered to the Golgi apparatus.

Alternatively, the uptake of HRP may, in part, be mediated by cell surface receptors whose function is to deliver mannan-rich ligands to the Golgi apparatus. A low affinity receptor-mediated uptake system with a specificity for glycoproteins having terminal sugars with the mannose or glucose configuration has been described in macrophages (Stahl et al., 1978; Sung et al.,1983), and some ligands that are endocytosed by receptor mediated mechanisms have been shown to accumulate in the Golgi complex (Bergeron et al., 1979; Josefsberg et al., 1979). Consistent with this hypothesis is the finding of Bunt and colleagues (1976) that periodate treatment of HRP abolishes its ability to serve

as an effective marker of axonal transport.

Further support for the adsorptive or receptor mediated endocytosis of HRP in these cells comes from the data presented in this chapter on the surface density of intracellular organelles. Whereas a marker of fluid phase endocytosis would not be expected to affect changes in cellular membrane distribution, intravitreally injected HRP had a dramatic effect on the surface density of all classes of organelles which contained HRP reaction product. The most distinct changes that were induced by intravitreal injection of HRP were increases in the surface density of small vesicles, lysosomal organelles, and SER, although an initial increase, and then latter a decrease in the surface density of the Golgi associated vesicles was also observed. These increases in surface density are not likely to be due to artifactual swelling of those organelles which contain HRP reaction product, since 1) the labeled organelles composed a very small percent of the total number of organelles that were analyzed for this study and 2) swelling would increase the volume, but not the surface area of labeled organelles. Thus, these increases in surface density most likely reflect the induction of organelles (increased size and/or increased number) for the uptake and subsequent degradation of the injected HRP. Findings similar to these have been previously reported by Chan et al. (1980). They found following exposure of cultured neuroblstoma cells to HRP isoenzyme C that there was a rapid and sustained increase in the total number vesicles, tubules, vacuoles, and dense bodies. Thus, it would appear that in some neuronal cell types HRP is not a benign probe of fluid phase endocytosis, but plays an active role in inducing its own uptake and

subsequent degradation.

It has been suggested that a number of mutivalent ligands, including lectins, may also play an active role in their cellular uptake, by cross linking receptors on the plasma membrane. The best documented example of this is the capping and subsequent endocytosis of membrane in macrophages and lympocytes following exposure to Con A (Yahara and Edelman, 1973; Goldman, 1974; Edelson and Cohn, 1974), although a similar process has also been described in cultured sympathetic neurons (Carbonetto and Argon, 1980). Moreover, the initial reason for conducting the the surface density study was to determine whether WGA-HRP had an effect on the membrane distribution of the retinal ganglion cells similar to that which has been described for Con A in these other cell types. The HRP injected animals were controls.

Similar changes in the surface density of organelles were observed following WGA-HRP injection as were observed following HRP injection, except that increases in the surface density of small vesicles, lysosomal organelles, and SER at three hours post-injection were somewhat greater in the WGA-HRP injected animals. Whether any of these changes are due to the direct interaction of the lectin portion of the conjugate with the ganglion cell is unclear at this time due to the dramatic effect of HRP on these cells. For example, the lectin portion of the conjugate may play an indirect role in enhacing the effect of HRP on these cells, by raising the effective concentration of HRP available to the ganglion cells, while not having an effect on these cells itself. To get at the answer to this question further experiments, including those in which chick retinal ganglion cells are exposed to native WGA, need to be conducted.

TABLE 11

RELATIVE DISTRIBUTION OF LABELED ORGANELLES FOLLOWING INTRAVITREAL INJECTION OF WGA-HRP AND HRP

% OF TOTAL LABELED ORGANELLES

Marker	Time	Small vesicle	Lysosomal organelles	Golgi cisternae	Golgi associated vesicles	SER	Mitochondia	RER
WGA-HRP	1 hr	29%	10%	12%	41%	8%	0%	0%
WGA-HRP	3 hr	25%	28%	7%	33%	7%	0%	0%
HRP	1 hr	43%	19%	2%	21%	15%	0%	0%
HRP	3 hr	43%	30%	2%	13%	12%	0%	0%

TABLE 12

ORGANELLE SURFACE DENSITY ($\mu m^2/\mu m^3$)

Marker	Time	Small vesicles	Lysosomal organelles	Golgi cisterns	Golgi associated vesicles	SER	RER	Mito-chondria	Unknown
PBS	1 hr	0.37	0.36	1.60	0.81	0.36	3.30	1.30	0.10
HRP	1 hr	0.59	0.53	1.73	1.20	0.32	3.20	1.51	0.15
WGA-HRP	1 hr	0.57	0.38	1.77	1.10	0.42	3.40	1.34	0.18
PBS	3 hr	0.28	0.41	1.53	1.04	0.42	3.58	1.56	0.12
HRP	3 hr	0.49	0.54	1.78	0.74	0.68	3.63	1.31	0.13
WGA-HRP	3 hr	0.61	0.64	1.54	0.97	0.89	3.60	1.49	0.15

SUMMARY

1) Following intravitreal injection, ^{125}I-wheat germ agglutinin is endocytosed by chick retinal ganglion cells and subsequently transported in an anterograde direction to their nerve terminals in the optic tectum.

2) The mechanism responsible for the uptake and anterograde axonal transport of ^{125}I-wheat germ agglutinin is selective and saturable.

3) The rate of anterograde axonal transport of ^{125}I-wheat germ agglutinin in chick retinal ganglion cells is 22-44 mm/day.

4) The uptake and anterograde axonal transport of ^{125}I-wheat germ agglutinin in chick retinal ganglion cells may be independent of the rapid axonal transport of newly synthesized protein.

5) Following intravitreal injection, wheat germ agglutinin conjugated to horseradish peroxidase is endocytosed by chick retinal ganglion cells and accumulates in a variety of intracellular organelles including cisterns and vesicles of the Golgi apparatus. The intracellular distribution of the wheat germ agglutinin conjugate is different than the intracellular distribution of native horseradish peroxidase.

APPENDIX

AN ISOTOPE DILUTION TECHNIQUE AS APPLIED TO AXONAL TRANSPORT

The technique of isotope dilution may be used to determine the steady state pool size of free amino acid in the chick eye which is available for synthesis into axonally transported protein. However, standard approachs to analyzing the data from an isotope dilution experiment cannot be used in this sytem, since intravitreally injected amino acid is rapidly cleared from the retina. A method for analyzing isotope dilution data as applied to the study of axonal transport is presented.

Consider $P_v^*(t)$, the pool size of ^{3}H-amino acid in the vitreous at a given time point, t, after intravitreal injection. Since radiolabeled amino acid is cleared from the vitreous in an exponential fashion (Nixon, 1980; Cuénod and Schonbach, 1971; McEwen and Grafstein, 1968), $P_v^*(t)$ may be expressed as function of the initial amount of injected ^{3}H-amino acid, $P_v^*(0)$, as well as an exponential decay constant L, in the following manner:

$$P_v^*(t) = P_v^*(0)\ e^{-Lt} \qquad (1)$$

Consider also, $P_v(t)$, the total pool size of amino acid in the vitreous (labeled plus native) at a given time point, t, after intravitreal injection. Assuming that homeostatic mechanisms act to maintain the pool size of proline in the vitreous at a steady state level, P_{vs}, then for all t:

$$P_v(t) = P_{vs} + (P_a)\ e^{-Lt} \qquad (2)$$

where P_a is the total amount of injected amino acid (labeled plus native). For the same μCi dose of labeled amino acid, P_a will be smaller at higher specific activities. When P_a is very small as compared to P_{vs}, equation (2) simplifies to:

$$P_v(t) = P_{vs} \tag{3}$$

Assuming that intravitreally injected ^{3}H-amino acid rapidly equilibrates with the native pool of free proline in the retina, and that the specific activity of newly synthesized axonally transported protein accurately reflects the specific activity of the amino acid pool available to retinal ganglion cells, then following intravitreal injection of labeled amino acid, the rate of deposition of ^{3}H-amino acid in the tectum, $\frac{dP_t^*}{dt}$, may be described as follows:

$$\frac{dP_t^*}{dt} = k \frac{P_v^*(t - D)}{P_v(t - D)} \tag{4}$$

where k is the rate of uptake of proline by ganglion cells for incorporation into protein destined for axonal transport, and D is the time delay in detecting transported ^{3}H-amino acid in the tectum following intravitreal injection. Substituting equations (1) and (2) into equation (4) one arrives at:

$$\frac{dP_t^*}{dt} = k \frac{P_v^*(0)\ e^{-L(t-D)}}{P_{vs} + (P_a)\ e^{-L(t-D)}} \tag{5}$$

and substituting the variable R for $\frac{P_a}{P_{vs}}$, equation (5) becomes:

$$\frac{dP_t^*}{dt} = \frac{k\ P_v^*(0)\ \ e^{-L(t-D)}}{P_{vs}\ \ (1 + R\ e^{-L(t-D)})} \tag{6}$$

which simplifies to:

$$\frac{dP_t^*}{dt} = \frac{k\ P_v^*(0)}{P_{vs}\ (R\ +\ e^{L(t-D)})} \tag{7}$$

Integrating equation (7) over the time course of arrival of ^{3}H-amino acid in the tectum (from time D, to the end of the experiment, time T), with the assumption that k remains constant, we find that:

$$P_t^*(T) = \frac{k\ P_v^*(0)}{P_{vs}} \left[\frac{T-D}{R} + \frac{1}{R\ L} (\log(R + 1) - \log(R + e^{L(t-D)})) \right] \tag{8}$$

which by regrouping may be expressed as,

$$P_t^*(T) = \frac{kP_v^*(0)}{P_{vs}\ RL} (L(T-D) + \log(R+1) + \log(e^{-L(T-D)}) - \log(1+Re^{-L(T-D)})) \tag{9}$$

However, since $\log e^{-L(T-D)} = - L\ (T-D)$, equation 9 reduces to:

$$P_t^*(T) = \frac{k\ P_v^*(0)}{P_{vs}\ R\ L} (\log\ (R + 1) - \log\ (1 + R\ e^{-L(T-D)})) \tag{10}$$

which may be expressed as

$$P_t^*(T) = \frac{k\ P_v^*(0)}{P_{vs}\ R\ L} \log \left[\frac{1 + R}{1 + R\ e^{-L(T-D)}} \right] \tag{11}$$

Equation (11) describes $P_t^*(T)$, the amount of radioactive amino acid (free + incorporated into protein) which has been transported to the tectum over a determined period of time, T, in terms of a decay constant, a delay, and the relative pool sizes of amino acid in the retina.

