THE DISTRIBUTION OF GLYCOCONJUGATES IN THE BASAL LAMINA AND ECM DURING ESOPHAGEAL MUSCLE FORMATION IN EMBRYOS OF THE STARFISH PISASTER OCHRACEUS AS REVEALED BY LECTIN HISTOCHEMISTRY

By

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ABSTRACT

Morphogenetic events consist of complex interactions of cells and extracellular materials resulting in the movement and rearrangement of groups of cells and their subsequent differentiation to form organs or organ systems. Although we can predict these movements for any given event, we have little understanding of how morphogenesis is controlled. In the starfish Pisaster ochraceus, assembly of mesenchyme cells on one particular region of the larval gut, the oesophagus, and their subsequent differentiation into muscle is an example of a simple morphogenetic event which is readily accessible for study. In their migration to the gut, the mesenchyme cells travel through a rich substrate of ECM. Upon their arrival at the presumptive esophagus, they come to settle on the BL underlying the endodermal epithelium. It is quite possible that interactions between the mesenchymal cells and the ECM/basal lamina are important in directing and regulating their differention into muscle.

The basal laminae and ECM of vertebrates and invertebrates is rich in glycoconjugates, including glycoproteins, proteoglycans and glycosaminoglycans. Ultrastructural studies of embryos of the asteroid Pisaster ochraceus have demonstrated that at the late gastrula stage, the endodermal basal lamina is thinner and less alcianophilic in the esophageal region. FITC and colloidal gold labelled lectins, which act as specific probes for carbohydrate moities, usually those at the terminal end of oligosaccharide chains, have been used to localize these sugars at the light and electron microscope levels. These studies show

that a heterogeneity exists with respect to terminal sugars in the basal lamina, i.e. lectin binding of the basal lamina is not uniform in all regions of the embyro. Specifically, a statistical analysis of lectin binding determined that labelling with the two lectins, Au_{25}-Con A and Au_{25}-LFA was significantly reduced in the esophageal region as compared with the other regions of the embyro, while labelling of the BL with Au_{25}-DBA showed a similar intensity in all areas of the embryo. These results confirmed the alcian blue results described above and suggest that there are some sugar containing molecules, perhaps specific glycoproteins, GAGs and/or proteoglycans, which are present in reduced quantities in this region. In addition, these studies show a distinct labelling pattern of the ECM through which the mesenchymal cells migrate on route to the esophagus. Different lectins label different regions of the ECM, however it can not yet been said whether there is a regionally distinct pattern in the area of the migratory path of mesenchymal cells to the esophagus.

Proteoglycans and GAGs are involved in cell movement in vertebrates and sulfated glycoconjugates have been shown to be necessary for mesenchyme cell movement in echinoids. A decrease in proteoglycans and GAGs in the esophageal BL could therefore help to direct movement of the presumptive muscle cells to the esophagus by providing a "stop" signal.

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LIST OF ABBREVIATIONS

aq	aqueous
Au_{25}	colloidal gold with mean particle size diameter, 25 nm
BL	basal lamina(e)
BM(s)	basement membrane(s)
C	carbon
CS	chondroitin sulfate
CPC	cetyl pyridinium chloride
DABCO	1,4 diazabicyclo [2.2.2] octane
DS	dermatan sulfate
dH_2O	distilled water
Ec	ectoderm
ECM	extracellular matrix
En	endoderm
Es	esophagus
FITC	fluorescene isothiocyanate
fuc	L-fucose
GAG(s)	glycosaminoglycan(s)
gal	galactose
galNAc	N-acetyl-D-galactosamine
glcNAc	N-acetyl-D-glucosamine
H	hydrogen
HA	hyaluronic acid
HL	hyaline layer

