LECTIN BINDING PATTERN IN *D. MELANOGESTER* EMBRYOS

CARBOHYDRATE DISTRIBUTION PATTERN IN *DROSOPHILA MELANOGASTER* EMBRYOS AS SURVEYED WITH A BATTERY OF LECTINS

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ABSTRACT:

Lectins were used to survey the distribution of complex carbohydrates in *Drosophila melanogaster* embryos. Lectins are a tool to study the glycobiology of developing tissues, like the development of the central nervous system (CNS). A battery of lectins from all five groups as outlined by Damjanov (1987) were used: glucose/mannose specificity (*Conavalia ensiformis* [Con A]); N-acetylglucosamine specificity (*Triticum vulgaris* [WGA]); N-acetylgalactosamine/ galactosamine specificity (*Dolichos biflorus* [DBA], *Glycine maximus* [SBA], *Bauhinia purpurea* [BPA], *Griffonia simplicifolia* I-B_4 [GSA-I] and *Arachis hypogaea* [PNA]); L-fucose specificity (*Ulex europaeus* [UEA-I]); and sialic acid specificity (*Limulus polyphemus* [LPA]). The N-acetylgalactosamine/galactosamine specific group was divided further into subgroups consisting of DBA, SBA, BPA and GSA-I in one group and PNA in another. PNA has been placed into its own group since it is the only lectin used in the survey which is specific for ß-galactose.

At pre-gastrulation PNA and LPA both labeled the entire embryo fairly intensely while Con A and UEA-I labeled but not as intensely. DBA, SBA, BPA and GSA-I expressed a peculiar binding pattern at this stage that bound the cells lightly but the yolk intensely while WGA did not stain at all. The pattern established by each lectin

remains by stage 11, except that WGA now also binds all cells. Stage 16 are the oldest embryos observed in this survey. By this period in their development most structures are nearly completely formed. Con A displayed intense signal on all structures including the ectoderm, CNS and yolk. By stage 16, WGA revealed only faint signal throughout the embryo. DBA, SBA, BPA and GSA-I exhibited signal throughout the embryo but was seen to be most intense and specific for the developing digestive system and trachea. At this stage, UEA-I binding was completely absent. LPA binding did persist until this stage and was seen on basically all organs and tissues. PNA is very specific for only the central nervous system at this stage. The binding patterns of all these lectins were also observed after the embryos had been pretreated with the enzymes neuraminidase or hyaluronase. Binding was judged as true if the signal was eliminated or severely reduced after the lectin had been exposed to a competing sugar.

The most interesting binding pattern revealed and the one studied in greater detail was that of *Arachis hypogaea* (PNA). This lectin specifically recognized ligands on the longitudinal tracts and the anterior and posterior commissures. PNA binding was further studied at the electron microscopic level. When staining was performed in the presence of detergent, mimicking conditions used for whole embryo staining, signal was seen in the neuropil and all membranes surrounding it, as well as the commissures. When staining was performed in the absence of detergent, thus leaving cells intact, the signal was localized to the basal lamina. More specifically, it was only present in the extracellular matrix adjacent to the glia of the CNS.

The pattern revealed in whole embryo staining with detergent, though not exactly, is similar to that of monoclonal antibody MAb BP102 (Klambt, 1991), which binds to an unknown glycoprotein on the axons and commissures. PNA has been used to purify *Drosophila* laminin and is known to bind to laminin's ß1 and ß2 subunits (Montell and Goodman, 1988 and 1989). Laminin is a potential ligand for this PNA probe, however, laminin is known to be more widely distributed throughout the embryo.

PNA labeling was further examined in a number of mutations known to affect the formation of the CNS axon tracts: *rho*^{7M43}, *spi*^{IIA}, *sim*$^{B13.4}$, *S*^{1}, *sna*^{IIG05}, *sli*^{IG107}, *twi*^{1}, *pnt*^{8B}, *otd*^{H1}, *hnt*^{E8}, *flb*^{IF26}, *stg*^{7M}, and *ph*^{505}. Since BP102 and PNA, as seen at the light level, have similar binding patterns in the whole embryo, PNA labeling was compared to that of BP102 in these mutants. In all instances the signal was neither eliminated nor enhanced, but rather, was similar and lighter than the pattern established by the BP102.

This thesis is dedicated to the memory my father
Francesco D'Amico.
It was his love of knowledge and school
that instilled the need for me to
persue higher education.
I hope he can be proud of this accomplishment.

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INTRODUCTION:

Glycobiology is a newly developing field whose aim is to understand the structure-function relationships of glycoproteins and glycolipids (Quiocho and Dwek, 1991). Glycosylation of proteins is thought to be one the of the most important post-translational modifications. Glycosylation, like phosphorylation and sulfation often determines the final three dimensional structure of a protein and directs transcellular traffic. Unlike other cellular polymers such as proteins, deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), carbohydrates are not restricted to a linear arrangement. Their branching structure imparts the molecule extraordinary variability and thus makes it a wonderful talent for roles such as receptor-ligand interaction, enzyme modification and cell recognition.

The focus of this thesis is to address the possible effects of carbohydrates on the latter phenomenon in a model system with well characterized development and genetics, *Drosophila*. The first step in this investigation consists of a survey of the carbohydrate distribution in the developing *Drosophila melanogaster* embryo. This is accomplished by using lectins as molecular probes for various species of glucoconjugates.

The term lectin was coined by W. C. Boyd, in the late 1940s, from the past participle of the Latin verb *legere*, which means to choose or select (Sharon and Lis, 1989). Lectins are carbohydrate binding

proteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates. In fact, they select and bind to very specific carbohydrates with high affinity. Typically, they possess at least two sugar binding sites and may be either soluble or membrane bound (Barondes, 1984; Damjanov, 1987; Drickamer, 1988). They have no enzymatic activity and are either of bacterial, plant or animal in origin (Barondes, 1984; Damjanov, 1988). Lectins can therefore be viewed as a useful and naturally occurring cytochemical and histochemical tool.

This tool will be exploited by conjugating the lectin, which binds to specific carbohydrates, to biotin, which binds very specifically to avidin. Avidin is, in turn, conjugated to horse radish peroxidase, which offers the experimenter an indirect means to visualize binding of the lectin to a carbohydrate. Damjanov (1988) reviews evidence that the addition of molecules such as biotin, agarose or fluorescein does not alter the lectin's ability to bind carbohydrate ligands.

Conjugated lectins have been capitalized by cell biology, pathology and many other fields. Lectins were first discovered in 1888, by Hermann Stillmark, who observed the haemagglutinating ability of castor bean extracts. More recently, Peter C. Nowell discovered the mitogenic capabilities of *Phaseolus vulgaris* (PHA) on lymphocytes in 1960 which revitalized the study of these molecules (Sharon and Lis, 1989). Of even greater importance to the scientific community was the 1963 discovery of Joseph C. Aub, showing *Triticum vulgaris* (WGA) preferentially promoted agglutination of malignant cells. The realization that researchers had in their hands an invaluable tool to study mitosis and cancer was astonishing in itself. However, this awareness also suggests that carbohydrates are playing some role in these and

possibly other cases.

The natural function of lectins was still largely conjecture until 1974, when Ashwell and Morel described the role of an animal lectin in the endocytosis of glycoproteins (Sharon and Lis, 1989). This event possibly marks the first implication that carbohydrates and lectins perform very specific functions in plants, bacteria and animals. What was also realized was a veritable treasure chest of tools, accumulated over the last eight decades. The lectins themselves can be used to dissect the distribution and function of carbohydrates and carbohydrate-protein interactions.

Other potential uses of lectins became apparent in 1970 when Aspberg, Lloyd and Porath used *Conavalia ensiformis* (Con A) to affinity purify glycoproteins (Sharon and Lis, 1989). This practice is still widely used today. In fact many lectin columns are commercially available. Also accessible are almost every lectin ever purified, and conjugated to some sort of detection system. Consequently, a researcher has at their disposal a means of detecting many carbohydrates in their system of study.

They are ideal markers for cellular components since organelles such as cell surface membranes, the golgi apparatus, the endoplasmic reticulum and organelles involved in the synthesis, storage or turnover of complex carbohydrates or the post-translational modification of proteins are rich in glucoconjugates (Damjanov, 1988). Lectins have also been used to study microdomains in cell membranes, to track intracellular traffic, identify specific cell types, as markers of differentiation and development and as an extracellular matrix marker (Damjanov, 1988). The later is of interest to those studying the

development of the nervous system. An example of a lectin that is a useful probe for the ECM is *Griffonia simplicifolia* I-B4, which is a good marker for basement membranes in murine tissues (Damjanov, 1987).

In the last decade, as more researchers are turning to lectins as novel markers in their systems, the list of useful lectin probes has mushroomed. By reviewing some of the recent discoveries made using lectins as probes in the nervous system of various organisms, one may uncover clues regarding how to study carbohydrates and their possible function in *Drosophila*, particularly with their role in the developing nervous system.

One example is *Ulex europeus* agglutinin I (UEA-I), which displays high affinity for freshly isolated rat Merkel cells (Rosati, Nurse and Diamond, 1984). *Arachis hypogaea* (PNA) binding was revealed when these cells were first treated with neuraminidase. This binding pattern was also observed in cryostat and paraffin sections.

Boya et al. (1991) used lectins to study controversial aspects of the origin and nature of rat microglial cells. They determined that *Griffonia simplicifolia* I-B4 (GSA-I) and *Ricinus communis* (RCA-I) labeled developing microglial cells. Using these novel lectin markers they demonstrated that the source of microglial cells in the rat are from both the meningeal connective tissue layer and the cavum septum pellucidum. Subsequently, Colton et al. (1992) studied rat CNS microglia *in vitro*. They first identified which lectins would be useful probes of this cell type. They also found that GSA-I and RCA expressed high affinity for these cells as well as WGA and Con A. Colton et al. (1992) continue to study the effects of agents such as

dimethylsulfoxide (DMSO), interleukin-1, (IL-1), interferon (IFN) and lipopolysaccharide (LPS) have on rat microglia using these lectins as markers.

Damjanov and Black (1987) also exploited lectins as probes in neurobiology. They were able to identify ciliated ependymal cells on paraffin embedded sections of rat spinal cord with *Solanum tuberosum* (STA) and *Triticum vulgare* (WGA) that were conjugated to fluorescein isothiocyanate (FITC).

Herken, Sander and Hofmann (1990) observed the localized binding of four lectins in the seven day old mouse embryo. After ultrastructural examination, they demonstrate WGA and RCA-I binding at the surface of the embryonic ectoderm, while *Glycine maximus* (SBA) and *Limax flavus* (LFA) had their binding sites on the basement membrane of the ectoderm. LFA was also shown to have affinity to the nucleoli of the ectodermal, the mesodermal and endodermal layer and the ribosomes of these cells.

Lectins are also used to observe the changes in carbohydrate distribution during development. An example of such a study is that of Griffith and Sanders (1991) who investigated the changes in oligosaccharides during the gastrulation and neurulation stages of the early chick embryo development using a battery of fifteen lectins. Ultrastructural observations demonstrate PNA bound to the extracellular matrix fibrils at gastrulation. Several lectins demonstrated affinity for the epiblast, mesoderm and endoderm layers at this stage. At neurulation, PNA bound only to the neural plate, and after pretreatment with neuraminidase also bound to the future floor plate. Hyaluronate lyase pretreatment completely eliminated PNA binding,

suggesting the ligand is associated with the extracellular matrix.

Hyaluronic acid is a simple molecule consisting of up to several thousands of the nonsulfated disaccharides glucuronic acid and N-acetyglucosamine arranged in a regular repeating pattern (Gilbert, 1988; Alberts et al., 1989). In chicks the compound is made by ectodermal cells and accumulates in the blastocoel (Gilbert, 1988). Several studies have demonstrated that hyaluronic acid is necessary to keep mesenchymal cells separated in order to facilitate proper migration (Fisher and Solursh, 1977). It has been found in varying amounts in all tissues and extracellular spaces but is most abundant in embryonic and wound repairing tissues. There is evidence that hyaluronic acid is involved in cell migration (Gilbert, 1988; Alberts et al., 1989) and hence is of great interest to those studying the development of the CNS.

Neuraminidase hydrolyzes galactose-N-acetylneuraminic acid at the terminal end of oligosaccharides. This carbohydrate is found at the terminus of many complex carbohydrates and, consequently, many researchers use this enzyme to uncover (or reveal) lectin ligands which may be masked by neuraminic acid (Rosati, D., Nurse, C.A. and Diamond, J., 1984; Griffith and Sanders, 1991).