For the special condition where P_a is very small as compared to P_{vs}, a much simpler relationship may be obtained for $P_t^*(T)$. For this special condition, equations (1) and (3) are substitued into equation (4) resulting in the following expression:

$$\frac{dP_t^*}{dt} = k \frac{P_v^*(0)\, e^{-L(t-D)}}{P_{vs}} \qquad (12)$$

Integrating equation (12) over the time course of arrival of ^{3}H-amino acid in the tectum (from the delay, time D, to the end of the experiment, time T), one finds that,

$$P_t^*(T) = \frac{k\, P_v^*(0)}{P_{vs}\, L} \left[1 - e^{-L(T-D)} \right] , \qquad (13)$$

which describes $P_t(T)$ in terms of D, L, and the ratio of pool sizes of amino acid in the tectum for the special condition where P_a is very small as compared to P_{vs}.

The amount of ^{3}H-proline that was transported to the tectum following intravitreal injection of different specific activities of ^{3}H-proline as detailed in chapter 5 may now be described by the functions derived above. First, consider case #1, the experimental situation presented in chapter 5 where 15 μCi of ^{3}H-proline are injected into the vitreous at a specific activity of 100 Ci/mmole. In this case $P_a = 1.5 \times 10^{-10}$ moles, which is likely to be significantly less than P_{vs}. Assuming that $P_a \ll P_{vs}$ (see final estimate for P_{vs}), then for this experimental situtation $P_t^*(T)$ may be described by equation (13).

Next, consider case #2, the experimental situation presented in

chapter 5 where 15 μCi of ^{3}H-proline at a specific activity of 100 Ci/mmole was co-injected with 1.5×10^{-6} moles of native proline, thus reducing the specific activity of the injected ^{3}H-proline to 10 mCi/mmole. In this case, $P_a \approx 1.5 \times 10^{-6}$ moles, which is not likely to be significantly less than P_{vs}. Thus, for this experimental situation, $P_t^*(T)$ may be described by equation (11).

As distinguished by subscripts, the ratio of the tectal accumulation of radioactive proline for these two cases, $\frac{P_{t2}^*(T)}{P_{t1}^*(T)}$, may be described by combining equations (11) and (13) as follows:

$$\frac{P_{t2}^*(T)}{P_{t1}^*(T)} = \frac{\dfrac{k\,P_v^*(0)}{P_{vs}\,R\,L}\log\left[\dfrac{1+R}{1+R\,e^{-L(T-D)}}\right]}{\dfrac{k\,P_v^*(0)}{P_{vs}\,L}\left[1-e^{-L(T-D)}\right]} \qquad (14)$$

where $R = \frac{P_{a2}}{P_{vs}}$.

Equation (14) simplifies to:

$$\frac{P_{t2}^*(T)}{P_{t1}^*(T)} = \frac{\log\left[\dfrac{1+R}{1+R\,e^{-L(T-D)}}\right]}{R\left[1-e^{-1(T-D)}\right]} \qquad (15)$$

and, by substituting $\frac{P_{a2}}{P_{vs}}$ for R in equation (15), the following relationship is obtained:

$$\frac{P_{t2}^*(T)}{P_{t1}^*(T)} = \frac{\log\left[\dfrac{1+P_{a2}/P_{vs}}{1+P_{a2}/P_{vs}\,(e^{-L(T-D)})}\right]}{P_{a2}/P_{vs}\left[1-e^{-L(T-D)}\right]} \qquad (16)$$

Given that T=25 hours, $P_{t1}^*(25)$ = 46,486 dpm, $P_{t2}^*(25)$ = 26,160 dpm, and $P_a = 1.5 \times 10^{-6}$ moles (see chapter 5), and that values for both D and L have been reported by others (Sjostrand et al., 1973; Cuenod and Schonbach, 1971), then equation (16) may be solved for P_{vs} numerically by succesive approximation.

Assuming a delay, D, of 2 hours (Sjostrand et al.,1973), and an exponential decay constant, L, of 0.385 half lives/hour [1] (Cuénod and Schonbach, 1971), then $P_{vs} = 7.72 \times 10^{-7}$ moles. Furthermore, this affirms the assumption in case #1 that $P_a << P_{vs}$.

As described by equations (1) and (3), a profile of the intravitreal pools of proline (native and labeled) available to retinal ganglion cells following intravitreal injection of ^{3}H-proline as described in case #1 may now be determined (see Figure A.1). Furthermore, the area under the curves in Figure A.1, from time = 0 to time = 23 hours (T-D), may now be calculated to determine the relative pool sizes of native proline and ^{3}H-proline available to retinal ganglion cells for transport to the tectum during the 25 hour time course of the experiment.

The area under the P_{vs} curve, A_{Pvs}, from time 0 to time (T-D) is simply described as:

$$A_{Pvs} = P_{vs} (T-D) \qquad (17)$$

Given that T-D = 23 hours and $P_{vs} = 7.72 \times 10^{-7}$ moles, and then solving equation (17) for A_{Pvs}, $A_{Pvs} = 1.77 \times 10^{-5}$ mole-hours. Furthermore, the area under the P_v^* curve, A^*_{Pv}, may be described by the integral of the function which describes the curve. Thus, integrating equation (1)

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[1] This corresponds to a half life of 1.8 hours.

for the limits t = 0 to t = T-D, one finds that:

$$A^*_{Pv} = P_v^*(0)\ L^{-1}\ (1 - e^{-L(T-D)}) \qquad (18)$$

Given that T-D = 23 hours, L = 0.385 half lives/hour, and $P_v^*(0) = 1.5 \times 10^{-10}$ moles, then solving equation (18) for A^*_{Pv}, $A^*_{Pv} = 3.89 \times 10^{-10}$ mole-hours.

Thus, over the time course of this experiment, the total pool of native proline available to the retinal ganglion cells for rapid transport to the tectum is 4.56×10^4 times the size of the total pool of injected ^{3}H-proline [2]. This means that the effective specific activity of ^{3}H-proline available to the retinal ganglion cells is reduced from 100 Ci/mmole to 2.43 mCi/mmole, and thus that 48,486 dpm of axonally transported label represents the transport of 8.85×10^{-9} moles of proline.

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[2] Thus, the ^{3}H-proline pool represents approximately 0.0022% of the total proline pool.

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Figure 2.1

Dark-field micrographs of autoradiograms of the optic tecta of a chick injected vitreally with affinity-purified ^{125}I-WGA. The optic tectum contralateral to the injected eye (a) contains radioactive label over the SO and layers a through f of the SGFS. The ipsilateral optic tectum (b) was unlabeled. Bars, 100 μm. x 170.

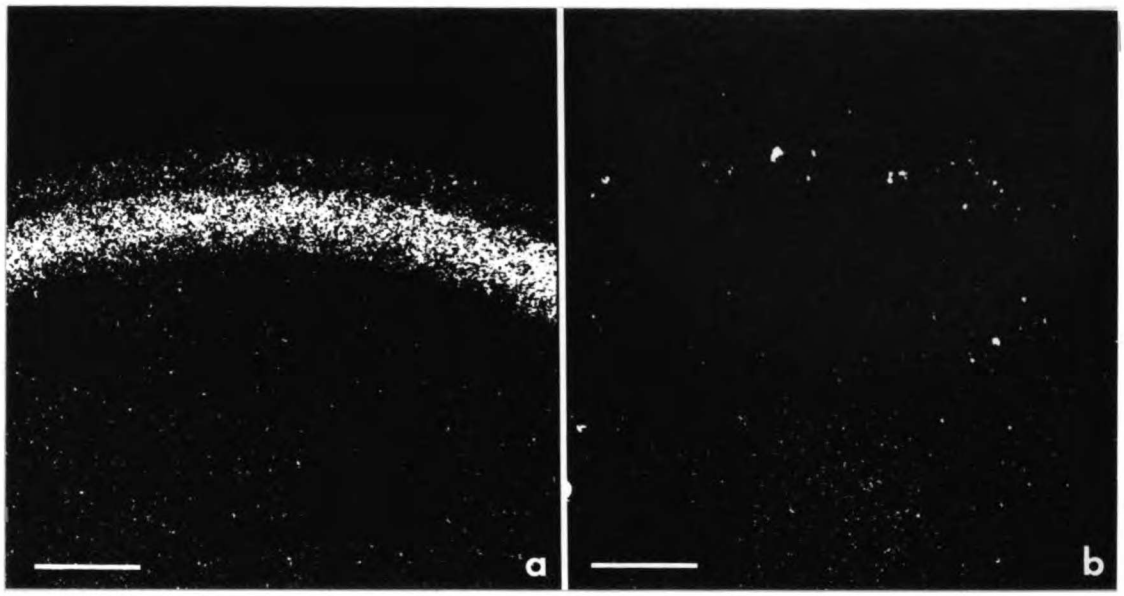

a
b

Figure 2.2

The density of labeling as a function of depth in the labeled optic tectum. The average number of grains is presented for six traverses across the autoradiogram. Layers d and a-b are more heavily labeled; layers c and f are less heavily labeled. The background level of radioactivity measured in adjoining midbrain tissue is indicated by the horizontal dashed line. The boundaries of layers a-f are indicated.