HSPG	heparan sulfate proteoglycan
^{3}H	tritium
KS	keratin sulfate
Lectins:	
Con A	concanavalin A
GSA-II	Griffonia simplicifolia agglutinin II
DBA	Dolichos biflorus agglutinin
LFA	Limax flavus agglutinin
PNA	peanut agglutinin
RCA	Ricinus communis agglutinin
UEA-I	Ulex europaeus agglutinin-I
SBA	soybean agglutinin
WGA	wheat germ agglutinin
sWGA	succinylated wheat germ agglutinin
LM	light microscopy
man	mannose
µCi	microCurie
mOs	milliosmole
M	molar
Me	mesenchyme
Mu	muscle
NC	neural crest
Neu5Ac	N-acetyl-neuraminic acid
Neu5Gc	N-glycololy-neuraminic acid
nm	nanometer

pI	isoelectric point
PBS	phosphate buffered saline
PF	paraformaldehyde
S	stomach
TBS	tris buffered saline
TEM	transmission electron microscopy

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I consider it a priviledge to have worked in Dr. Bruce Crawford's lab. I feel that because of him, I have come to learn what research is "all about". It was a great benefit to be able to rely on his time and expertise whenever required, and because of this, I have learned a great deal of research techniques and research methodology. I would also like to thank my parents for their support and constant encouragement in my academic endeavors.

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1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Morphogenesis involves the organization of sub-populations of cells into specific arrangements, which ultimately results in the development of a mature organism. In the organization of cell populations, a series of controlled or directed events take place: these are cell division, cell migration, cell death, cell adhesion, and cell differentiation (Edelman, 1985). Our knowledge of the molecular bases of these processes has increased greatly over the past decade, but the strategies of how the primary processes are interconnected in multicellular organisms are still unknown. Although these developmental events are ultimately under genomic control, it is thought that cell and tissue interactions such as epithelial-mesenchymal interactions, may be the primary regulatory mechanisms for the assembly of cells during morphogenesis (Bernfield et al., 1973). These interactions involve recognition and communication among participating cells and include direct cell-cell interaction, direct cell-matrix interactions as well as indirect cell communication with other cells and the matrix via the production of diffusible substances.

1.2 EPITHELIAL-MESENCHYMAL INTERACTIONS

The importance of epithelial-mesenchymal interactions for morphogenesis and cellular differentiation is well established (Rudnick, 1933; Auerbach, 1960; Taderera, 1967). For example, during the development of normal skin, the epidermal cell layer interacts closely with the underlying mesenchyme, and appears to control the differentiation, growth & spreading, and orientation of the overlying

epidermis (McLoughlin, 1968). A similar situation is present during salivary gland development, during which salivary epithelium undergoes characteristic morphogenesis only when it is in the presence of (its own specific kind of) mesenchyme (Grobstein, 1968). In some cases, the requirement appears to be less restrictive, so that while epithelia still maintain a developmental requirement for mesenchyme, they are able to continue their characteristic morphogenesis in the presence of a mesenchyme which is not their own, (i.e. pancreatic epithelium, Grobstein, 1968).

While the interdependency of epithelium and mesenchyme seems certain, there has been some controversy with regards to the exact nature of this interaction, which has centered around the question of whether actual direct cell-cell contact is required, or whether short-range interactions (matrix-mediated) are sufficient to induce differentiation. The classic experiments of Grobstein (1956) and his associates (Koch & Grobstein, 1963; Kallman & Grobstein, 1966) suggested the presence of diffusible molecules which lead to "embryonic induction" in the absence of direct epithelial-mesenchymal contacts. However, reports by Nordling et al. (1971) and Saxen (1972) have indicated that the length of time required for induction to occur between interacting cell populations in a transfilter situation tends to rule out diffusion of molecules as a mechanism for information transfer. They postulated that inductive information might be transmitted by direct cellular contact or extracellular materials which were bound to the cell periphery. Both of these hypotheses require that the plasma membranes of cells from the interacting tissues be brought into relatively close apposition, and furthermore, that the basal lamina (BL) in this region would be degraded or changed in some way to allow for this contact.