Roth et al. (1992) employed a lectin, *Limax flavus* (LFA), to demonstrate that sialic acids are also developmentally regulated in *Drosophila melanogaster*. Sialic acids were detected in the fruit fly from blastoderm to third instar larvae stage. LFA binding was observed to be most intensely at the pole cells in the blastoderm and the golgi and cytoplasmic vesicles of blastoderm cells. As the embryo develops they observe staining in most cells but describe the most intense signal in the nervous system. A second phase in this research

was to look at homopolymers of α2,8-linked sialic acid (or polysialic acid, PSA) with the monoclonal antibody MAb 735. Polysialic acids also appear to be developmentally regulated since they are only present in 12-18 hour old embryos. This result is of particular importance because the polysialic acids detected have been shown to play an important role in regulating the adhesive properties of N-CAM during development (Rutishauser et al. 1988).

In another study, Zipser and Cole (1991) exhibit a role for carbohydrates in regulating neuronal architecture. They demonstrated that defasciculation of axons in the leech CNS is mediated by a mannose containing glycoprotein. Defasciculation was inhibited using three approaches. Firstly, by blocking the Lan3-2 epitope which contained the mannose oligosaccharides in question with antibodies; secondly, by cleaving the carbohydrate moieties with glycosidase N-glycanase; and thirdly, by competing with the putative mannose binding protein with mannose-BSA.

Krull, Morton and Tolbert (1991) performed a lectin screen as a first step to address what glucoconjugates are present in the developing olfactory system of the moth *Manduca sexta*. They discovered that *Arachis hypogaea* (PNA) bound to neurons in the antennal lobe while other lectins displayed no signal. Western blot analysis revealed multiple protein bands. This is another example of how a lectin screen can be used as a first step in unraveling the effects and importance carbohydrates play in biology, more specifically, in developing nervous systems. Also note the recurrence of PNA (or its galactose ligand) as a marker in the nervous system in various organisms which are phylogenitically very distant.

Glucoconjugates on adhesion molecule's are also gaining prominence as functional subunits which are playing meaningful roles in the molecule's overall purpose. Carbohydrates have been shown to facilitate axon growth in culture (Zhang, Miller and Rutishauser, 1992). This group studied the neurite outgrowth from chick retinal neurons on embryonic chick tectal tissue which contains N-CAM. They observed the length of processes which formed on this substrate and after the substrate and neurons were pretreated with neuraminidase which removes polysialic acids. Observations revealed a 25% drop in the average length of neurite outgrowth after polysialic acid residues were eliminated. Polysialic acid is a major component of an axon's surfaces and Zhang, Miller and Rutishauser (1992) propose that it is also a negative regulator of cell-cell interactions. They argue that it limits the degree of receptor-receptor interactions and thus allows optimal axonal growth.

It has been demonstrated that for proper axonal development glucoconjugates are necessary on neurons. It is logical to assume they are also necessary in the substrate or extracellular matrix. This is in fact the case, as Dean, Chandrasekaran and Tanzer (1990) demonstrate with the extracellular matrix molecule, laminin.

Laminin is a highly glycosylated extracellular matrix molecule (ECM) (Timpl and Rohde, 1979; Martin and Timpl, 1987; Fessler and Fessler, 1989; Dean, Chandrasekaran and Tanzer, 1990) and is worth reviewing since much is known about laminin's structure and function and more recently, about the function of some of laminin's glucoconjugates. In 1979, Timpl and Rohde first isolated a large noncollagenous glycoprotein from a mouse tumour that produced

basement membrane. They then knew it consisted of at least two chains, 220 and 440 kDa in size and was part of the basement membrane. The model at the time was that collagenous and noncollagenous proteins fashion a matrix which form barriers between tissues.

Over time many details have been elucidated making this model much more complex. For example, it is known that laminin binds to type IV collagen, heparin, heparan sulfate proteoglycan, entactin, the 140 kDa integrin complex and to itself (Martin and Timpl, 1987; Fessler and Fessler, 1989; Dean, Chandrasekaran and Tanzer, 1990). This is the most abundant protein in basement membranes and is structurally and biologically active. It is found in significant quantity in the ECM around epithelial tissues, fat cells, muscles and nerves and is the first matrix molecule detected during embryogensis (Martin and Timpl, 1987). The model now holds that the basement membranes create barriers that allow embryonic cells to segregate and differentiate into specific tissues.

Laminin is a heavily glycosylated protein. In most cases, 13-15% of the molecule's weight is carbohydrate with some subpopulations up to 25-30% carbohydrate by weight. It consists of three subunits A, B1 and B2, which are 440 kDa, 225 Kda and 205 kDa respectively in the mouse (Martin and Timpl, 1987; Dean, Chandrasekaran and Tanzer, 1990) and are 400 kDa, 220 kDa and 180 kDa in *Drosophila* (Montell and Goodman, 1988, 1989). In mouse the A chain has 46 potential N-glycosylation sites while the B1 and B2 have 11 and 14 each (Dean, Chandrasekaran and Tanzer, 1990). Studies to date show only N-linked oligosaccharides exist on laminin and that carbohydrates are not required for interaction with heparin nor do they protect the molecule

from proteolytic digestion (Dean, Chandrasekaran and Tanzer, 1990). Another fact worth noting is that this molecule has many unique features of oligomers, such as, α-glycosidically linked galactose at the nonreducing end.

Laminin's functions are enhancing binding and spreading of cells, promoting axonal process outgrowth from neurons, inducing endothelial cells to form capillary-like structures and exhibiting growth factor-like activity. Carbohydrates are suggested to be necessary for cell spreading and cell migration on basal lamina. The peptide on the B1 chain YIGSR has been identified as major cell binding domain, while the long arm of the B2 chain is the region responsible for promoting neurite outgrowth (Dean, Chandrasekaran and Tanzer, 1990).

Dean, Chandrasekaran and Tanzer (1990) delved into the problem of demonstrating the role of carbohydrates on laminin using two approaches. The first has been demonstrated in this review as a very powerful and useful tool, lectins. The experimenters coated petri dishes with purified laminin and observed the differentiation of the neuron-like rat pheochromocytoma cell line PC12. They expressed PC12 typical appearance of binding to the substrate and then becoming flattened with several processes emanating from the cell body. They then blocked specific carbohydrates on laminin with lectins. *Griffonia simplicifolia* I (GSA-I) obstructed the binding of PC12 to the substrate in a dose dependant manner while *Conavalia ensiformis (Con A)* did not diminish binding. Con A did, however, prevent neurite outgrowth. Therefore, by masking some carbohydrates on laminin, PC12 differentiation is prohibited.

A second line of evidence was acquired by using unglycosylated

laminin. Laminin was purified from lysates from cultures of a mouse embryonic carcinoma derived cell line M1535 B3 with an anti-Engelbreth-Holm-Swarm (EHS) laminin monoclonal antibody immunoaffinity column. Unglycosylated laminin was attained by treating the culture with tunicamycin and with an additional purification step of a GSA-I column to remove any laminin that did possess glucoconjugates. When the differentiation of PC12 was compared between cells plated on glycosylated and unglycosylated laminin, the unglycosylated cells appeared as those which were plated on laminin treated with Con A; they did not differentiate. This established that once cells bind to laminin the carbohydrate residues, probably in the neurite extension region of the molecule, enable the cells to spread and extend neurite processes. Several laboratories (Dean, Chandrasekaran and Tanzer, 1990; Begovac, Hall and Shur, 1991) believe that galactosyltransferase (GalTase) functions as the receptor during cell migration and neurite extension on the basal lamina.

Begovac, Hall and Shur (1991) ascertained that ß1,4 galactosyltransferase functions as a laminin receptor and promotes neurite outgrowth in PC12 cells. They identified that the fragment E8 bound to GalTase by immunoprecipitating purified fragments with radiolabelled antibodies to that fragment. E8 had higher affinity than any other fragment or the intact molecule. Coincidentally, the E8 fragment spans the region of laminin which is also known to be responsible for promoting neurite outgrowth (Martin and Timpl, 1987). It is not surprising then, that when E8 fragments are used as a substrate they promote neurite outgrowth and that this ability is blocked if the E8 fragment was previously exposed to an excess of free GalTase. These

observations were not noted when other fragments were examined. Moreover, anti-GalTase also hindered neurite outgrowth.

This brings attention to another facet of carbohydrates as functional biological molecules. If they guide some process, the organism must have a means to detect and identify them. GalTase is one potential receptor for a carbohydrate and is an example of how carbohydrates can affect cellular processes. However, another molecule exists which is very well suited to operate as a carbohydrate receptor, namely, lectins. Several very recent discoveries offer more proof that carbohydrates are biologically active, and more importantly, that they are necessary for the proper development of the nervous system.

These lectins can be subdivided into two groups; C-type or S-type, depending on whether they are membrane bound or soluble (Barondes, 1984; Drickamer, 1988). The family of lectins termed S-type are widely distributed in animal tissues in the ECM, cell surface and intracellular compartments depending on the cell and developmental stage. They primarily bind ß-galactosides (Barondes, 1984; Drickamer, 1988). The function of S-type lectins is as yet unclear, however, it is known they are developmentally regulated and in some cases are related to metastasis.

Zhou and Cummings (1990) purified an S-type lectin from the calf heart, giving it the appropriate name, calf heart agglutinin or CHA. They went on to shown that this lectin binds to laminin. Zhou and Cummings suggest that the carbohydrates on laminin are participating in some functional manner.

Woo et al. (1990), have discovered a non-integrin laminin binding protein in murine macrophages. Protein sequencing has shown

this 35 kDa molecule to be identical to carbohydrate binding protein 35 which is galactose specific. Their work suggests that non-integrin LBPs may contribute to laminin adhesion through protein-carbohydrate interactions.

Another example of an animal lectin is the carbohydrate binding 30 kDa (CBP30) from baby hamster kidney cells. This lectin binds type I or II Galß(1-3(4))GlcNAc chains (Sato and Hughes, 1992). Sato and Hughes (1992) also reveal that CBP30 binds to Engelbreth-Holm-Swarm (EHS) tumour laminin. Examination of which fragments of laminin bound CBP30, they found that E8 was the only one which bound that also supported cell attachment and spreading.

There are many other galactose binding proteins which have recently been discovered. Among them are the 15 kDa calcium dependant galactose binding protein from the tunicate *Polyandrocarpa misakiesis* (Suzuki et al., 1990); the 16 kDa (C-16) (Sakakura et al., 1990) and 14 kDa (C-14) (Barondes, 1984) ß-galactose binding lectins from chicken; and the bovine 14 kDa ß-galactose binding protein (Abbott and Feizi, 1991). This list of examples indicates that nature does have a means of using carbohydrates and it must have developed this capability early in evolution since such a wide variety of life share these features.

Once a lectin such as those mentioned is discovered the next question to address is what that lectin's function(s)? Wilson, Carrow and Levitan (1992) discovered a novel galactose binding protein from *Aplysia californica* which is a dimer of 65 kDa with two 34 kDa subunits named *Aplysia* gonad lectin (AGL), indicating the lectin's source. They then tested AGL's affect on *Aplysia* neurons in a primary

culture system. AGL was found to enhance neurite outgrowth and viability of these neurons *in vitro*. These results suggest that AGL possesses some function in the normal differentiation and maintenance of *Aplysia* neurons. Wilson, Carrow and Levitan (1992) offer three potential roles that AGL may be fulfilling. They postulate that AGL's affect on cultures may be the result of one of the following three reasons: (i) non-specific, resulting from an altered turnover rate of some neuronal protein due to the binding of AGL; or, (ii) due to a better adhesion of the neurons to the substrate, increasing the longevity of the neurites; or, (iii) that AGL is somehow modulating a protein such as a growth factor that affects neurite survival. Whatever the explanation, one thing is certain, carbohydrates and carbohydrate-protein interactions are responsible for the results.

One point which all the various systems listed here have in common is that they are difficult to manipulate for one reason or another. They also lack readily available mutants which affect systems like the nervous system and processes like development. What is required is a means of experimentally manipulating carbohydrate-protein interactions once one is aware of the players involved.

The fruit fly, *Drosophila melanogaster*, has been used for over eighty years as an experimental organism, since it was first introduced in the study of genetics by T. H. Morgan in 1909. *Drosophila* have several features which make them amenable to genetic study, such as, their small size, short life cycle and a genome which is one twentieth that of a typical mammalian genome (Thomas et al., 1984; Rubin, 1988). Since its inception as an experimental organism a host of genetic, biochemical and molecular techniques have been developed

which exploit these attributes. Eighty years of study has also uncovered thousands of mutants, which either occurred naturally or were induced by the experimenters. The development of this organism is also mapped in great detail, both cytologically and genetically (Campos-Ortega and Hartenstein, 1985). Packaged together, this makes the fruit fly a very versatile and powerful tool for multidisciplinary scientific study. These features also provide excellent reasons to select *Drosophila* to study glycobiological phenomena.