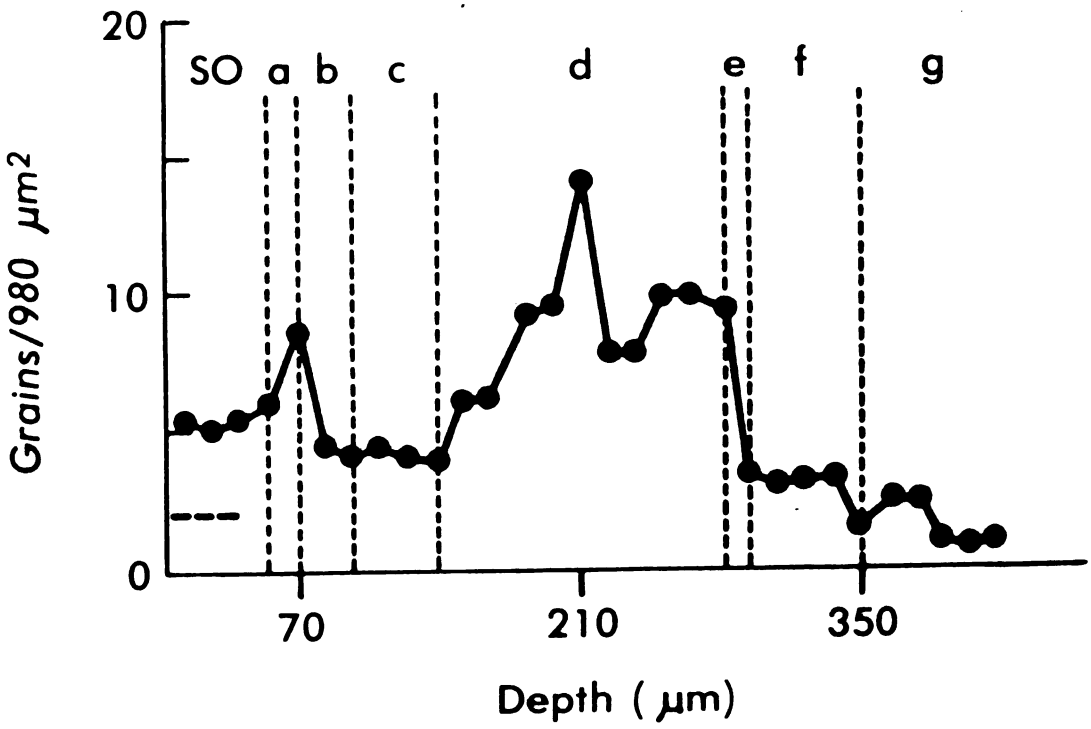

20
10
0
SO
a
b
c
d
e
f
g
70
210
350
Grains/980 μm2
Depth (μm)

Figure 2.3

Densitometric tracings of autoradiograms of SDS gels. (a) Grain density of autoradiogram of affinity-purified fraction of ^{125}I-WGA. (b) Density of grains found over autoradiograms of the nonspecific fraction, B. (c) Density of grains found over autoradiogram of SDS gel of homogenates of the experimental optic tectum. (d) Density of grains found over autoradiogram of the gel of the control optic tectum. The arrow indicates the 18,000 dalton monomer of ^{125}I-WGA.

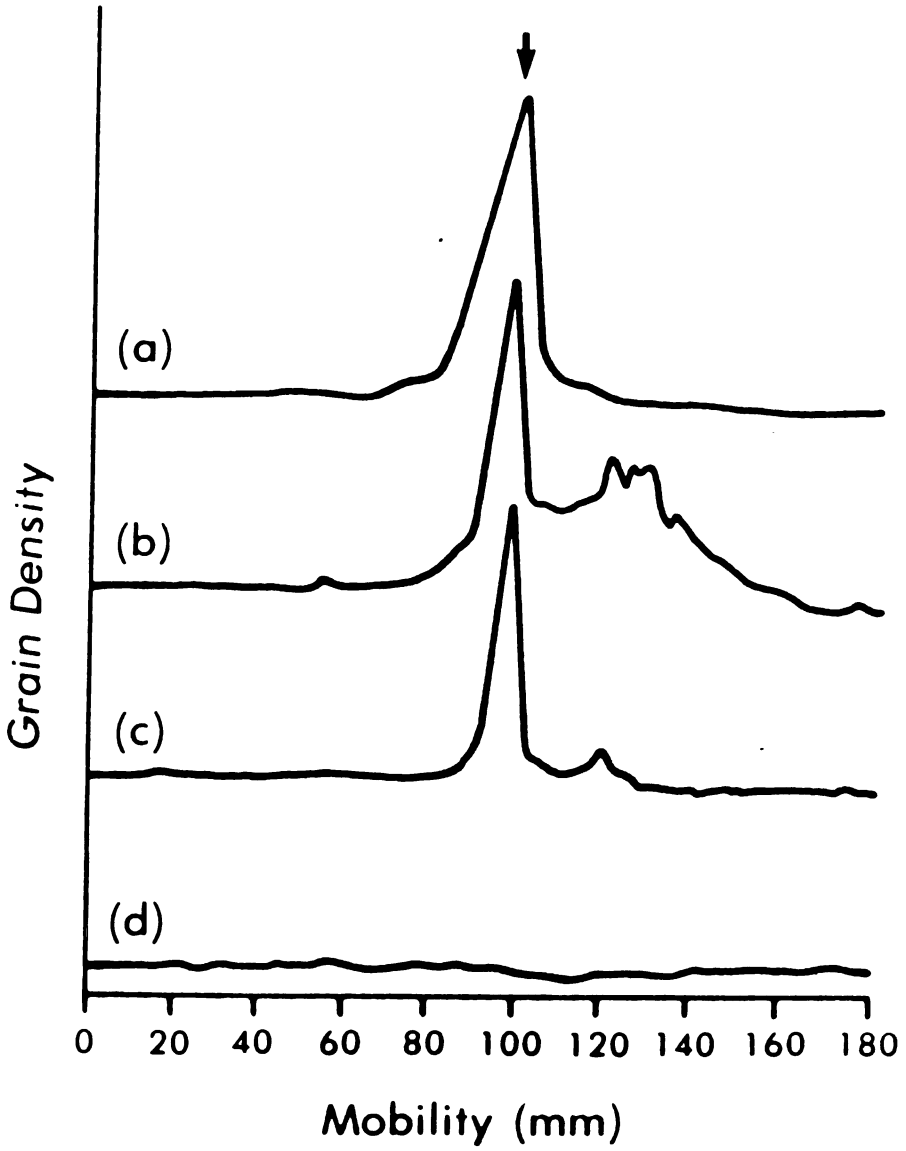

(a)
(b)
(c)
(d)
Grain Density
0
20
40
60
80
100
120
140
160
180
Mobility (mm)

Figure 2.4

Composite SDS gel patterns: a-d, autoradigrams of the gels; e, Coomassie Blue staining pattern. The affinity-purified fraction (a) contained a single radioactive band that co-migrated with both the native 18,000 dalton monomer of WGA (e) and the major radioactive band recovered after anterograde transport to the experimental tectum (c). No radioactive bands were observed in gels of the control tectum (d). The nonspecific fraction (b) consisted of multiple radioactive bands, the strongest of which co-migrated with WGA. Molecular weight standards are indicated on the left.