Reports by Wartiovaara et al. (1974), Lehtonen (1975), and Saxen et al. (1976) indicate that direct epithelial-mesenchymal contacts are essential for the induction of kidney tubule morphogenesis. Several transfilter embryonic induction studies of kidney tubules using Nucleopore filters have demonstrated that heterotypic cell processes approximate one another and make direct contact (Wartiovaara et al., 1974), but that interactions take place only across filters having pores which allow the formation of cytoplasmic processes (Lehtonen et al., 1975). More recent studies have added embryonic lung (Bluemink et al., 1976), tooth germs (Slavkin & Bringas, 1976), rat submandibular gland (Cutler & Chaudhrey, 1973; Cutler, 1977) and embryonic mouse mammary gland (Heuberger et al, 1982) to the growing list of tissues in which transient epithelial-mesenchymal contacts are seen. In all cases, these contacts seem more closely associated with functional differentiation of cells rather than morphogenesis of the structure. Despite this evidence, there does, however, appear to be cases where direct contact between epithelium and mesenchyme is not necessary for induction of differentiation. During epithelial initiation of bone formation in the mandible, Hall (1987) has shown strong evidence to support short range matrix-mediated interaction as the means of epithelial-mesenchymal induction.

The molecular basis of induction following epithelial-mesenchymal interactions is not known, for once the two tissues have come into contact, various types of interactions can take place. For example, a signal molecule may directly interact with the nucleus or genome of the responding cell, or alternatively, the signal may be passed via a cytoplasmic factor, i.e. a chemical mesenger, as occurs when steroid hormones act on animal cells. Since these interactions involve a local

signal transduction from the mesenchyme to the epithelium and vice versa, an extracellular matrix component secreted and deposited by the mesenchyme would be a natural candidate for a signal transducer. Recently, the discovery of the extracellular matrix protein tenascin, which is expressed by mesenchyme, has provided some strong evidence to support this idea. Tenascin is a protein which has an unusually restricted expression pattern in the developing embyro (Chiquet-Ehrismann et al., 1986). In several organs, it is found in the dense mesenchyme that immediately surrounds budding and growing epithelia, but is absent from other parts of the mesenchyme (Chiquet-Ehrismann et al., 1986). In addition, it has a rather dramatic temporal expression, in that it appears to be directly stimulated by epithelial-mesenchymal interactions; in the developing kidney, it appears shortly after the first epithelial-mesenchymal interaction (Aufderheide et al., 1987). The distribution of tenascin, coupled with its expression in development suggests that it is important for either mesenchymal condensation, for epithelial growth, or both.

1.3 ROLE OF THE BL IN EPITHELIAL-MESENCHYMAL INTERACTIONS

Recently, attention has been focussed on the role that the BL plays in epithelial-mesenchymal interactions. The BL is a structurally ordered and chemically specialized region of the extracellular matrix (ECM), usually forming a continuous sheet closely applied to the basal surfaces of epithelia (Kefalides et al., 1979; Madri et al., 1984). It thus occupies a strategically important position as it separates the epithelium from the underlying mesenchyme, and would appear to act as a physical barrier between the two.

In addition to several types of collagens and glycoproteins

(fibronectin, laminin, entactin, nidogen), the BL contains a large quota of anionically charged carbohydrates in the form of glycosaminoglycans (GAGs), such as chondroitin sulfate, heparan sulfate and hyaluronic acid. (Pratt et al., 1975; Weston et al., 1978; & Erickson & Weston, 1983). GAGs are the carbohydrate components of proteoglycans, and consist of repeating disaccharide units. Each disaccharide unit consists of a hexosamine, usually in its N-acetylated form, as well as a nonnitrogenous sugar, D-glucuronic acid or L-iduronic acid (Schrevel et al., 1981). The GAG composition and arrangement of the BL varies, however, from tissue to tissue. For example, the BL of the corneal epithelium contains primarily chondroitin sulfate, which is organized in a regular array in 2 planes on either side of the lamina densa (Trelstad et al., 1974). In the mouse embryo salivary gland, the BL contains hyaluronate and chondroitin sulfate (Cohn et al., 1977); in the pregnant mouse mammary epithelia, hyaluronate and heparan sulfate (Gordon & Bernfield, 1980), in the chick embryo notochord & neural tube, chondroitin sulfate and heparan sulfate (Hay & Meier, 1974), and in the mature rat glomerular BL as well as chick embryo lens capsule, mainly heparan sulfate (Hay & Meier, 1974; Kanwar & Farquhar, 1979).