In order to initiate a study of *Drosophila* glycobiology we used lectins to dissect the carbohydrate distribution patterns in embryonic development. This survey may provide novel histochemical and cytological markers for the study of *Drosophila's* CNS or other systems. It may also lend clues revealing which carbohydrates are of functional importance. Lis and Sharon's (1991) review of lectin-carbohydrate interactions, states that lectins are exemplary candidates to function as mediators of cell recognition since they seem to be ubiquitous on cell surfaces and are highly specific for carbohydrates. As all the examples reviewed express, a survey is a valuable launching pad for numerous avenues of glycobiological investigations.

Monosaccharide specificity of lectins is determined by observing which sugar has the most inhibitory affect on the lectins ability to haemagglutonate red blood cells. The binding of many lectins is effectively inhibited by more than one sugar, while some sugars have less affect and others none at all. This test describes the proposed ligand for a lectin but does not necessarily reflect the exact nature of the carbohydrate that has the highest affinity for a particular lectin. Hence, when interpreting data from a lectin screen one must show caution and

not make definitive conclusions with regards to a certain lectin's ligand.

The affinity of some lectins to accept more than one species of carbohydrate can be explained by its capability of tolerating some variation at the C-2, very little variation at C-3 and no variation at C-4 of the pyranose ring (Damjanov, 1988). An example of this is *Conanvalia ensiformis* (Con A), a mannose lectin that can also bind glucose because of the structural similarities of these three carbon atoms (Damjanov, 1988). Another point one must note is that although most lectins preferentially react with the nonreducing end of a carbohydrate chain, some do react with internal components (Damjanov, 1988). For example, internal N-acetyl-galactosamine residues are typical of hyaluronic acid and are recognized by *Triticum vulgare* (WGA). Therefore, to distinguish between actual and presumptive ligands to which a lectin is binding, extensive enzymatic and biochemical controls must be performed.

This thesis will take the first step in a study of carbohydrate function in *Drosophila* by performing a survey of carbohydrate distribution in developing embryos. Embryos will be collected from various stages, then fixed and then exposed to lectins conjugated to biotin. To visualize binding, avidin conjugated to horse-radish-peroxidase, which will bind with high affinity to biotin, will be used. Embryos from pregastrulation to stage 16 will be examined for lectin binding.

To address the obstacles outlined, three types of control tests will be performed in this study. Firstly, in order to ensure that lectin binding observed was due to a true signal, the lectin was preincubated with the sugar which is known to provide the most inhibition in

agglutination assays. This should severely reduce or completely eliminate any signal that is not a result of the lectin's binding carbohydrates. Secondly, embryos will be pretreated with hyaluronate lyase to remove hyaluronic acid. This will eliminate any signal which is a result of some component of this residue or the entire residue. It may also unmask new potential ligands. Thirdly, embryos will also be pretreated with neuraminidase for the same purpose as mentioned for hyaluronate lyase. These biochemical tests are crude but will begin to assess if binding of a particular lectin is real and may narrow the potential ligands to which it is associating with.

To our knowledge, no one has completely surveyed *Drosophila melanogaster* embryos for lectin binding. Montell and Goodman (1988) did perform a partial survey because they were in search of a means to purify *Drosophila* laminin and enroute discovered that PNA bound laminin. The primary goal of our study was to explore the carbohydrate distribution pattern throughout the embryos development utilizing a battery of lectins. These lectins were selected to be specific for a broad range of carbohydrates representing all the five groups outlined by Damjanov (1987). This survey addresses two questions: firstly, what is the carbohydrate distribution in the *Drosophila* embryo and does it change through the various stages of development; and secondly, are any lectins highly specific for particular cells or tissues which could be exploited as histochemical markers or tools to probe functions like adhesion recognition.

Our laboratory is interested in the development of the embryonic central nervous system (CNS), but such a survey may uncover markers which can be potentially useful to researchers studying other organ

systems in *Drosophila*. Therefore, a lectin which selectively binds the CNS axons or glial cells or any other component of the CNS could be highly useful and will be explored further. Once the lectins which show affinity for the CNS are determined, we can then ask where specifically does it bind? The answer to this problem clears the path for many other questions which could be pursued.

Once such a histochemical marker for the CNS is described one can quickly survey mutations that affect this area. By assessing a mutant's affect on the binding pattern one can begin the first steps of defining the lectin's ligand potential role in the fruit fly, if any exists. Embryos which have mutations in genes that are known to have an affect in either determining neuronal phenotypes or differentiation and/or are transcription factors within the CNS represent a logical place to begin this survey. One can ascertain whether such a presumptive ligand is under the control of these genes or is involved in neuronal determination if it is either eliminated or severely up regulated in these mutants. This survey represents the first step to more focused glycobiological studies in *Drosophila melanogaster*.

Materials and Methods:

Fly strains

The lectin screen was performed on *Drosophila melanogaster* wild type Oregon-R strain, which was maintained on modified banana-yeast media. Most of the mutant alleles used were isolated in a large scale screen of cuticle morphogenesis (Nusslein-Volhard, C., Wieschaus, E. and Kluding, H. 1984) and are nulls unless otherwise indicated. The alleles *rho*^{7M43}, *spi*^{IIA}, *S*^{1}, *sna*^{IIG05}, *sli*^{IG107}, *twi*^{1}, *pnt*^{8B}, *otd*^{H1}, *hnt*^{E8} were all obtained from the Bowling Green Stock Centre. Other alleles used were *sim*$^{B13.4}$ (provided by S. Crews), *flb*^{IF26} a hypomorphic allele (provided by C. Nusselin-Volhard), *stg*^{7M} (provided by P.H. O'Farrel) and *ph*^{505} (provided by J-M. Dura).

Reagents

Several biotinylated lectins were obtained from E-Y Laboratories Inc. (San Maeo, CA). They included; *Conavalia ensiformis* (Con A), *Triticum vulgaris* (WGA), *Dolichos biflorus* (DBA), *Glycine maximus* (SBA), *Bauhinia purpurea* (BPA), *Griffonia simplicifolia* I-B$_4$ (GSA-I), *Arachis hypogaea* (PNA), *Ulex europaeus* (UEA-I), and *Limulus polyphemus* (LPA). Additional biotinylated PNA was obtained from the Sigma Chemical Co. To detect lectin binding horse radish peroxidase conjugated to avidin was used (E-Y Laboratories, San Maeo, CA). To verify that labeling observed was real, each lectin was

preincubated with its competing sugar. The sugars were acquired from the Sigma Chemical Company were: D-glucose (Con-A), N-acetyl-D-glucosamine (WGA), D-galactose (DBA, SBA, BPA, GSA-I and PNA), a-L-fucose (UEA-1) and N-acetylneuraminic acid (LPA). Hyaluronate lyase (from *Streptomyces hyalurolyticus*) and neuraminidase (from *Vibrio cholerae*) were procured from Sigma Chemical Company. The monoclonal antibody BP102 was a gift from C. S. Goodman. The antibody goat-anti-mouse horse radish peroxidase was obtained from Jackson Immunological Laboratories. Osmium tetroxide was acquired from Marivac while thiocarbohydrazide was obtained from Ted Pella Inc. (Redding, CA).

Lectin and antibody staining of Drosophila embryos

Embryos up to stage 16 were collected on apple juice agar plates and dechorinated with 3% hypochlorite for 5 minutes. They were then washed thoroughly with distilled and deionized water and fixed at the heptane:phosphate-buffered saline (PBS) interface for 30 minutes. The PBS contained 4% paraformaldehyde. The fix solution was then removed and replaced with 85% ethanol. Embryos were violently agitated to facilitate vitelline removal. Devitellinized embryos were then collected and washed several times with fresh 85% ethanol. At this stage the embryos were either immediately stained or stored up to 48 hours at 4°C in 85% ethanol. The ethanol was replaced during several long washes on a rotator with PBS and 0.1% Triton X-100 (PBT). The embryos were then blocked for a minimum of 30 minutes with 5% normal goat serum (NGS) in PBT. Then 50 µg/ml of a biotinylated lectin was added and incubated at room temperature for 60 minutes.

After this primary reaction the embryos were washed with several changes of PBT for a minimum of 2 hours or a maximum of 12 hours. Once again they are blocked with 5% NGS for 30 minutes and then the secondary avidin conjugated to horse radish peroxidase (Av-HRP) is added at a dilution of 1:500. This is allowed to react at room temperature for 60 minutes. The embryos were once again thoroughly washed as described. To visualize binding PBT was replaced with 0.05% diaminobenzidine (DAB) and given 10 to 15 minutes to perfuse through the embryo. Then 0.03% hydrogen peroxide was added to initiate the reaction. The DAB reaction was stopped by dilution of substrate within two minutes.

To establish that lectin staining was specific control experiments were performed. Each lectin was incubated with 0.2M solution of a sugar with which the lectin has a high affinity, for 30 minutes at room temperature. This solution was then used as the primary as described in the protocol. The lectin signal was judged to be positive if this control experiment severely reduced or eliminated the signal.

Antibody labeling was performed on whole mount embryos of similar ages, as described by Patel, Snow and Goodman (1987).

Neuraminidase and Hyaluronate Lyase Pretreatment of Drosophila embryos

After being devitellinized, some embryos were treated with neuraminidase or hyaluronate lyase prior to being exposed to a lectin. Exposure to neuraminidase will remove N-acetyl neuraminic acid (NeuAc) residues, which can either eliminate the lectins ligand if it is linked through this residue or reveal new ligands which were masked by

NeuAc. Hyaluronate lyase will cleave hyaluronic acid type residues potentially achieving the same type of effect as neuraminidase. Neuraminidase pretreatment was carried out by incubating the embryos in 0.17 U/ml for 24 hours at 37°C. Hyaluronate lyase pretreatment was performed by incubating the embryos in 50 U/ml for 2 hours at 37°C. The embryos in both cases were then washed with PBT and then treated with the lectins as already described.

Visualization of lectin and antibody binding to Drosophila embryos

Embryos were staged under a compound Zeiss Axioskop according the specifications as outlined by Campos-Ortega and Hartenstein (1985). To examine embryos prepared as described a Zeiss dissecting microscope was used to establish the likely binding pattern of a large number of embryos to asses the representative staining pattern. For more detailed observations and photomicroscopy the higher magnification of the compound Zeiss Axioskop equipped with Nomarski optics was used.

Electron microscopy

Fixation and embedding for electron microscopy were performed as described by Jacobs and Goodman (1989a,b). Preparation of Drosophila dissected central nervous system was performed essentially as described by Bieber et al. (1989), with a few modifications. Once dissected the embryos were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde prior to staining. Some embryos were exposed to 0.1% of the detergent Triton X-100 during the staining protocol while others were not exposed. After postfixation, the DAB reaction product was

intensified using the Tomlinson and Ready (1987) thiocarbohydrazide method.

RESULTS:

Lectin binding in *Drosophila melanogaster* embryos

Embryos spanning stages from pregastrulation to stage 16 were fixed and stained with various lectins conjugated to biotin. These lectins have been divided into five groups depending on their carbohydrate binding specificity as outlined by Damjanov (1987). A summary of the lectins used in this study can be seen in table 1. To detect binding, avidin conjugated to horseradish peroxidase (Av-HRP) was used, followed by reaction with diaminobenzidine (DAB). The principal findings of this survey are summarized in table 2. For each lectin, binding was observed and noted, especially for staining in the tissues mentioned in table 2, namely, the trachea, gut, ectoderm and central nervous system. Specifically, any unique carbohydrate binding pattern associated with the central nervous system was of particular interest to this study. The results from this screen are summarized below and are illustrated in figure 1 (A-R).

To confirm that the binding observed was true signal and not background, lectins were preincubated with the appropriate competing carbohydrate specified in table 1, and then used to stain embryos. Staining was judged to be true if the signal was severely reduced or completely eliminated (Roth, 1978). This is demonstrated in figure 2 (C, F, I, L, O, and R).