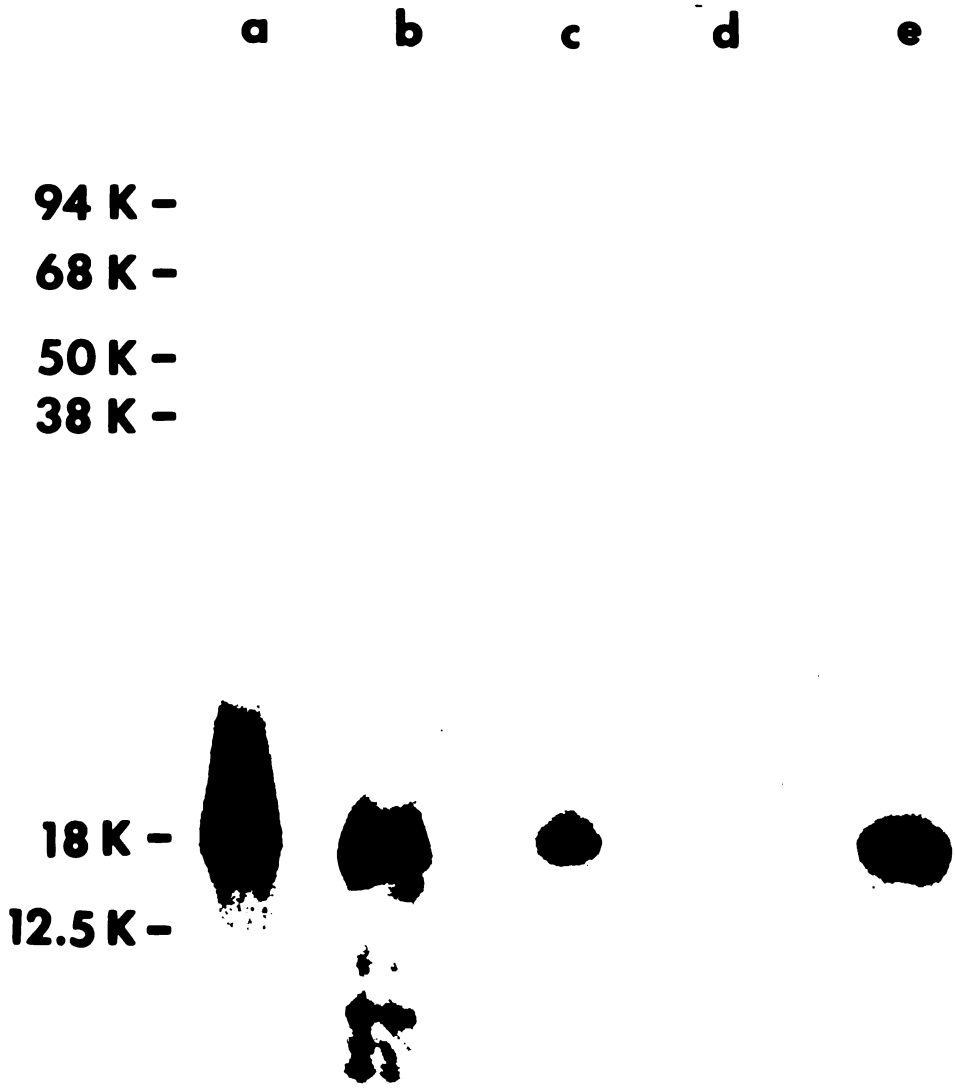
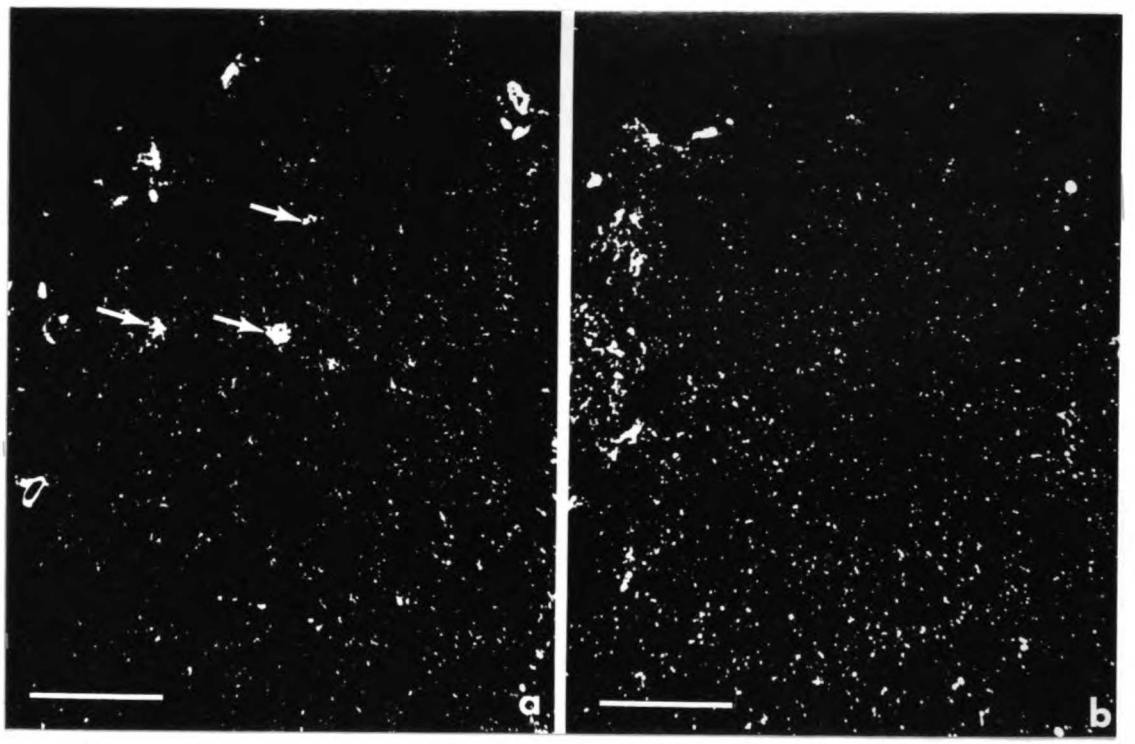

a
b
c
d
e
94 K -
68 K -
50 K -
38 K -
18 K -
12.5 K -

Figure 2.5

Dark-field micrographs of autoradiograms of the superior cervical ganglia after vitreal injection of the nonspecific fraction, B. Two labeled neuron cell bodies are indicated by arrows in the ipsilateral ganglion (a). Endothelial cells (arrowhead), glia, and pericytes were also found labeled in both the ipsilateral and contralateral ganglia. No labeled neurons were found in the contralateral ganglia (b). Bars, 150 μm X 100.



Two
teral
s were
glia.
Bars,

Figure 3.1

Dose response relationship for the anterograde axonal transport of affinity purified ^{125}I-WGA (circles), inactive ^{125}I-WGA (squares) and ^{125}I-BSA (triangles). Chicks were intravitreally injected with varying doses of iodinated protein and twenty-five hours later their optic tecta were assayed for radioactive label. The ordinate represents the amount of radioactive label axonally transported to the optic tectum contralateral to the site of injection, i.e., contralateral tectal dpm minus ipsilateral tectal dpm. The abscissa represents the dose of iodinated protein which was injected. Each point is the mean $\pm$ SEM (n= 2-20).

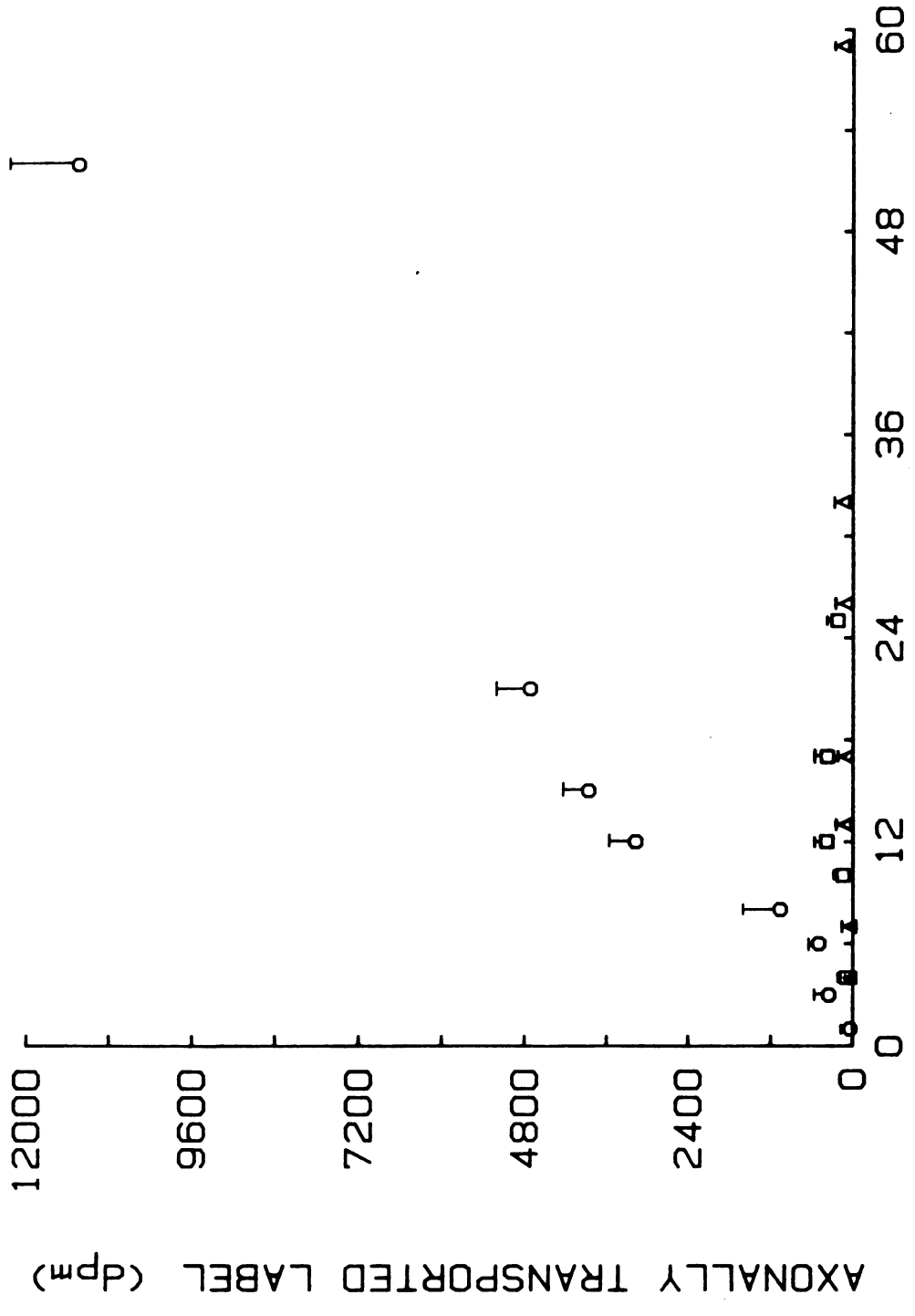

AXONALLY TRANSPORTED LABEL (dpm)
12000
9600
7200
4800
2400
0
0
12
24
36
48
60
INJECTED DOSE (μCi)

Figure 3.2

Competition for the uptake and transport of ^{125}I-WGA by excess unlabeled lectin. Chicks were intravitreally injected with 15 μCi of ^{125}I-WGA in a 1.1 mM solution of either native WGA, UEA-I, or SBA. Twenty-five hours later their optic tecta were assayed for radioactivity, and the amount of radioactive label which had been axonally transported to the contralateral tectum of each animal was calculated by subtracting ipsilateral dpm from contralateral dpm. Each bar represents the mean $\pm$ SEM (n = 6-20).