It seems likely that during epithelial-mesenchymal interactions in which direct cell-cell contact is made, the barrier between the two, i.e. the BL, must be somehow overcome. Neural crest (NC) migration provides us with an in vivo system in which to study the BL in morphogenetic interactions. The migratory behavior of NC crest cells is characterized first by a loss of cell to cell attachments, followed by movement of cells to a fenestrated BL and thereafter penetration through it. The BL, in this case, appears to form a physical barrier to cell migration, and the premigratory NC cell population will not emmigrate

unless the BL perforated or absent (Martins-Green et al., 1986; Newgreen and Erickson, 1986).

The epithelial-mesenchymal interations which occur during branching morphogenesis (i.e. during lung, salivary gland and kidney development), have been studied extensively, and an interesting observation has been made in that the BL is thinned or discontinuous at the tips where active growth is taking place (Bernfield et al., 1973; Cutler & Chaudhry, 1973; Coughlin, 1975; Lehtonen, 1975; Gallagher, 1986a). Direct cell to cell contacts between the epithelial and mesenchymal cells appear to occur through these thinned BL. There is also evidence from studies on the formation of hairs, particularly the specialized whiskers of the rodent snout, that holes appear in the specialized BL on which the superficial epithelial cells rest, and that processes from mesenchymal cells penetrate these holes to establish direct contact with the epithelial cells (Goldberg & Hardy, 1983). Such contact immediately precedes the inductive interaction which occurs between epithelium and mesenchyme in the formation of these whiskers, a timing which is consistent with cell-to-cell communication being required to mediate the interaction.

In vitro experiments have attempted to focus on the role which the BL plays in epithelial-mesenchymal interactions. In lung organoid cultures, fetal lung epithelial and mesenchymal cells differentiate into type II pneumocytes and connective tissue cells respectively; it has been shown that cell contacts between these two cell types are necessary in order for differentiation to occur, and that the formation of a BL by the epithelium is not a requirement for pneumocyte differentiation to occur (Zimmerman, 1987).

These observations have lead to the proposal that developmentally

regulated BL degradation may be a general mechanism for controlling morphogenetic tissue interactions by the timing of direct cell contacts. (Bluemink et al., 1976; Goldberg & Hardy, 1983). The BL is therefore ascribed more than just a passive structural role in morphogenesis but rather a dynamically changing informational role which influences the migration of cell populations and the nature of epithelial-mesenchymal interactions (Sanders & Prasad, 1983).

1.4 ECM-STRUCTURE & ROLE IN MORPHOGENESIS

In addition to epithelial-mesenchymal interactions, morphogenetic processes appear to be influenced strongly by cell-ECM interactions. Together with mesenchymal cells, ECM comprises the connective tissue of the early embryo, and its role in guiding cell migration during morphogenesis has been studied widely in several embryonic systems including NC and primordial germ cell migration (Heasman & Wylie, 1981; Toole, 1981; Turner et al, 1983; Erickson, 1987a). The ECM through which NC cells migrate has been described ultrastructurally: tissue fixed in the presence of cationic dyes or tannic acid appears to consist of a stained granular material associated with matrix fibrils (Hay, 1978). The ECM has also been analyzed by histochemical and immunochemical methods and includes various collagen types (Von Der Mark, 1980; Wartiovarra et al., 1980), fibronectin (Mayer et al., 1981; Newgreen & Thiery, 1980) and various GAGs such as hyaluronic acid, chondroitin sulfate and heparan sulfate (Kvist & Finnegan, 1970; Pratt et al., 1975; Weston et al., 1978;). Results of studies using hyaluronidase to digest away GAGs have lead some to speculate that the stained granular material is high in hyaluronic acid (HA) or proteoglycans (Sanders, 1979; Solursh et al., 1979), while the fibrils

are composed of collagens (Singley & Solursh, 1989).