TABLE 1*:

Abbreviations	Lectins/Source	Normal carbohydrate specificity	Inhibitor carbohydrate used
I. Glucose/Mannose group			
Con A	*Conavalia ensiformis* (jack bean)	αMan > αGlc > GlcNAc	D-glc
II. N-acetylglucosamine group			
WGA	*Triticum vulgaris* (wheat germ)	GlcNac(ß1,4GlcNAc)$_{1-2}$ > ßGlcNAc > Neu5Ac	GlcNAc
III. N-acetylgalactosamine/galactosamine group			
DBA	*Dolichos biflorus* (horse gram)	GalNAcα1,3GalNAc >> αGalNAc	D-Gal
SBA	*Glycine maximus* (soybean)	α and ßGalNAc > αGal	D-Gal
BPA	*Bauhinia purpurea* (camel foot tree)	α and ßGalNAc > αGal	D-Gal
GSA-I	*Griffonia simplicifolia* I-B$_4$	αGal	D-Gal
PNA	*Arachis hypogaea* (peanut)	Galß1,3GalNAc > α and ßGal	D-Gal
IV. L-fucose group			
UEA-I	*Ulex europaeus* (gorse seed)	αL-Fuc	αFuc
V. Sialic acid group			
LPA	*Limulus polyphemus* (horse crab)	Neu5Ac (or Gc)α2,6GalNAc > Neu5Ac	Neu5Ac

*Modified from Damjanov 1987.

The effect of removing specific carbohydrate moieties on the lectin binding pattern was also explored. Some embryos were pretreated with hyaluronate lyase to remove hyaluronic residues. The effects on the binding patterns of each lectin was then assessed and can be viewed in figure 2 (A, D, G, J, M, and P). Some embryos were pretreated with neuraminidase to remove N-acetyl neuraminic residues. These results are in figure 2 (B, E, H, K, N, and Q).

Lectin screen, enzyme pretreatments and controls:

(i) Group-I lectin

The lectin *Conavalia ensiformis* (Con A), specific for glucose/mannose and branching mannose residues binds to all membranes throughout the embryo's development. This is demonstrated in figure 1 (A-C). At pregastrulation we observe uniformly light stain in the cellular region and in the yolk. By stage 11 all developed structures are staining lightly but the ectoderm stains more heavily. This pattern as well as granules dark with Con A staining in the yolk, and some light staining in the nervous system persists and becomes much more intense by at least stage 16.

When embryos are treated with the enzyme hyaluronate lyase binding was nearly eliminated throughout the embryo and further restricted to a weak signal in the ectoderm. Neuraminidase pretreatment also reduced Con A signal with the remaining label only in the ectoderm and a very light staining of the epiderm ventral to the nervous system. This is demonstrated in figure 2 (A and B, respectively).

lectins have prospective ligands in common, one would expect that when used in this screen they would have similar results. This is, in fact the case. Therefore, only the results from one representative lectin in this group, GSA-1, are listed in figure 1(G-I).

At pregastrulation there is only very weak observable staining in the cellular region of the embryo, but their is very heavy staining in the yolk. By stage 11 we see staining on the ectoderm. The dark staining in the yolk persists. At stage 16 the staining is very heavy, especially in the trachea and developing hind and foregut. This is illustrated by a montage of two focal planes of a GSA-I stained embryo in figure 1 (I). DBA is the only variant in this group. DBA has additional very light staining of the central nervous system. With light level examination, DBA does not appear specific to any structures, but rather binds diffusely throughout the central nervous system (data not shown).

When embryos are pretreated with either hyaluronate lyase or neuraminidase no lectins in this group exhibited any new labeling. For DBA and GSA-I all binding was greatly reduced under both these conditions which suggests that the ligands for these lectins are joined via uronic and neuraminic acid conjugates (data not shown). SBA and BPA binding, however, appeared to be more resistant to exposure to these enzymes. SBA ligands appear to have been largely eliminated by hyaluronate lyase (data not shown) but not by neuraminidase (see figure 2H). SBA ligands present in the ectoderm were eliminated but the ligands in the gut and trachea survived exposure to neuraminidase. BPA manifested the opposite result. When embryos were pretreated with hyaluronate lyase all binding in the gut and trachea was reduced while binding in the ectoderm survived (see figure 2G). Conversely, all

binding was eliminated when treated with neuraminidase (data not shown). This demonstrates that binding of BPA in the ectoderm is independent of uronic acid but may be linked via or dependent upon neuraminic acid as seen by the observed binding in the gut and trachea (see figure 2H). This also illustrates that SBA binding in the gut and trachea are independent of neuraminic acid whereas the signal in the ectoderm is dependent. All other binding is dependent upon hyaluronic acid. It should be noted that although these lectins belong to the same group, they differ slightly in their specificities. They may share ligands but after that ligand has been altered by an enzyme, each lectin may possess a differing tolerance to that alteration.

When GSA-I, SBA, DBA and BPA were each preincubated with D-galactose binding to all tissues was nearly eliminated. An example of this control result is DBA in figure 2 (I).

(iv) Group-III(B) lectin

Arachis hypogaea (PNA) has been placed into group-III(B) because unlike the lectins of group III(A) it is also specific for ß-galactose while lacking some of the specificity's possessed by group III(A). This unique specificity appears to have conferred to this lectin a very distinct binding pattern as is demonstrated in figure 1(J-L). At pregastrulation the whole embryo stains, including the yolk and the cellular region. By stage 11 the stain becomes restricted to the ectoderm and the developing central nervous system(CNS). The CNS binding has a distinctive ladder pattern of axon tracts similar to that revealed by the monoclonal antibody BP102 (see figure 5, A and B). PNA labeling becomes darker and more restricted by stage 16 while

staining in the ectoderm is lost. Similar to group-III(A), this lectin has binding in the yolk and some signal in the gut.

When embryos are pretreated with hyaluronate lyase and then stained with PNA all CNS, yolk and gut binding is eliminated, however, the early ectoderm binding is not eliminated (see figure 2J). This evidence would suggest that the CNS binding is hyaluronic acid dependant while the ectoderm binding may be hyaluronic acid independent. PNA binding is completely unaffected by neuraminidase, indicating that PNA's ligands are not linked via neuraminic acid (see figure 2K). Neither enzyme reveals new PNA binding.

PNA preincubation with D-galactose severely reduces all binding as shown in figure 2(L).

(v) Group-IV lectin

Ulex europaeus (UEA-I) is specific for L-fucose and belongs to group-IV. This lectin bound lightly to the cellular region and germ band at pregastrulation and stage 11, but by stage 16, all specific binding was lost. This can be seen in figure 1(m-o).

Preincubating the embryos in neuraminidase did not reveal any new staining patterns (see figure 2n). However, preincubation with hyaluronate lyase did change the binding pattern as shown in figure 2(m). Hyaluronate lyase uncovers UEA-I ligands in the ectoderm, CNS, gut and yolk. This suggest that uronic acid masks L-fucose in a Drosophila carbohydrate.

Preincubating UEA-I with a-fucose eliminated all binding (see figure 2o).

Figure 1: Representative binding of lectins conjugated to biotin detected with avidin conjugated to horse radish peroxidase and then reacted in the presence of diaminobenzidine. All embryos are oriented with the anterior facing left and ventral side down. The only exception is window J which has the embryo's anterior facing right. Each vertical column represents a different stage of the embryos development. The column commencing with: A are pregastrulation embryos; B are stage 11 embryos; C are stage 16 embryos. The lectins used were: Con A in row A, B and C; WGA in row D, E, and F; GSA-I in row G, H and I; PNA in row J, K and L; UEA-I in row M, N and O; and LPA in row P, Q and R. The stage 16 embryo stained with GSA-I in window I is a montage of two focal planes of this embryos, displaying a parasagital plane in the left half and a sagittal plane in the right half. These embryos were photographed at a final magnification of 360x under a compound microscope with Nomarski.

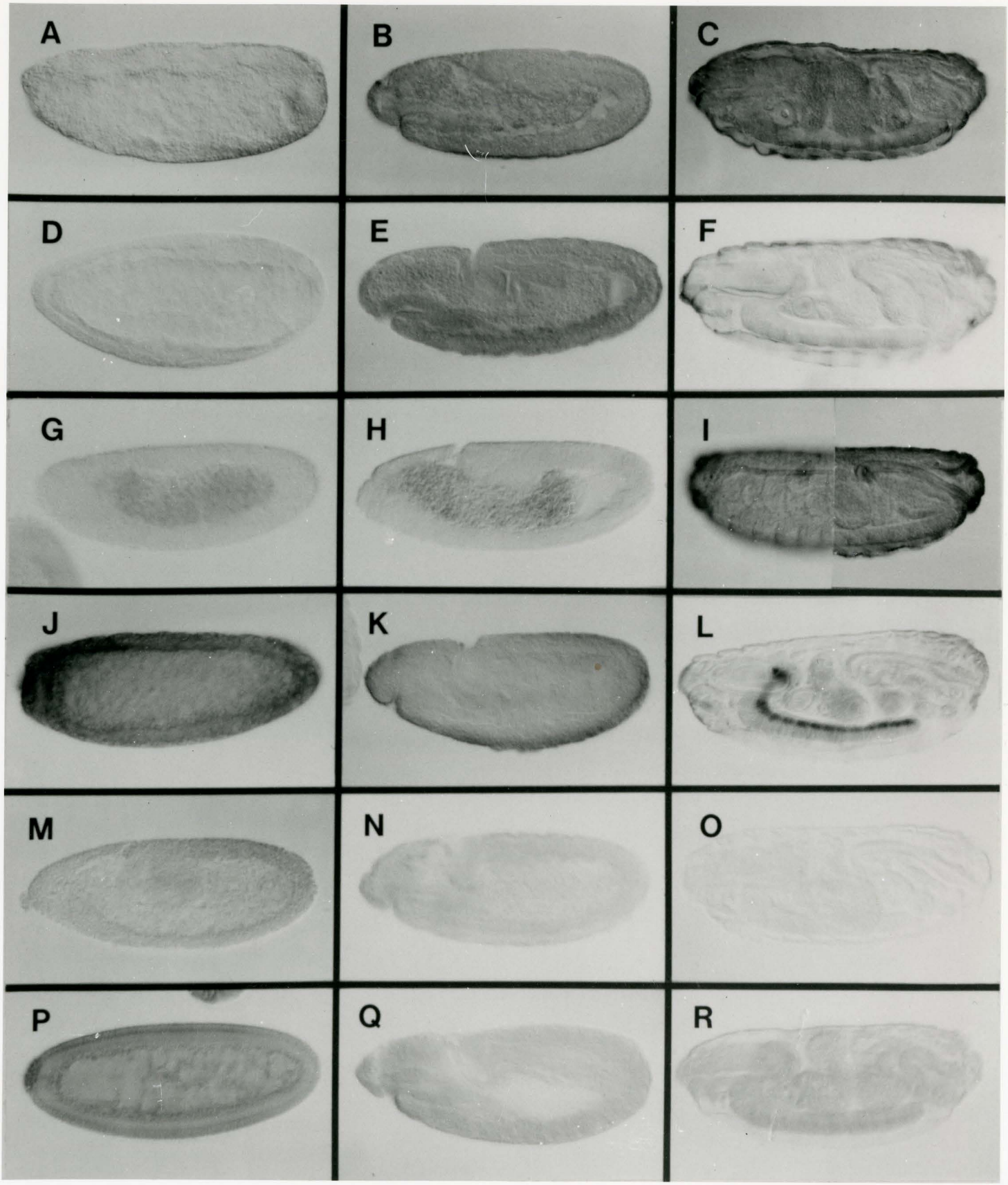
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Figure 2: Representative binding of lectins conjugated to biotin detected with avidin conjugated to horse radish peroxidase and then reacted in the presence of diaminobenzidine. All embryos are oriented with the anterior facing left and ventral side down. All embryos in this panel are at stage 16 of development. Each vertical column represents a different control experiment. The column commencing with: A are embryos which were pretreated with hyaluronate lyase; B are embryos that were pretreated with neuraminidase; C are embryos that were stained with lectins which were preincubated with the appropriate competing sugar. The lectins used were: Con A in row A, B and C; WGA in row D, E, and F; GSA-I in row G, H and I; PNA in row J, K and L; UEA-I in row M, N and O; and LPA row in P, Q and R. These embryos were photographed at a final magnification of 360x under a compound microscope with Nomarski.