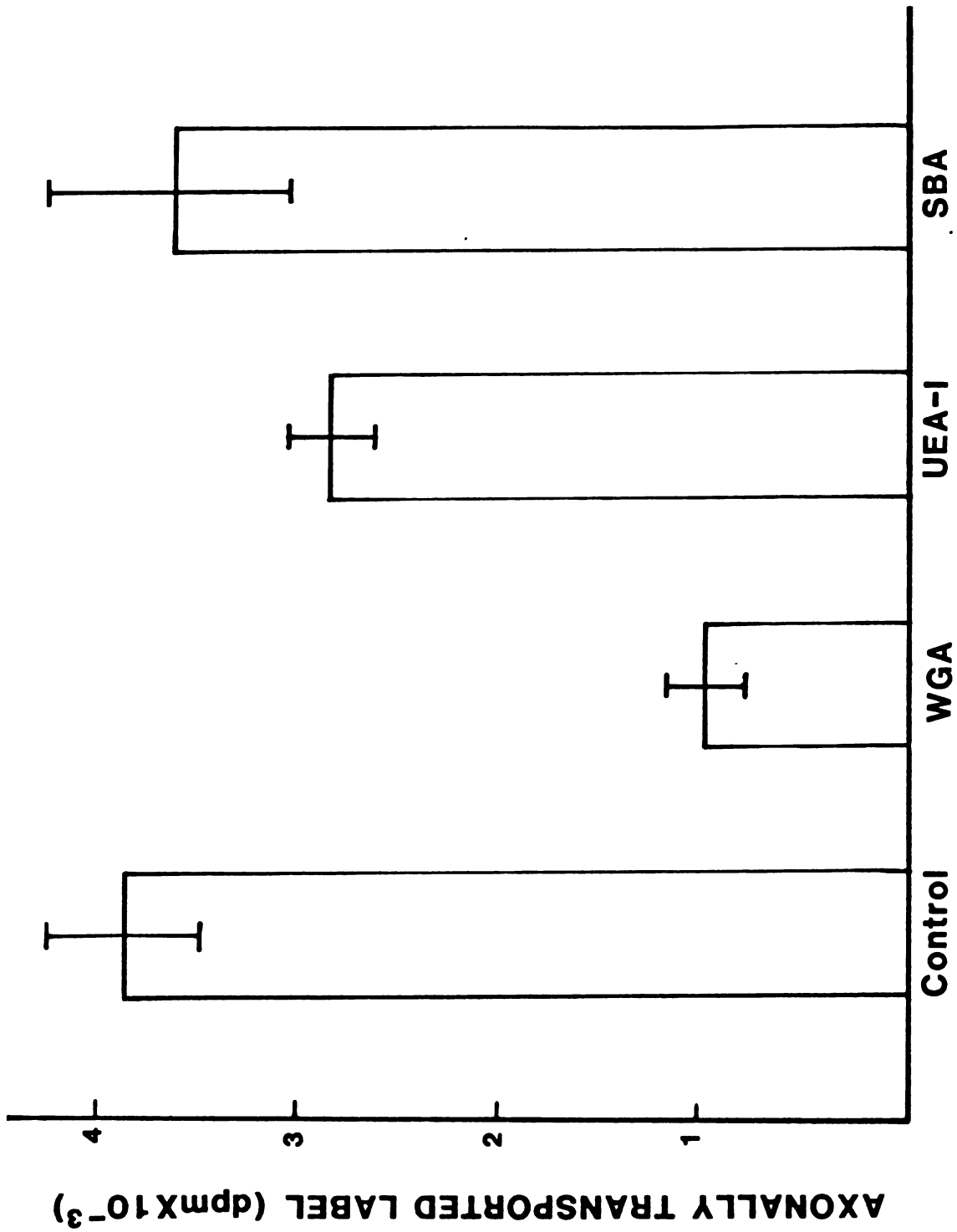

SBA
UEA-I
WGA
Control
4
3
2
1
AXONALLY TRANSPORTED LABEL (dpmX10^-3)

Figure 3.3

Effect of co-injection of varying doses of either BSA or native WGA on the uptake and anterograde transport of ^{125}I-WGA. Chicks were each injected with 15 μCi of ^{125}I-WGA in 15 μM to 1.1 mM solutions of either native WGA (closed squares) or BSA (open squares). Twenty-five hours later their optic tecta were assayed for radioactivity. The ordinate represents the amount of radioactive label which had been axonally transported to the contralateral tectum of each animal and was calculated by subtracting ipsilateral dpm from contralateral dpm. The ordinate represents the log of the concentration of the protein which was co-injected with the iodinated lectin. Each point is the mean $\pm$ SEM ($n = 5-12$).

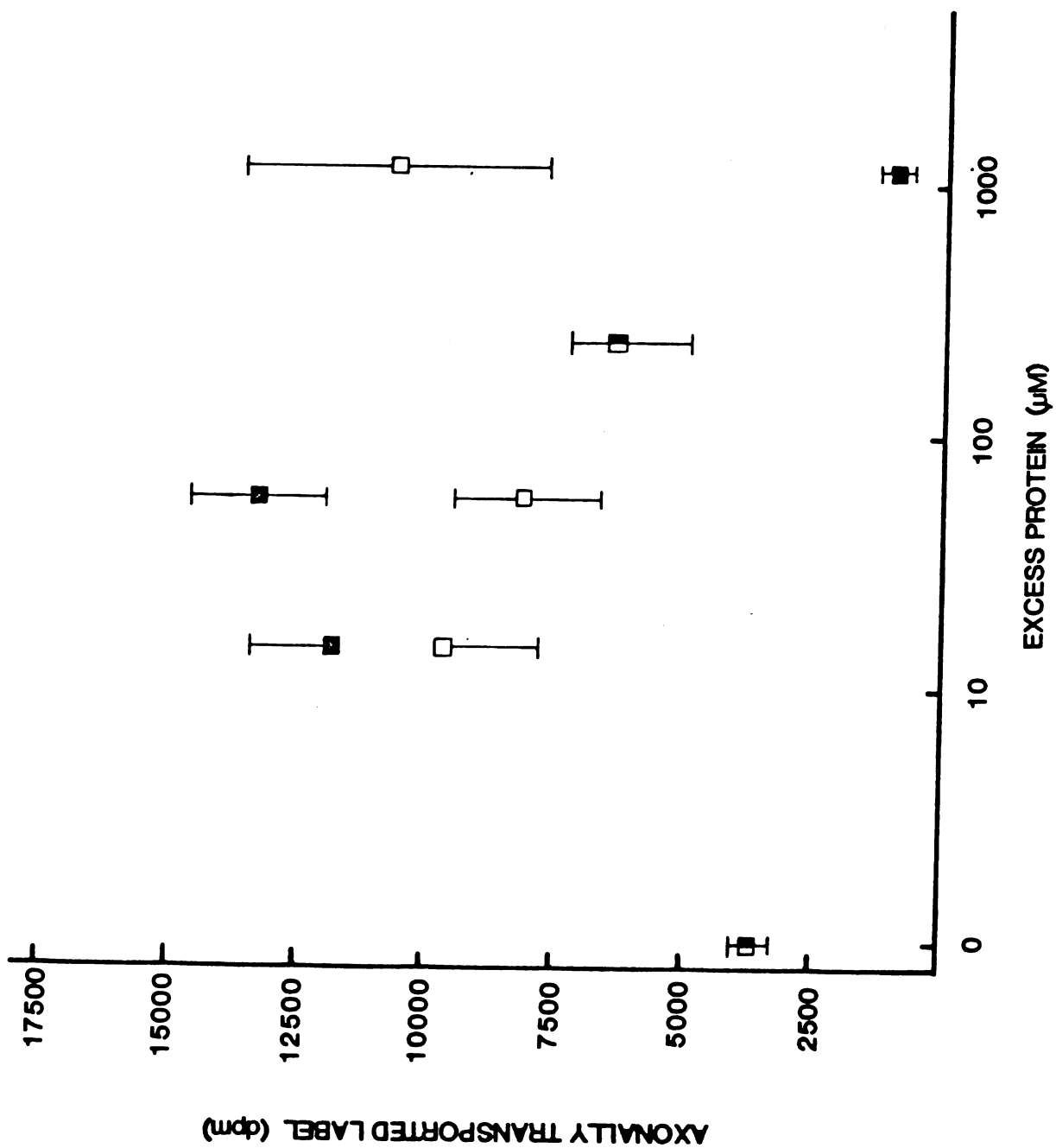

AXONALLY TRANSPORTED LABEL (dpm)
17500
15000
12500
10000
7500
5000
2500
0
10
100
1000
EXCESS PROTEIN (μM)

Fig. 4.1

Time-course of accumulation of radioactivity in the ipsilateral optic tectum (open symbols) and of axonally transported label (ATL) to the experimental optic tectum (closed symbols) after intravitreal injection of affinity purified ^{125}I-WGA. The line of best fit by linear regression analysis is indicated by the dashed line. Inspection of the slope of the line indicates that radioactivity accumulates in the tectum at a rate of 156 dpm/hr. Different symbols represents different groups of animals. Each value is the mean (and S.E.M.) of 3-5 tecta, except for the opened and closed triangles at 23 hours which represent the mean of 2 tecta.