During morphogenesis, the ECM is considered to aid the organization of mesenchymal cells in two ways: Firstly, ECM enlarges the available migratory spaces for cells to travel through because it is rich in HA, which, in its hydrated state, is high in volume (Toole et al, 1972; Meier & Hay, 1973; Trelstad at al, 1974; Pratt et al, 1975; Derby, 1978; Toole et al, 1984). Secondly, the ECM provides a substratum, that is, a physical support or meshwork for the movement and migration of not only mesenchymal cells, but also more specialized cells such as primordial germ cells and NC cells (Sanders, 1986a).

1.5 ECM AS A DIRECTOR IN MORPHOGENESIS

The role that ECM plays in cell migration has been studied widely in NC cell migration (Duband & Thiery, 1982; Thiery et al, 1982; Le Douarin et al, 1984; Brauer et al., 1985; Bronner-Fraser, 1986; Erickson, 1987b). NC cells follow a well defined pathway during their migratory route. They arise in the ectoderm, and at neurulation are positioned at the top of the neural folds. From here, they break through their BL and migrate individually as mesenchymal cells through an ECM-rich area, and eventually differentiate into a large number of derivative tissues (Weston, 1970, 1983; LeDouarin, 1980, 1982). Considerable effort has gone into identifying and investigating the organization of matrix substances in the ECM migratory pathways of NC cells, largely because NC cells select a particular pathway which varies depending on their axial (anterior-posterior) location. For example, in the cranial region, NC cells enter the dorsal-lateral pathway and avoid the ventral pathway, whereas in the trunk region, the majority of the NC cells enter the ventral pathway (i.e. between the somite and neural

tube), and not the dorsal-lateral pathway (Anderson & Meier, 1981).

It is believed that one determining factor in directing NC cell migration is the actual composition of the ECM in these pathways (Weston et al., 1978; Bolender et al., 1980; Newgreen & Thiery, 1980; Erickson & Turley, 1983; Brauer et al., 1985). Recent studies (Brauer & Markwald, 1988) have revealed that the initial pathway taken by NC cells at either axial level is enriched in fibronectin-containing particles and lacks sulfated polyanions. Because fibronectin particles are also present in areas that NC cells do not enter, fibronectin alone can not be accredited for directing the paths of cell movement; additional factors are probably involved in this process, and one such factor could be the presence of sulfated proteoglycans (Kvist & Finnegan 1970). In vitro studies have shown that sulfated proteoglycans inhibit cell attachment and migration in fibronectin-rich substrates (Rich et al., 1981; Newgreen et al., 1982). It is therefore possible that sulfated components of ECM in some unknown way block or inhibit the fibronectin-NC cell interaction and in doing so may direct or select the pathway for NC cell migration.

Future advances in understanding the intricate ways in which the ECM influences cells during morphogenesis will come as a result of identifying and localizing candidate macromolecules in the matrix. In addition to antibody techniques, several techniques which visualize glycoconjugates specifically are currently being used to characterize components of the ECM. These will be discussed next.

1.6 TECHNIQUES USED TO VISUALIZE GLYCOCONJUGATES

Morphological and histochemical studies of ECM have, in the past, largely relied upon the use of cationic dyes such as alcian blue

incorportated into fixatives or used after the processing of tissue to stain ECM components. Cationic dyes are successful because of the charged nature of ECM, due primarily to GAGs. However, more recently, use of these low resolution cationic dyes has been replaced by techniques in which individual glycoproteins and carbohydrates are mapped both spatially and temporally during morphogenetic processes. One technique which is used is autoradiography of ^{3}H-sugars. In this method, radioactively labelled sugars placed in the culture medium are taken up by cells and are incorporated in the various different carbohydrate containing structures present in the organism. Following chases in "cold" medium, the localization of these radioactively labelled sugars can be determined, as well as their rate of metabolism in the organism. Autoradiographic studies using ^{3}H-glucosamine have been used in the past to identify epithelial surface-associated GAGs of the salivary gland, lung and ureteric bud (Bernfield & Banerjee, 1972).