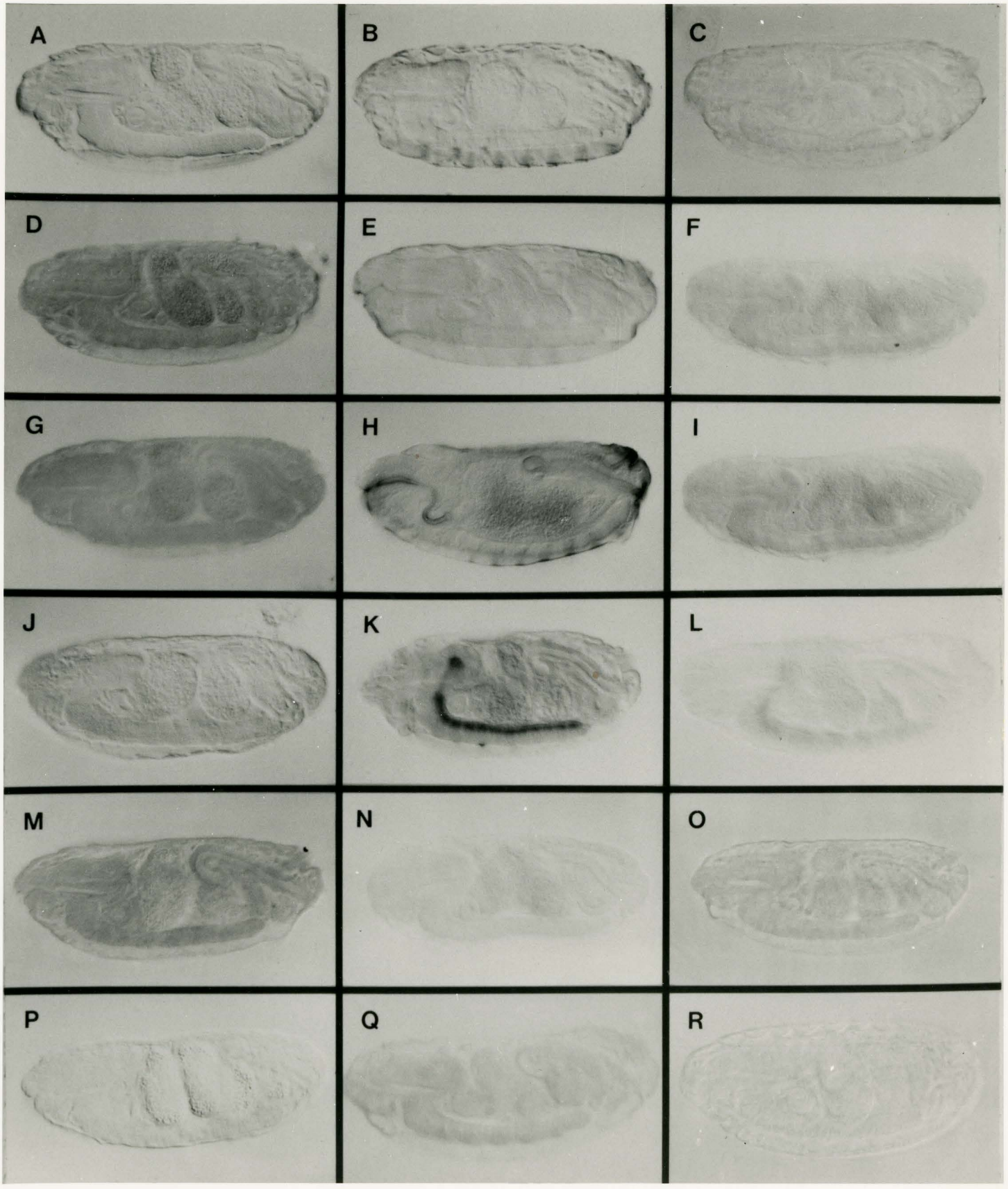
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Table 2:

Structures	Experiment	Lectins					
		Group-I	Group-II	Group-III(a)	Group-III(b)	Group-IV	Group-V
Trachea	lectin	+	-	++	-	-	+
	neuraminidase	-	-	(+)	-	-	-
	hyaluronase	-	-	(w)	-	-	-
	control	-	-	-	-	-	-
Gut	lectin	-	w	++	+	-	+
	neuraminidase	-	-	(+)	+	-	-
	hyaluronase	-	w	(w)	-	+	-
Ectoderm	lectin	++	+	+	+	-	+
	neuraminidase	w	-	-	+	-	-
	hyaluronase	w	-	(w)	-	+	-
CNS	lectin	+	-	-	+++	-	+
	neuraminidase	-	-	-	+++	-	(w)
	hyaluronase	-	w	(w)	-	+	-

Group-I is specific to glucose/mannose, *Conavalia ensiformis* (Con A); group-II is specific to N-acetylglucosamine, *Triticum vulgaris* (WGA); group-III(A) is specific to N-acetylgalactosamine/ galactosamine, *Dolichos biflorus* (DBA), *Glycine maximus* (SBA), *Bauhinia purpurea* (BPA) and *Griffonia simplicifolia* I-B4; group-III(B) has the general specificity as group-III(A), *Arachis hypogaea* (PNA); group-IV is specific to L-fucose, *Ulex europaeus* (UEA-I) and group-V is specific to sialic acids, *Limulus polyphemus* (LPA). Symbols indicating relative strength of stain: + staining, ++ intense, +++ very intense, - no staining, w weak

(vi) Group-V lectin

Limulus polyphemus (LPA) is a group-V lectin which is specific for sialic acid residues. LPA stained the cellular region and the germ band at pregastrulation and stage 11, respectively. By stage 16 it also seemed to bind to trachea, CNS, ectoderm and gut. This can be seen in figure 1(P-R).

Both hyaluronate lyase and neuraminidase eliminated or drastically reduced all staining observed with LPA (see figure 2 P and Q).

Preincubating LPA with N-acetylneuraminic acid abolished any staining previously observed (see figure 2R).

Lectin binding to embryonic cells in tissue culture

Lectins were tested on primary cell cultures, to assess their potential as probes that could identify cell type. Drosophila embryonic cells were cultured as described in Appendix A and then stained with the entire battery of lectins. The carbohydrate distribution pattern characterized *in situ* appears to be conserved *in vitro*.

Group-I lectin, Con A, bound to all cells and to some cellular debris in culture paralleling this observation *in situ*. Group-II, WGA, which is similar to Con A *in situ*, also had an affinity for most cells in culture. Group-III(A) formed a class of lectins that bound to the trachea and gut, except for DBA which lightly stained the CNS and BPA that possessed an affinity for the ectoderm. In accordance with the *in situ* observations, BPA stained most cells in culture lightly while SBA, DBA, and GSA-I displayed no significant staining. Group-III(B)

consisted of PNA and was very specific for the CNS. *In vitro* this lectin appeared to stain cell aggregates with one or more processes which suggests that these cells are derived from the CNS. Group-IV lectin, UEA-I had a very low signal as seen *in situ*, while Group-V lectin, LPA only bound to very few cells in culture. This data is only preliminary and awaits a proven primary culture system for *Drosophila* cells. Tissue culture data are not shown.

PNA binds to laminin and is restricted to the extracellular matrix surrounding the glial cells in the CNS.

The most interesting binding pattern was that of *Arachis hypogaea* (PNA). As seen under the light microscope in figure 5 (B), PNA binds exclusively to the axons in the central nervous system (CNS). This includes the longitudinal tracts and the anterior and posterior commissures in each segment. It now seemed prudent to examine precisely where PNA was binding (i.e. the cytosol, the membrane, etc.). This was accomplished by electron microscopic observations of PNA's binding pattern when used on embryos with or without exposure to detergent.

Figure 3 illustrates PNA binding in the CNS at low magnification. Figure 3 (A), which was stained without detergent shows binding on the surface of the glial cells and the basil lamina. It also reveals stain in the neuropil. Figure 3 (B), which is tissue exposed to detergent, illustrates heavy binding in the neuropil only. Figure 3 (C), which was not

Figure 3: Electron microscopic analysis of PNA binding in the CNS. Pictured are micrographs of cross sections of the *Drosophila* embryonic CNS. The asterisk (*) illustrates the neuropil while the solid arrowhead points to the region dorsal to the axons and the open arrowhead focuses attention to the basement membrane. Panels A and C are from embryos that were not exposed to detergent while panels B and D are from embryos that were stained in the presence of detergent. Panels A and B are stained with PNA. Panel A, which was not exposed to detergent displays signal in the basement membrane and glial membranes. Panel B which was exposed to detergent exhibits further staining in the neuropil and all other staining seen previously is eliminated. Micrographs C and D are respective controls, in which the PNA was previously incubated with 0.2M D-galactose to saturate the carbohydrate binding domains of PNA. Both panels, with or without prior exposure to detergent illustrate that PNA binding in all tissue is drastically reduced. Scale bar is 5 μm. Note: Dr. J. R. Jacobs performed the dissections which exposed the CNS, the sectioning and electron microscopy.