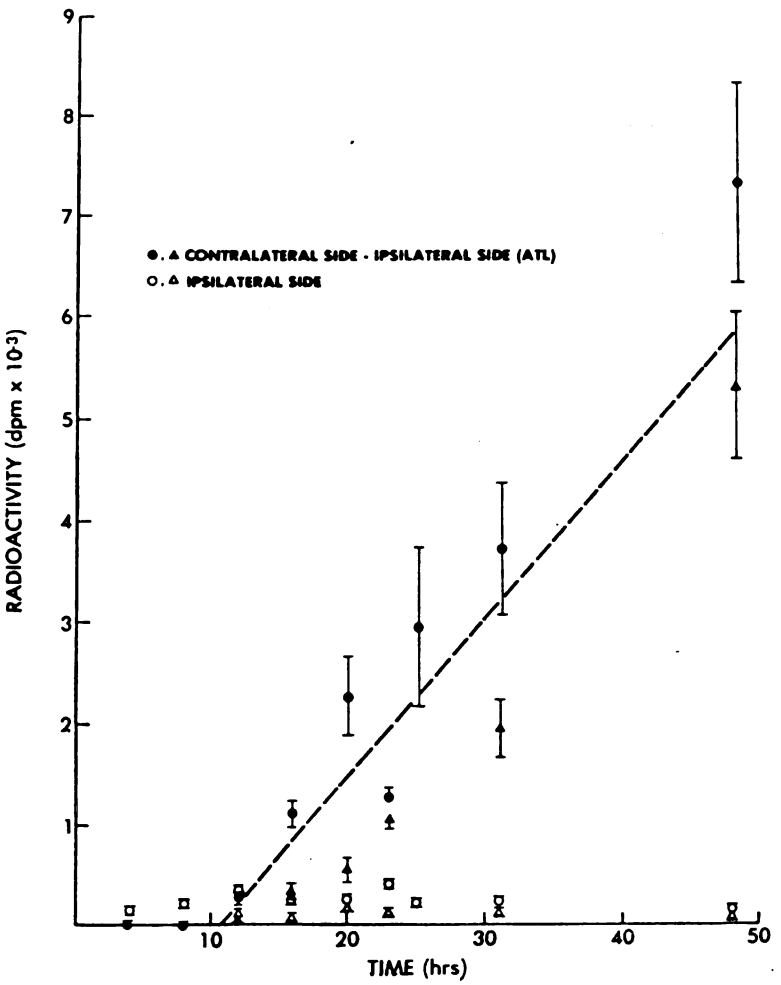

●, ▲ CONTRALATERAL SIDE - IPSILATERAL SIDE (ATL)
○, △ IPSILATERAL SIDE
RADIOACTIVITY (dpm x 10⁻³)
TIME (hrs)
9
8
7
6
5
4
3
2
1
10
20
30
40
50

Figure 5.1

Chicks were intravitreally injected with varying doses of CHX followed 1 hour later by intravitreal injection of 15 μCi of ^{3}H-proline or 15 μCi of ^{125}I-WGA. Twenty-five hours after the injection of the radioactive marker, the optic tecta were assayed for transported radioactivity. Control animals were pre-injected with PBS. Results are expressed in percent of control (= 100%). Open bars represent the relative amount of axonally transporte label following injection of ^{3}H-proline. Stipled bars represent the relative amount of axonally transported label following injection of ^{125}I-WGA. Each bar represents the mean ± SEM (n = 2-10).

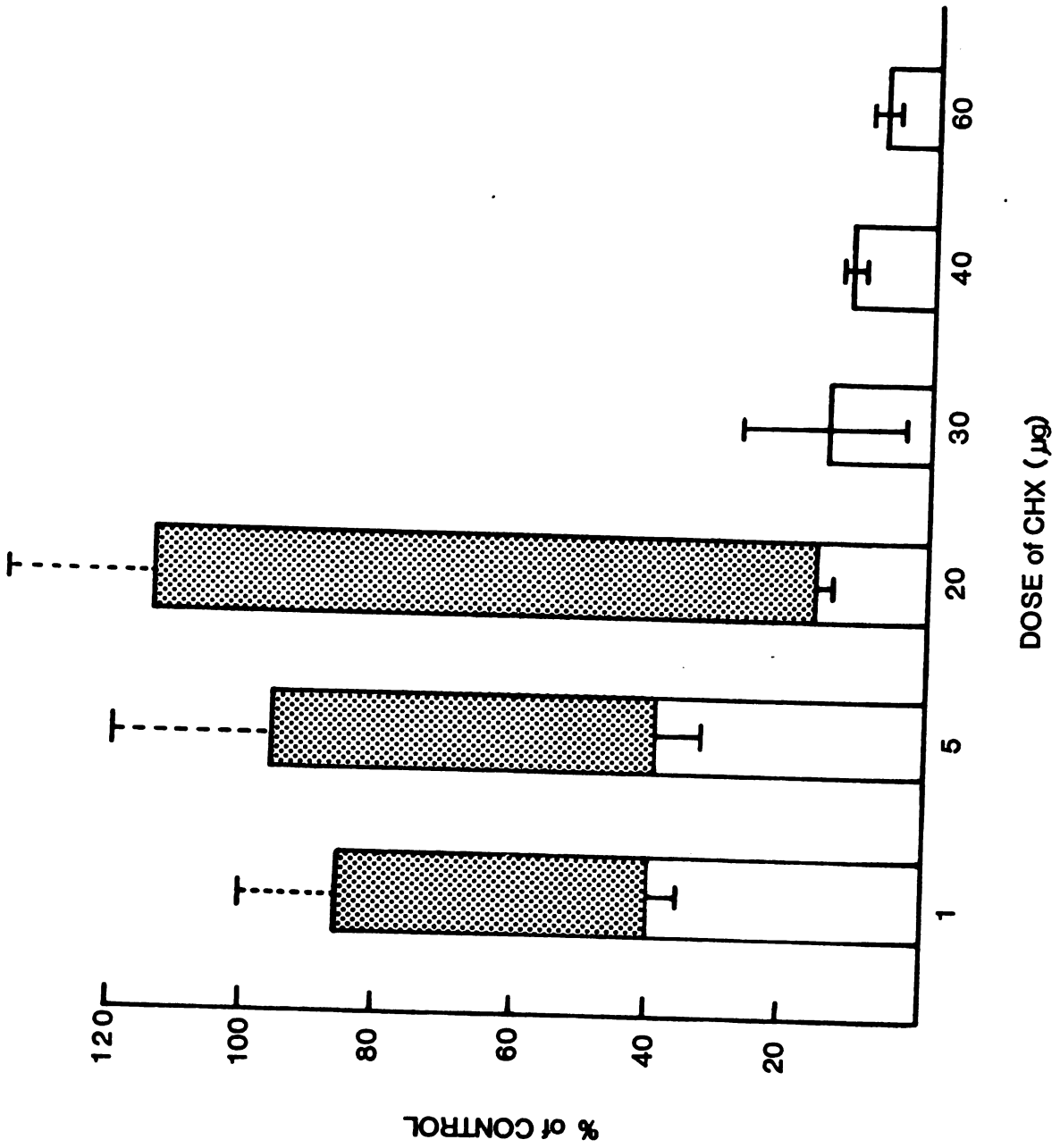

% of CONTROL
120
100
80
60
40
20
1
5
20
30
40
60
DOSE of CHX (µg)

Figure 6.1

Electron micrograph of the inner layers of the chick retina 1 hour after intravitreal injection of WGA-HRP. Reaction product extends throughout the extracellular space of the inner limiting membrane (ILM), optic fiber layer (OFL), ganglion cell layer, and inner plexiform layer (IPL) of the retina. Also note the intracellular accumulation of reaction product in the retinal ganglion cell (RGC) (arrows). Vit = vitreous, bar = 1 μm, magnification = 18,500 X.