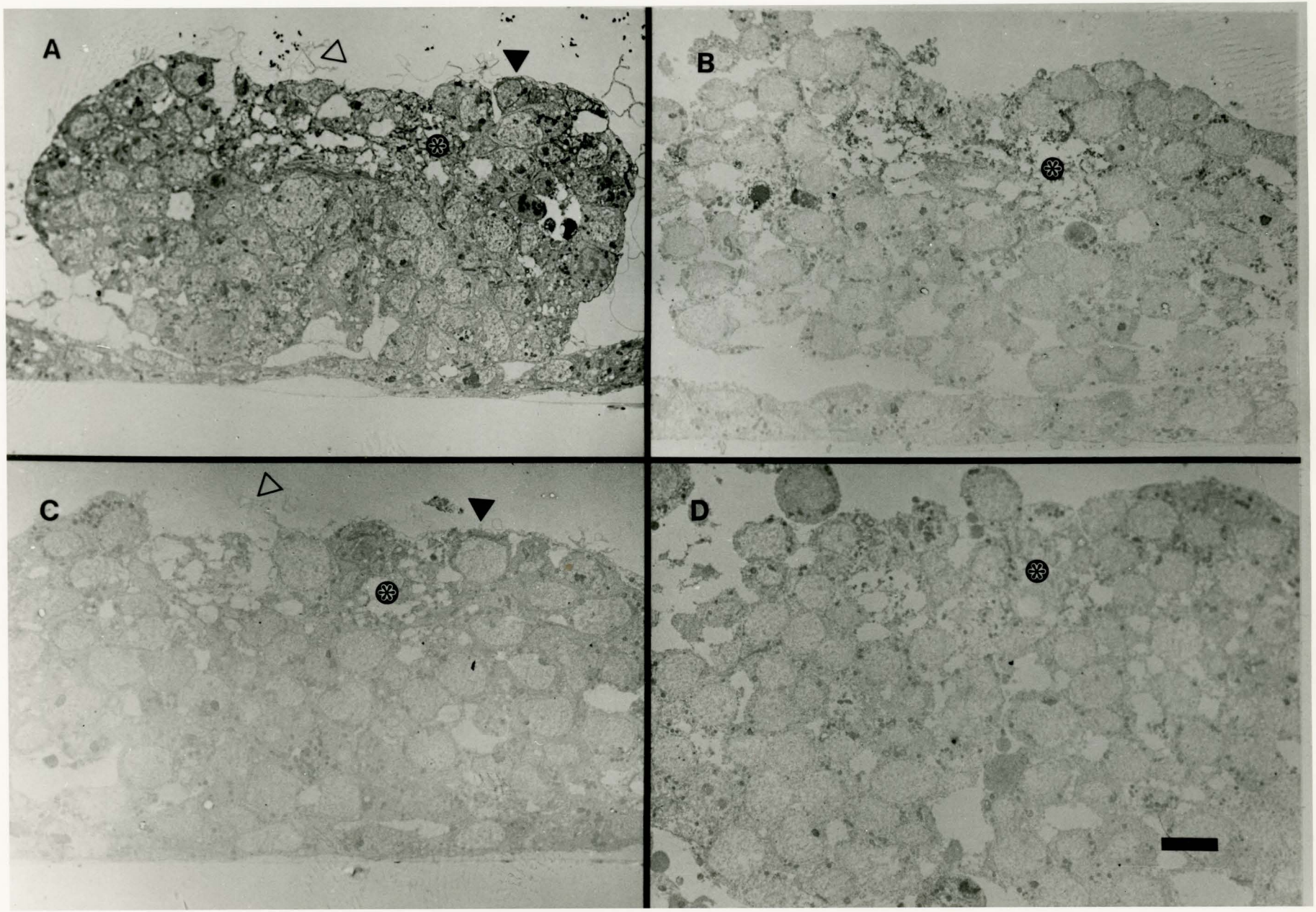
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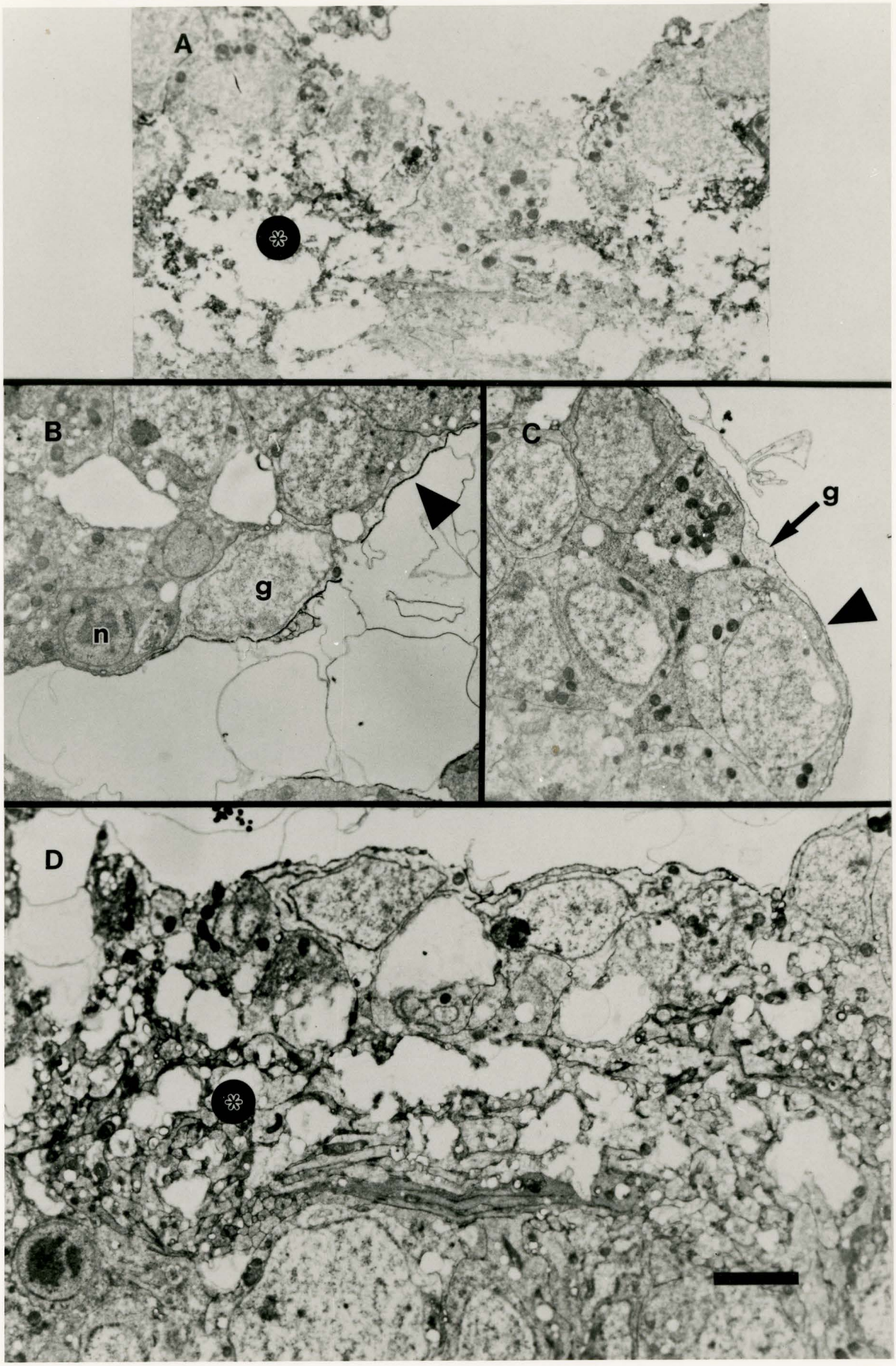
Figure 4: Electron microscopic analysis of PNA binding in the CNS at a higher magnification. Pictured are micrographs of cross sections of the CNS. The asterisk (*) illustrates the neuropil, the shaded arrowhead points to the extracellular matrix adjacent to the glial (g) cells on top of a neuron (n). Glial cells are identified by their distinct morphology and their electron translucent properties. Micrographs A and D are from embryos that were exposed to detergent while windows B and C are from embryos that were not exposed. All micrographs are from embryos that were stained with PNA. Binding is observed in the basil lamina and the commissures in windows A and D respectively and the glial membranes adjacent the extracellular matrix in windows B and C. Scale bar is 5 μm. Note: Dr. J. R. Jacobs performed the dissections which exposed the CNS, the sectioning and electron microscopy.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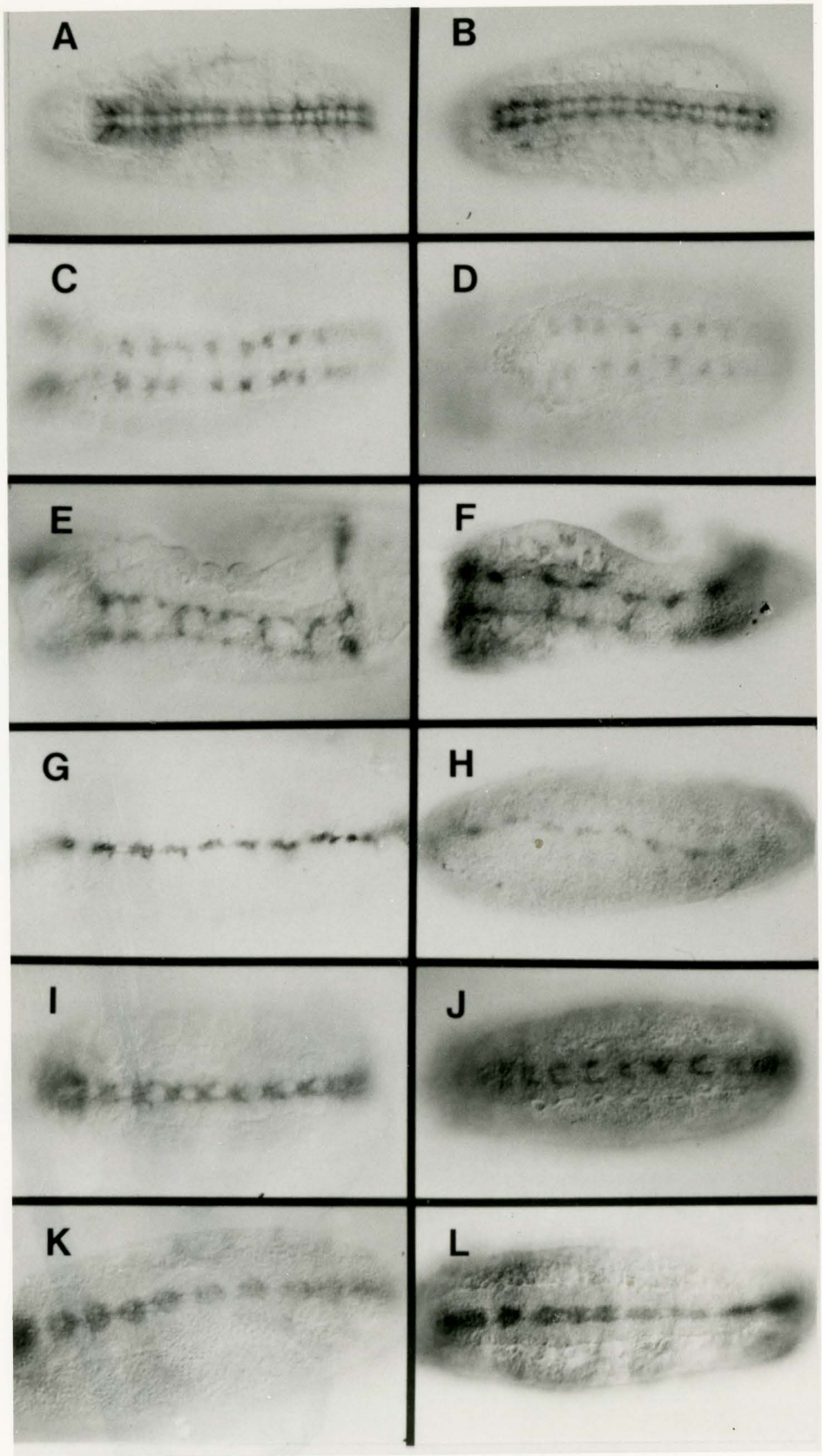
exposed to detergent and figure 3 (D) which was exposed to detergent, were both controls. They each were stained with PNA that was previously incubated with D-galactose. Neither condition displayed any signal.

Figure 4 (A-D) examine PNA binding much more closely. Figure 4 (A and D) displays PNA binding to the remaining tissue fragments of the neuropil after it was exposed to detergent. This demonstrates the signal is in the longitudinal axons and commissures after exposure to detergent. Figure 4 (B and C) both illustrate PNA binding without detergent present. At this magnification it is plain that the PNA ligands are in the basal lamina. Furthermore, these ligands are most evident in the extracellular matrix adjacent to the glial cells of the central nervous system. Figure 4 (B) clearly demonstrates that the signal is on the basal lamina adjacent the electron translucent cell which is ensheathing a neuron. This signal abruptly stops at either end of the glia's processes.

PNA's binding pattern in mutants that affect the CNS

The fruit fly has many mutations which are known to affect the development of the CNS. PNA has been shown to bind to specific areas within the CNS. One strategy that can be utilized to study the structural and functional significance of carbohydrates, would be to observe their distribution in mutants. PNA binding pattern is identical to that of the monoclonal antibody BP102 in wild type *Drosophila* embryos. We examined if PNA behaved as BP102 in various mutants which affect the CNS organization. With PNA as a probe for one or more prospective

Figure 5: Binding pattern of PNA in wild type and several mutants that affect the midline. The vertical column commencing with A represents the binding pattern as seen with the MAb BP102 while the column commencing with B represents the binding pattern as observed with PNA. The embryos are oriented with their ventral side down and the anterior side facing left. Windows A and B are of wild type embryos stained with BP102 and PNA, respectively. Mutations that follow are listed in the chronological order in which they are known to affect development of the midline. The mutants that are illustrated are representative of all mutants used in this screen. The mutants displayed are: ph^{505} in C and D; sna^{IIG05} in E and F; $sim^{B13.4}$ in G and H; rho^{7m43} in I and J; and S^{1} in K and L. All embryos were photographed on a compound microscope with Nomarski optics at a magnification of 400x.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ligands in the region, we can asses two possible outcomes. Firstly, if a mutant eliminates the PNA signal this would suggest that the ligand is dependent on the element for the ligands expression, and may somehow be involved in the mutation. Secondly, if the PNA signal is enhanced or displays a staining pattern other than that of the expected BP102 pattern, may indicate that some control mechanism of this carbohydrate is missing. In any event, this is a first step in utilizing the novel CNS probe in unraveling the mystery of processes like axon pathfinding which are theorized to use a labeled pathway (Goodman and Bastiani, 1984). This pathway may use carbohydrates as sign posts.

The mutants used in the survey affect the CNS at different stages of embryonic development. Mutants whose wild type product is a nuclear protein are: hnt^{E8}, $sim^{B13.4}$, sna^{IIG05}, twi^{1}, pnt^{8B}, ph^{505} and otd^{H1}. The mutants used whose products are transmembrane proteins are rho^{7M43}, spi^{IIA}; and that of Flb^{IF26} which is epidermal growth factor; while one known to be a component of the extra cellular matrix is sli^{IG107}. The missing product of stg^{7M} is a cyclin while the product of S^{1} was recently discovered to have a putative transmembrane domain and interacts with the *Drosophila* EGF receptor (Kolodkin et al., unpublished). Knowledge of the protein's function could help in the interpretation of PNA staining of these mutants.

Figure 5, demonstrates representatives from these groups, comparing MAbBP102 (figure 5 A, C, E, G, I and K) to PNA (figure 5 B, D, F, H, J and L) labeled embryos. Throughout the entire survey, no discernible difference was detected between these two very different staining methods. BP102 stained heavier than PNA in wild type and every mutant studied in this screen. Therefore, the mutants examined

probably do not interact or directly affect PNA's ligand(s).

DISCUSSION:

Carbohydrates are proving to be among the most important molecules involved in development. Unlike other cellular polymeric type molecules, carbohydrates possess the ability to branch, imparting to them an extraordinary variety of three dimensional structures. This consideration, and the fact that there are a great many different number of species of carbohydrate as building blocks, make this molecule an ideal candidate as a cellular recognition factor.

If cells are in fact going to use carbohydrate moieties for any purpose there must be a mechanism to identify and interact with these molecules. Nature accomplishes this with a carbohydrate recognizing protein known as a lectin. These molecules are very specific types of carbohydrates and a great many have been discovered during the last decade performing various functions in a very divergent group of organism. Hence, evidence is becoming available which justifies these speculations.