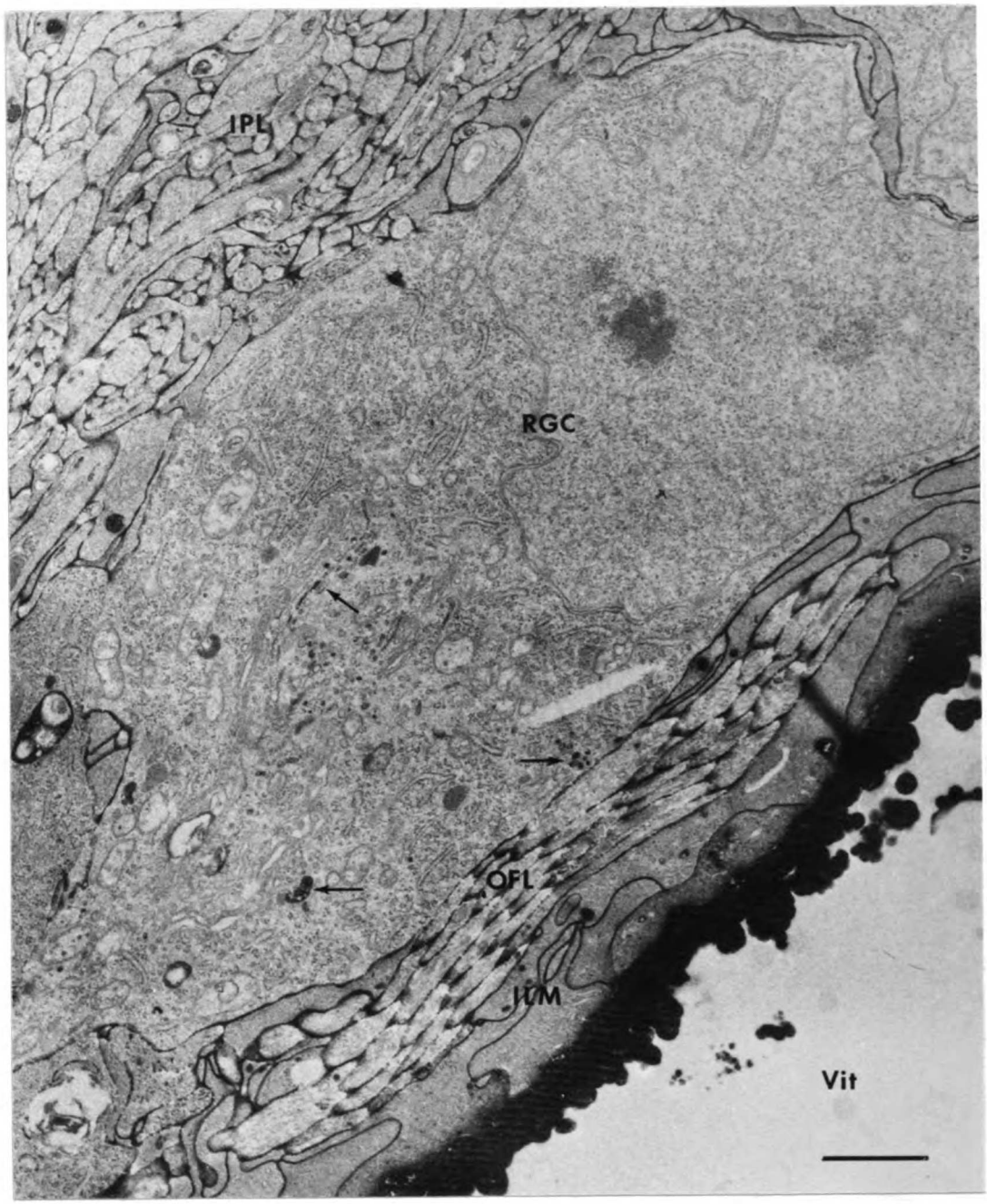
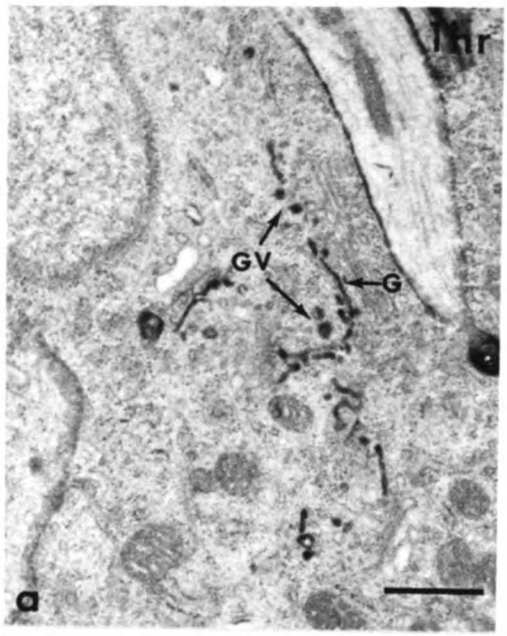
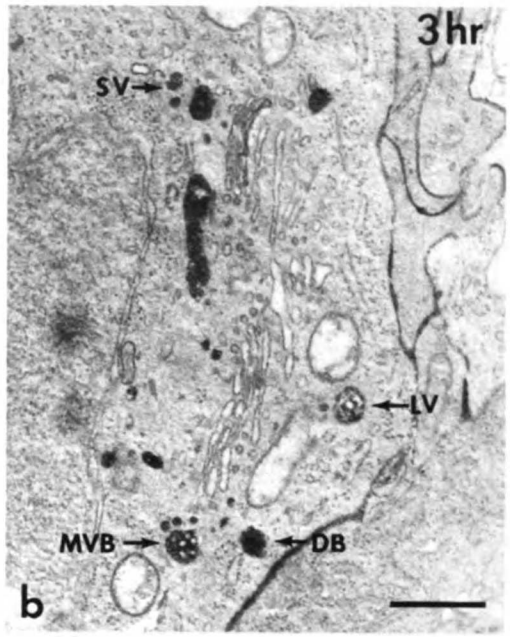

IPL
RGC
OFL
IUM
Vit

Figure 6.2

Electron micrographs of peroxidase labeled organelles in chick retinal ganglion cells at 1 hour (a) and 3 hours (b,c) after intravitreal injection of WGA-HRP. Reaction product was found to accumulate in small vesicles (SV), large vesicles (LV), dense bodies (DB), multivesicular bodies (MVB), tubules of the smooth endoplasmic reticulum (SER), Golgi cisterns (G), and Golgi associated vesicles (GV). a) bar = 0.5 μm, magnification = 28,000 X; b) bar = 1 μm, magnification = 27,000 X; c) bar = 0.5 μm, magnification = 32,000 X.


1hr
GV
G
a


3hr
SV
LV
MVB
DB
b

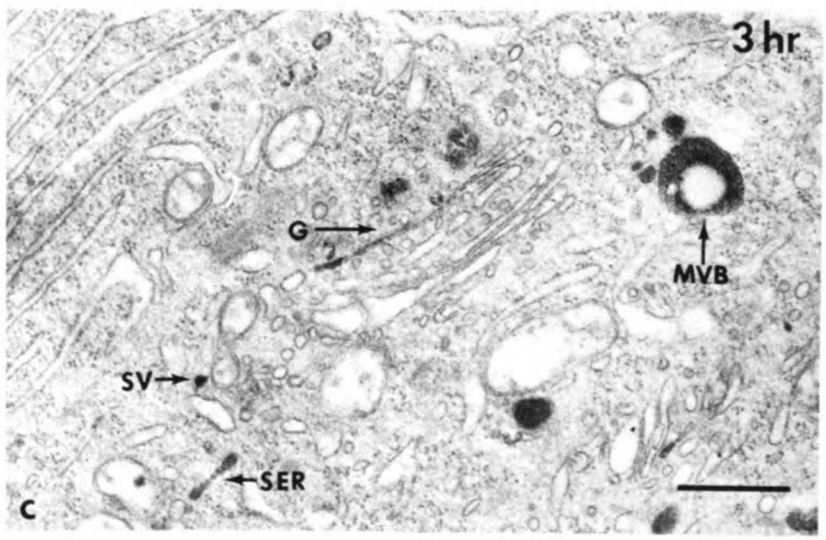
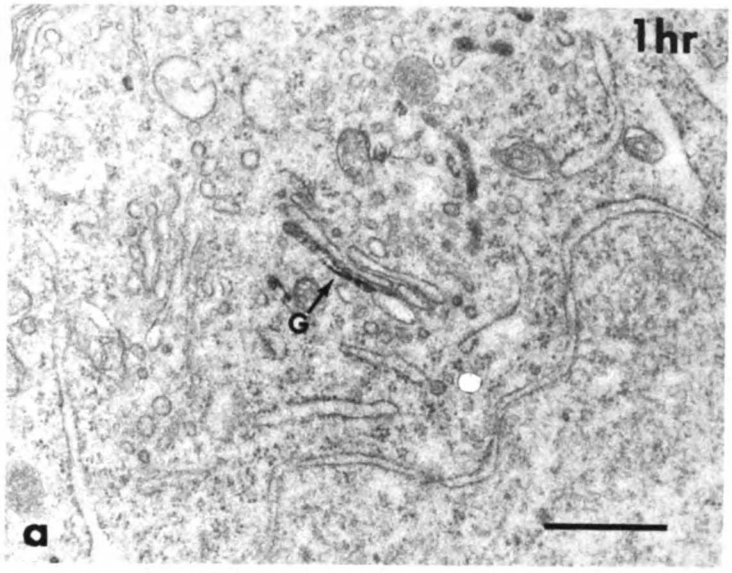

3hr
G
MVB
SV
SER
c

Figure 6.3

Electron micrographs of peroxidase labeled organelles in chick retinal ganglion cells at 1 hour (a) and 3 hours (b) after intravitreal injection of HRP. Arrows point out accumultion of reaction product in cisterns of the Golgi apparatus (G). a) bar = 0.5 μm, magnification = 32,000 X; b) bar = 0.5 μm, magnification = 37,000 X.


1hr
G
a

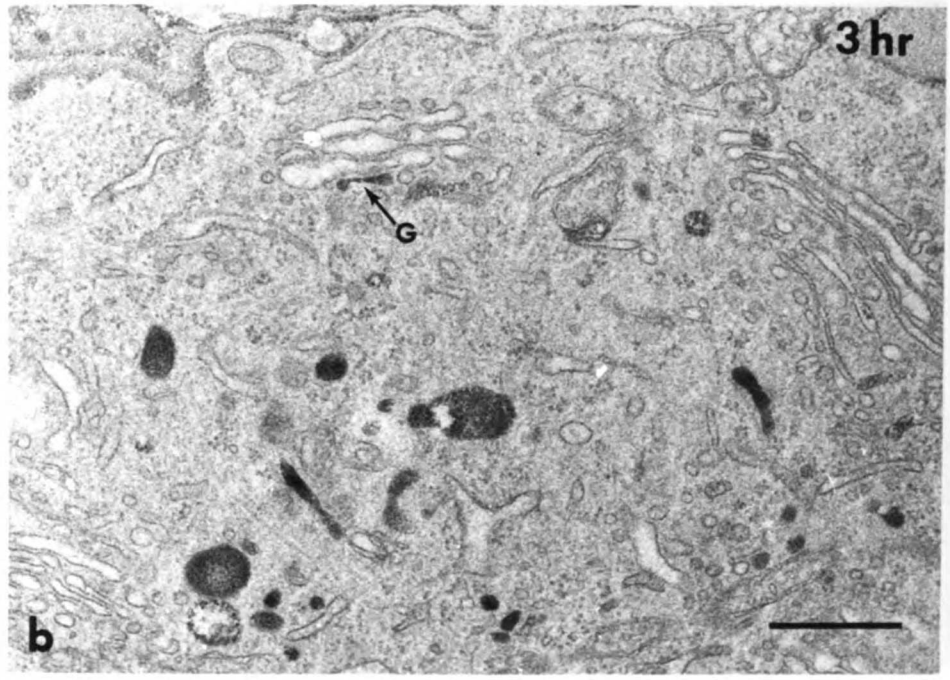
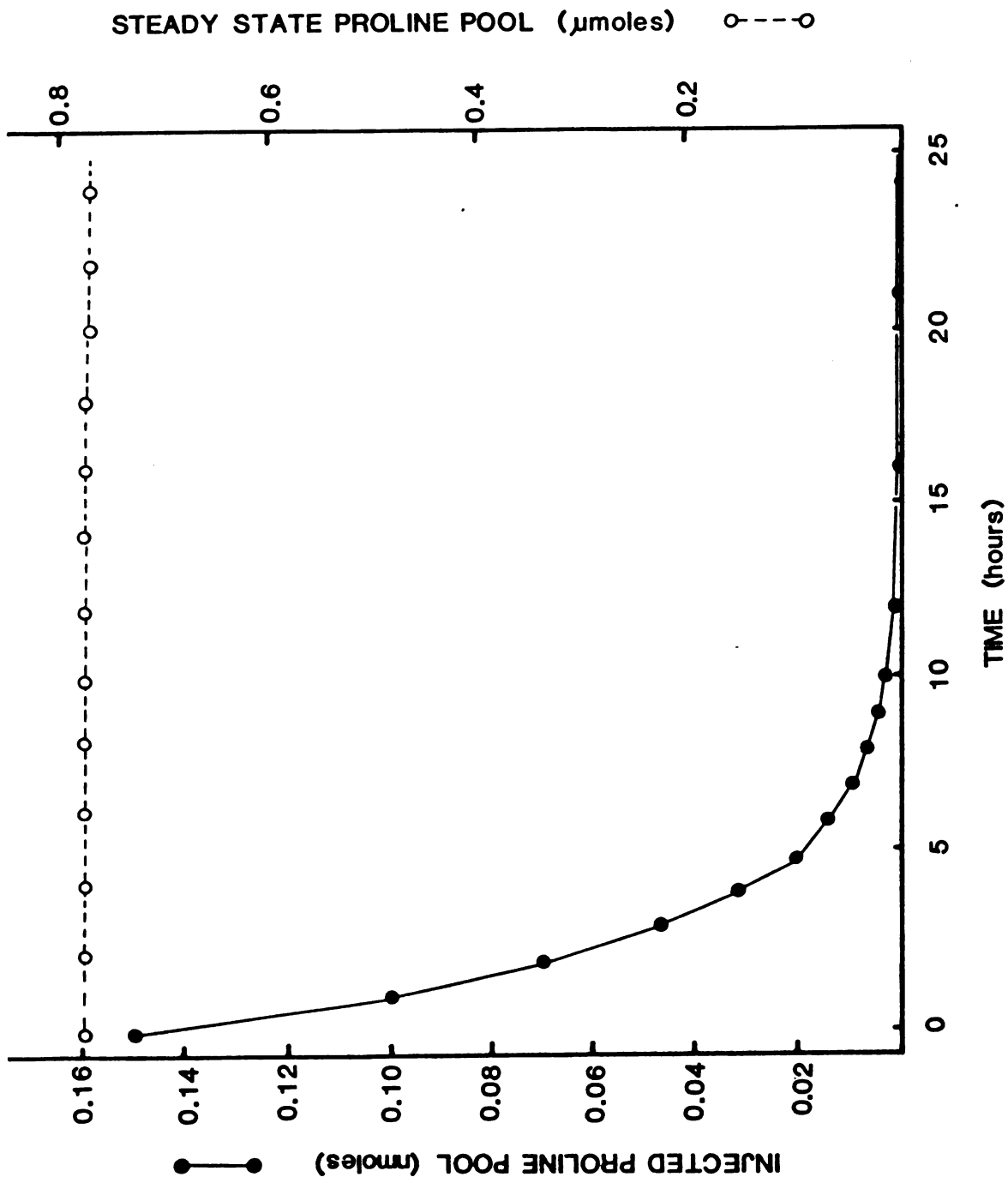
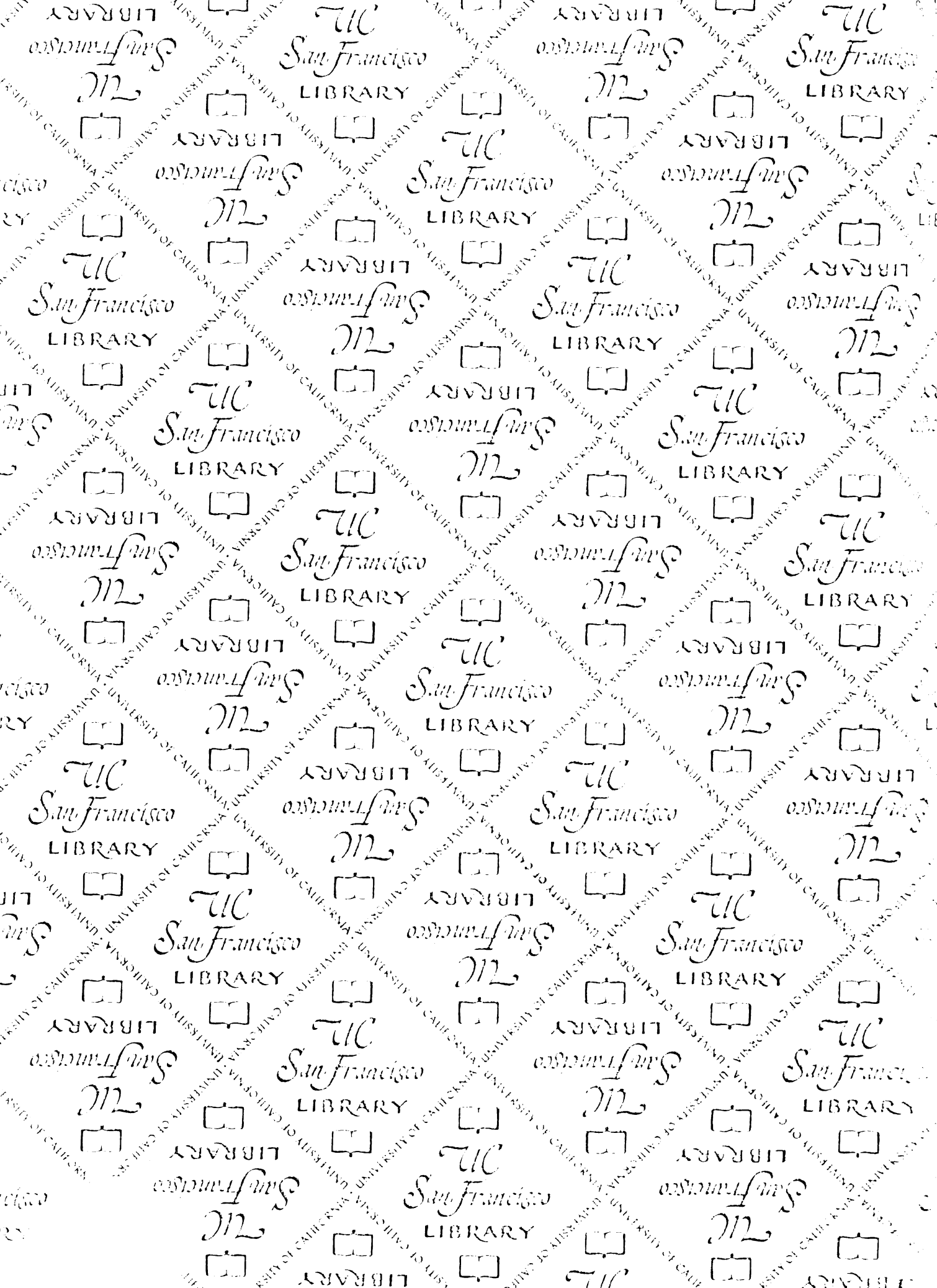

3hr
G
b

Figure A.1

Pool sizes of proline in the chick vitreous as a function of time following intravitreal injection of 15 μCi of ^{3}H-proline at a specific activity of 100 Ci/mmole. The data presented here are derived from theoretical considerations, and are described mathematically by equation (1), dotted line, and equation (3), solid line.


STEADY STATE PROLINE POOL (µmoles)
0.8
0.6
0.4
0.2
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
INJECTED PROLINE POOL (µmoles)
0
5
10
15
20
25
TIME (hours)


UC
San Francisco
LIBRARY
UNIVERSITY OF CALIFORNIA, UNIVERSITY OF CALIFORNIA