Lectins are also ideal probes for cellular components, cell types and tissues. Most discoveries of carbohydrate function and/or lectin function were preceded by a lectin survey of the carbohydrate distribution in a model system. Such screens have lead to knowledge of the structural (Rosati, Nurse, and Diamond, 1984; Damjanov and Black, 1987; Herken, Sander, and Hofmann, 1990; Colton et al. 1992), functional and developmental (Griffith and Sanders, 1991; Zipser and

Cole, 1991; Boya et al. 1991) roles of carbohydrates, glycolipids, and glycoproteins. In Drosophila embryos, this examination has been rather limited, with sialylated oligosaccharides (Roth et al. 1992) among the few under study. To our knowledge, this is the first examination of lectin binding patterns in Drosophila embryos with a wide range of lectins at varying stages of embryonic development. This can be used as a first step to begin addressing glycobiology in the developing embryo and more specifically the embryonic nervous system. Lectins can also add valuable new histochemical markers for the study of most systems in the Drosophila embryo.

Lectin Binding Patterns

The group-I lectin, Con A, specific for glucose/mannose and branching mannose residues binds throughout the embryo at all three stages of development observed (pregastrulation and stages 11 and 16). It has affinity for the trachea, CNS and especially for the ectoderm. Of all lectins studied, Con A demonstrates the most general and heavy binding pattern throughout the embryo. This suggest that Con A ligands are ubiquitously present in the embryos at all stages of embryonic development. This should not be a surprise since Con A affinity columns have long been used as a step in glycoprotein purification (Sharon and Lis, 1989). Con A binding was eliminated by neuraminidase and hyaluronate lyase everywhere except the ectoderm, which resists enzymatic removal, suggesting a different nature and/or greater abundance of the Con A ligands in this tissue.

WGA, which belongs to the group-II lectins with specificity for

N-acetylglucosamine, displays a signal of varying intensity throughout the developmental stages studied. At pregastrulation there was no staining and at stage 16 the structures which stained, such as the ectoderm and developing gut possessed only weak signal. However, the signal present at stage 11 was very heavy. Although we cannot quantify the amount of stain exhibited between embryos from different experiments, this comparison is still indicative of relative signal intensities since the reaction time for all lectins was the same as was the exposure to the lectins and Av-HRP. This suggests that at stage 11, some molecule(s), probably a glycoprotein(s), with WGA ligands is either: expressed transiently, in greater abundance, differentially glycosylated or with an altered structure revealing the presumptive ligand. Neuraminidase eliminated the weak signal. Interestingly, WGA ligands were revealed when the embryo was pretreated with hyaluronate lyase. WGA labels ependymal cells in rat spinal cord (Damjanov and Black, 1987) and the ectoderm of seven day old mouse embryos (Herken, Sanders and Hofmann, 1990).

Group-III lectins, specific for N-acetylgalactosamine/ galactosamine, but as shown in table 1, the first four members listed are specific for α-galactose and acetylgalactosamine groups, while only the last has a further specificity for ß-galactose. Therefore DBA, SBA, BPA and GSA-I have been placed into group-III(A). Since this group has similar specificity one would expect that when used in this screen they would have similar results. This assumption proved to be true.

DBA, SBA, BPA, and GSA-I form the group which bind heavily to the gut and trachea. At pregastrulation there is no detectable signal in the cellular region but an intense signal in the yolk. This persists

throughout development until the yolk has disappeared. Lectins of group-III(A) are the only group to show any signal in the yolk and can potentially be exploited as an effective histochemical tool. The gut and trachea both acquire an intense stain as they develop. Group-III(A) are the only to bind these structures with such an intensity and avail themselves to further use in the study of these systems both as histochemical probes and as tools to ascertain the potential function of their ligands. DBA showed in addition, a very weak and diffuse binding to the CNS.

Neuraminidase and hyaluronate lyase did not reveal any new signals but either eliminated or decreased the signal depending on the lectin. For example, GSA-I and DBA had all their respective signals removed under these conditions while some signal survived for SBA and BPA. SBA binding was eliminated by hyaluronate lyase but neuraminidase only removed the signals in the ectoderm leaving the trachea, fore and hindgut signal intact. BPA binding had the opposite reaction when used on embryos pretreated with these enzymes. Neuraminidase removed all signals, while following pretreatment with hyaluronate lyase, ectodermal staining survived.

PNA was placed into group-III(B) because unlike the other members of group-III, it has slightly different carbohydrate specificity including ß-galactose. It also demonstrates a completely unique binding profile. Although the yolk and the cellular region also stain with this lectin by stage 11 this signal is restricted to the germ band. Staining is further restricted as the embryo develops to a weak signal in the gut, yolk and ectoderm and a very intense and specific signal in the CNS. The binding pattern in the CNS is almost identical to that of MAb

BP102 (Klämbt, 1991). The longitudinal tracts and the anterior and posterior commissures label. Pretreating the embryos with hyaluronate lyase completely removed all CNS and gut binding and reduced the signal present in the ectoderm. This is evidence that the stain observed with PNA is probably in the ECM since that is where hyaluronic acid is known to be in high quantities. Interestingly, PNA binding was completely unaffected by neuraminidase. This discovery not only uncovers another potentially useful histochemical marker but can also begin to address the role of glycoconjugates in the developing CNS.

Members of group-III as a whole have long been recognized as effective markers of structures like basement membranes (GSA-I) (Damjanov, 1987), (SBA) (Herken, Sanders and Hofmann, 1990), glial cells (GSA-I and RCA-I) (Boya et al., 1991), neurons (PNA) (Krull et al. 1991), Merkel cells and some epidermal cells (PNA) (Rosati, Nurse and Diamond, 1984) and the ECM (PNA) (Griffith and Sanders, 1991). Consequently, the binding patterns established by these lectins in *Drosophila* should not come as a surprise.

Group-IV lectin UEA-I, specific for L-fucose, demonstrates very weak signal in the cellular and germ band of pregastrulation and stage 11 respectively. By stage 16 there is no observable signal. Neuraminidase pretreatment also revealed no new signal, however, hyaluronate lyase uncovered UEA-I ligands in the ectoderm, CNS gut and yolk. Again, this lectin may lend itself as a histochemical marker or tool for dissecting the glycobiological implications to those who study these systems. UEA-I has been used as a histochemical marker in Merkel cells (Rosati, Nurse and Diamond, 1984).

Group-V lectin LPA, which is specific for sialic acid residues,

also binds to the cellular and germ band at pregastrulation and stage 11, while also demonstrating a weak signal in the trachea, CNS, gut and ectoderm at stage 16. This is similar to a previous study (Roth et al. 1992), which also used a group-V lectin, *Limax flavus* (LFA), to study sialic acids in *Drosophila*. Pretreatment with neuraminidase and hyaluronate lyase removed all signal, except for a weak signal in the CNS which neuraminidase failed to eliminate.

In all control experiments, each lectin was incubated with its competing sugar. Binding was seen to be either completely eliminated or severely reduced. This indicates that all observations are likely due to true signal, namely the carbohydrate binding domain of a particular lectin binding to a ligand.

MAb BP102 versus PNA Binding in Wild Type and Mutant Embryos

Since many mutants that affect the CNS are available in Drosophila, a comparison was done between the staining pattern of MAb BP102 and PNA. The only difference between these two probes was that PNA exhibited fainter staining in wild type and all mutants studied. This suggests that the PNA ligand is not directly regulated by or interacts with the mutants studied. More importantly, it demonstrates that PNA staining is a characteristic property of the CNS. PNA correctly identified the axons in mutants which conferred the incorrect identity or misguided axonal development.

PNA Binding in the Central Nervous System

PNA has been shown to bind to the ß1 and ß2 subunits of Drosophila laminin (Montell and Goodman, 1988 and 1989). *Drosophila*'s laminin is a 800 kDa, three subunit molecule that was first purified from *Drosophila* Kc cell cultures (Fessler et al., 1987). The physical properties, homology, expression pattern and developmental regulation of this molecule strongly suggests that *Drosophila* laminin has a similar role to that in mouse neuronal development including promoting cell migration, growth and axon extension and differentiation (Fessler and Fessler, 1989). Montell and Goodman (1988) purified laminin by affinity chromatography using PNA bound to agarose beads. The proteins were eluted with 0.5M galactose. Specifically, the 400, 220 and 180 kDa subunits represent A, ß1 and ß2 subunits, respectively. These are similar to the mouse 440, 225 and 205 kDa subunits, and share similar structural properties of its homology. In fact, domains III and V, which are known to play functional roles in the mouse (Martin, 1987), have 55% similar amino acids, while domains I, II and IV have only a 25% similarity compared to the mouse homologue (Montell and Goodman, 1988). Figure 6, summarizes much of what is known about laminin, and represents the molecule subdivided into the regions that are discussed.

Although the function of invertebrate laminin is largely speculated to be similar to that of its vertebrate homologue based on structural similarities, they do have more in common. For instance, *Drosophila* also possess position-specific (PS) antigens which are homologous to vertebrate integrins in structure and function (Brower,

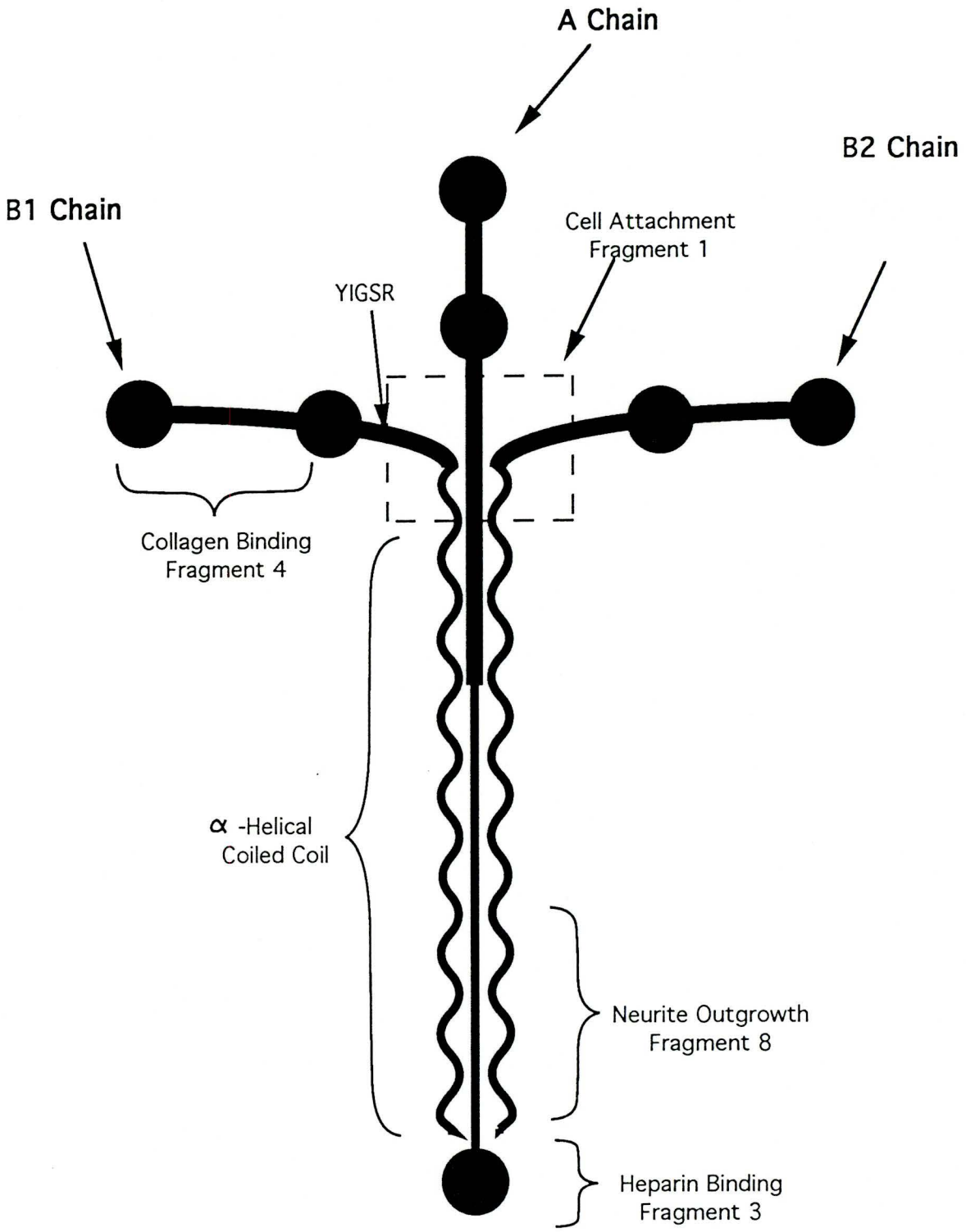
Piovant and Reger, 1985; Leptin et al., 1989). Integrins are cell surface proteins that act as receptors for components of the ECM and may be involved in processes such as cell-cell recognition. Increasingly, this system is presenting itself as a much more malleable model to study laminin's physiological and developmental role.

Since Timpl and Rohde (1979) isolated the first subunits of a noncollagenous glycoprotein, laminin, this extracellular matrix molecule has been the subject of much interest. Laminin is the most abundant noncollagenous protein in basement membranes, especially in the extracellular matrix (ECM) surrounding epithelial tissues, nerves, fat cells, smooth, striated and cardiac muscle. The ECM also includes collagen IV, various heparin sulfate proteoglycans and entactin/nidogen, but laminin is the first ECM molecule during embryogenesis. Martin (1987) reviews laminin's physical properties and the theory that basement membranes create barriers that allow embryonic cells to segregate and differentiate into specific tissues and later in the adult to serve as molecular filters as in the capillaries and glomeruli. If this is in fact the case, and laminin is the first molecule to appear, it has an enormous potential to guide developmental processes. Laminin has been shown to promote cell adhesion, secretion, axon extension, cell migration, growth, differentiation and to be a potent mediator of neurite outgrowth (Martin, 1987; Dean, Chandrasekaran and Tanzer, 1990). Figure 6, illustrates the specific domains of laminin which are known to be responsible for each of these functions.

Interestingly, laminin is a highly glycosylated molecule with up to 13% of laminin's weight coming from carbohydrate modifications with subpopulations of laminin up to 25% carbohydrate (Martin, 1987; Dean,

Figure 6: Laminin structure and proposed function are summarized (modified Martin, 1987). Mouse laminin (Mr=850 kDa), consisting of three subunits A, B1 and B2 (Mr= 440, 225 and 205 kDa, respectively). *Drosophil*a laminin (Mr= 800 kDa) is a trimer of the same subunits (Mr= 400, 220 and 185 kDa, respectively). Laminin is the most abundant non-collagenous protein found in the extracellular matrix that surrounds epithelial tissues, fat cells, smooth, striated and cardiac muscles and nerves. Laminin, along with molecules like collagen IV, heparan sulfate proteoglycans and nidogen form basement membranes. When this molecule is viewed under the electron microscope it displays three distinct short arms varying in length from 30 to 37 nm and one long arm approximately 77 nm, in both mouse and *Drosophila*. Each arm contains two rod like regions and two globular regions. Protease derived fragments have been individually tested to assess the functions of the various regions of the molecule. The figure summarizes regions of particular importance. A YIGSR integrin binding site is situated near the base of the short arm formed by B1. Fragment 1, is derived from the center of the cruciform and is responsible for cell attachment. Fragment 3, is found at the distal end of the long arm and has been shown to bind heparin. Fragment 4, is the collagen binding domain and found on the short arm formed by the B1 subunit. Neurite inducing abilities have been isolated to fragment 8. Laminin consists of 13-15% N-linked carbohydrates which are clustered in the certain regions of the molecule and seem to be linked to function. Carbohydrates roles are not yet known but they do not confer stability against proteases. In the mouse there are

46, 11 and 14 potential N-glycosylation sites on A, B1 and B2, respectively. Fragment 8 is known to be heavily glycosylated. Many oligosaccharide structures on laminin have the unique feature of α-glycosidicly linked galactose at the non-reducing end.


A Chain
B2 Chain
B1 Chain
Cell Attachment
Fragment 1
YIGSR
Collagen Binding
Fragment 4
α -Helical
Coiled Coil
Neurite Outgrowth
Fragment 8
Heparin Binding
Fragment 3

Chandrasekaran and Tanzer, 1990). The fact that some laminin possess a greater or lesser amount of carbohydrate is a clue that carbohydrates are playing a functional role in the molecule that can be modulated spaciotemporally. What is more interesting is that the carbohydrates are clustered in certain regions, as in the region which is known to promote cell spreading. Laminin also possess many unique features of oligomers, such as α-glycosidic linked galactose at the nonreducing ends. There are 46, 11 and 14 potential N-glycosylation sites on the A, ß1 and ß2 molecules, respectively (Dean, Chandrasekaran and Tanzer, 1990). Studies to date show only N-linked oligosaccharides and that carbohydrates are not required for interaction with nor protection from proteolytic digestion.

A bridge is now developing between the known function and the carbohydrate content of this molecule. Dean et al. (1990) used lectins to block specific carbohydrates on laminin dried onto a plastic dish and purified glycosylated and unglycosylated laminin to demonstrate the functions of carbohydrates on laminin. They found that PC12 cells failed to spread and extend neurites when the laminin was exposed to lectins and that certain lectins also impaired the cell's ability to attach to the substrate in a dose dependent manner. Unglycosylated laminin was seen to have the same effect. In the former case, lectins could elicit their effect by steric hindrance; however, the second experiment strongly suggests that the carbohydrate post-translational modification of laminin plays a direct role in enhancing binding and spreading of cells on a laminin substrate and promoting axonal process outgrowth from neurons. Possibly a better test would be to plate PC12 cells with an excess of sugar. Ideally, this should saturate any prospective

receptors and give the same results.

It was later demonstrated (Begovac, Hall and Shur, 1991) that PC12 cell migration and neurite outgrowth on laminin is facilitated by ß1,4 galactosyltransferase acting as a receptor for laminin. Several other groups have recently established that carbohydrate moieties do have an affect as a neuronal substrate (Farmer et al. 1991; Zhang, Miller and Rutishauser, 1992).

Drosophila availed itself as a model to study the relationship laminin's functions and glycoconjugates for the same reasons *Drosophila* has been used as a successful model system in many other fields (Rubin, 1988). When embryos were stained with the monoclonal antibody MAb 8E6 which recognizes A and B1 subunits of Drosophila laminin, binding was observed in and over the central nervous system, the peripheral nervous system, along the longitudinal axon pathways, the commissural axons, the intersegmental and segmental nerve roots, the dorsal basement membrane covering the central nervous system, many of the glial cells and the pair of midline mesodermal cells at the segmental border (Montell and Goodman, 1989).

PNA appears to bind to the axons in the CNS, including the longitudinal tracts, the anterior and posterior commissures in each segment. Study with the electron microscope reveals that the PNA binding pattern is dependent on whether detergent is present. In the absence of detergent the signal is seen in the lamina on surfaces adjacent to glial cells only. When the histochemistry is performed in the presence of detergent, signal is seen throughout the neuropil. The detergent allows the PNA access to the inner CNS structures. The fact that in the absence of detergent PNA binds only in the ECM suggests

that PNA may be binding to a carbohydrate on a molecule like laminin. The possibility remains that PNA is recognizing another molecule, since Montell and Goodman (1988) found more than one protein when they purified laminin with a PNA column. The other molecules PNA has affinity for may cause the signal we have described. The only possible means that these unknown proteins can be contributing to this pattern is if they are themselves ECM molecules. Either way, some effort should be made to identify these ligands.

The most intriguing result is that the PNA signal in the absence of detergent is not present throughout the ECM, but only in the dorsal basement membrane adjacent to the glial cells surrounding these axons. The glia are identified by their characteristic electron translucence and morphology and their positions ensheathing the axons of the CNS (Jacobs and Goodman, 1989a). The developmental role of laminin has been clearly documented and may hold the key to many more phenomena. It is not unreasonable to suggest that laminin is performing the same function in *Drosophila*. Patthy (1991) suggests that laminin plays an important role in the morphogenesis of ectodermal tissues. Since laminin is consistent throughout these areas and the only signal we observe is that between the dorsal basement membrane and glial cells, we can surmise that either this is some other molecule, or that laminin is differentially glycosylated or the PNA ligand on laminin in the remaining unstained areas is covalently or sterically masked. This is a potential mechanism which can guide the glial scaffold to their appropriate positions (Jacobs and Goodman, 1989a) and/or provide the essential growth and differentiation ques for neuronal and glial development.

Although this model may answer some questions, it has yet to address how the information from glycosylated laminin in the ECM is relayed to the cells. There has recently been an explosion in the number of ß-galactose binding lectins discovered (Barondes, 1984; Suzuki et al. 1990; Sakakura et al. 1990; Abbott and Feizi, 1991; Wilson, Carrow and Levitan, 1992; Sato and Hughes, 1992), some of which have been shown to have an effect on cultured neurons. In fact the 35 kDa, galactose specific lectin discovered by Woo et al. (1990), is a non-integrin laminin binding protein. Their study suggests that non-integrin LBPs may contribute to laminin adhesion through protein-carbohydrate interactions. The lectin Zhou and Cummings (1990) isolated was also shown to have an affinity for laminin and suggested that it participates with the carbohydrates in a functional manner. Sato and Hughes (1992) isolated a 30 kDa lectin from baby hamster kidney cells that is specific for type I or II Galß(1-3(4))GlcNAc chains and also binds to EHS tumour laminin. More specifically this lectin bound to fragment 8 which is responsible for neurite extension. These lectins belong to the S-type which classifies them as soluble (Barondes, 1984). Other characteristics of lectins belonging to this group are a wide distribution in the ECM of animal tissues, cell surfaces, and intracellular compartments depending on the cell and its developmental stage and they are seen to primarily bind ß-galactose (Barondes, 1984; Drickamer, 1988). Wilson, Carrow and Levitan (1992) also isolated a 65 kDa dimer galactose binding protein from *Aplysia californa* and found that it enhanced neurite extension in cultured *Aplysia* neurons. One can begin to envision an ECM which guides neuron-glial development and a possible mechanism.

The notion that endogenous lectins are performing functional roles in nature such as guiding neuronal development is gaining strength as more evidence comes to light from a myriad of systems. Lectins are seen as possible cell recognition molecules and the terms LEC-CAM and selectins are now in common use (Sharon and Lis, 1989; Brandley, Swiedler and Robbins, 1990; Springer and Lasky, 1991; Sharon and Lis, 1993).

In our system, PNA's ligand may have a structural or functional role. We have seen in numerous examples how these glycobiological processes guide cellular functions. It has also been demonstrated that nature has provided a means to detect and interact with specific carbohydrates through endogenous lectins or proteins like β1,4-galactotransferase. It is conceivable that *Drosophila* may have one or more endogenous lectins that have yet to be isolated. The highly specific and restricted distribution of PNA's ligands implies that this type of interaction is likely. The annal of examples suggests that one exists. If it does it may hold many answers to processes like neuronal differentiation and axon pathfinding.

It would also be prudent to see if the fruit fly possesses an endogenous lectin which is specific for galactose. There are many strategies one can take to pan the Drosophila embryo for a β-galactose binding protein. One possible method is the way PNA itself was isolated (Lotan, et al. 1975). Protein can be collected from fruit fly embryos and suspended in 0.9% NaCl. After several purification steps involving centrifugation and washing the supernatant with 60% ammonium sulfate followed by dialysis to remove insoluble material the a galactose binding protein can be isolated by applying the supernatant

to a Sepharose-ε-aminocaproyl-β-galactopyranosylamine column. The prospective *Drosophila* lectin can then be eluted with a solution of galactose in 0.9% NaCl.

Another strategy is that used by Wilson et al. (1992) when they isolated the Aplysia gonad lectin (AGL). The method of protein collection and purification was similar to that discussed. However, they used a Sepharose CL-4B-200 column in order to isolate AGL. This protocol takes advantage of the free poly-D-galactose-3,6-anhydro-L-galactose residues allowing this matrix to be used for the purification of galactose binding proteins. Again, any bound protein can be eluted by washing with a galactose solution.

A more interesting and possibly functional approach would be to use a purified *Drosophila* laminin conjugated to Sepharose. This is similar to the method employed by Woo et al. (1990) to isolate a non-integrin laminin binding protein from macrophages. This would be much more labour intensive then the first two approaches but would immediately yield answers regarding whether the presumptive lectin actually associates, at least *in vitro*, with laminin.

Drosophila tissue culture is still in an infancy stage after more than twenty years of trials. Cell survival is very low and differentiation is highly irreproducible using current protocols. It is possible that a or the missing component required for *Drosophila* primary cell culture is a properly glycosylated laminin or a laminin fragment. Further experiments should be done to ascertain the effect of Drosophila laminin on cell survival and differentiation in *Drosophila* tissue culture.

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