Another technique which has been very successful in localizing tissue carbohydrates is lectin histochemistry.

Lectins-General

Lectins are defined as proteins or glycoproteins which bind to carbohydrates in a very specific manner. The majority of lectins are isolated from plants, although lectins have also been isolated from bacteria, sponges, snails, the sera of fish, and the hemolymph of lobsters. Lectins have at least two sugar-binding sites, and their specificity is usually defined in terms of monosaccharides or oligosaccharides that inhibit the agglutination reaction.

Because of the similarities lectins and antibodies share in the binding mechanisms they employ, lectins have also been defined "loosely"

as antibodies specific exclusively for carbohydrates, as apposed to being specific for a protein. There are some fundamental differences between lectins and antibodies however, which leave lectins in a catagory all of their own. The primary difference is that lectins are of non-immune origin, while antibodies are a direct result of an immune reaction.

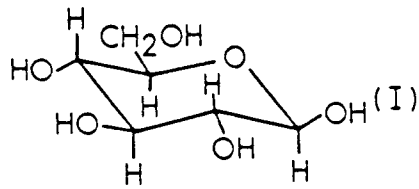
Unlike antibodies, the exact function of endogenous lectins is not clear, however several roles have been proposed. For plant lectins, these include (a) the possible involvement in defense against pathogens, particularly during early seed germination, where they may act as fungistatic or bacteriostatic agents (Mirelman et al., 1975); (b) a possible role in the packaging or mobilization of the storage materials that accumulate in seeds prior to dormancy (Boyd et al., 1958), and (c) a possible function in cell wall elongation (Labavitch, 1981). For vertebrate lectins, several roles have also been proposed. Soluble lectins are thought to participate in the organization of extracellular glycoconjugates, for example, chicken-lactose lectin-I interacts with glycoconjugates in the ECM between cells (Barondes, 1984). Membrane lectins are generally believed to participate in shuttling soluble glycoproteins from the outside to the interior of the cell and within intracellular compartments (Ashwell & Morell, 1974). Although there is no definitive proof for any of these proposed functions, studies on the properties, distribution and regulation of lectins as well as on their endogenous receptors will likely lead to an ultimate elucidation of their various roles.

Lectins-Specificity for Carbohydrates

Although some lectins bind exclusively to one carbohydrate moiety, most lectins bind to two or three different sugars which are structurally similar. Usually, however, one sugar binds to the lectin with a greater affinity than do its structurally similar counterparts. The reason that there is a degree of variability among many lectin-carbohydrate interactions has to do with the areas of the sugar with which the lectin interacts. For example, as a general rule, lectins tolerate very little variation at Carbon-3 (C-3) of the sugars they bind, and consequently a lectin which binds to a sugar with an α-OH group at C-3 (eg, D-glucose) will not bind to a carbohydrate possessing a ß-OH group at C-3, such as L-fucose (table 1) (Goldstein & Poretz, 1986). The C-4 hydroxyl group is also critically involved in directing lectin-binding specificity. This is demonstrated in that lectins which bind mannose/glucose, residues, sugars which have ß-OH groups at C-4, do not interact with galactose, which has an α-OH group at C-4 (table 1). It follows that galNAc-binding lectins do not bind to glcNAc residues for the same reasons (Allen et al., 1973).

Variations at C-2 impart some degree of specificity to lectin binding as well. For example, Con A binds primarily mannose (α-OH at C-2), but it will also bind to glucose and glcNAc (ß-OH at C-2), although to a lesser extent. Likewise, lectins which preferentially bind galNAc also react with galactose. Lectins differ markedly with respect to their anomeric specificities, that is, the orientation of the C-1 hydroxyl group. Some lectins, for example Con A, have anomeric specificity and bind only to the α-anomers (Smith & Goldstein, 1967), whereas others, like SBA and RCA bind to both α and ß-anomers (Lis et al., 1970).

TABLE 1: STRUCTURE OF MONOSACCHARIDES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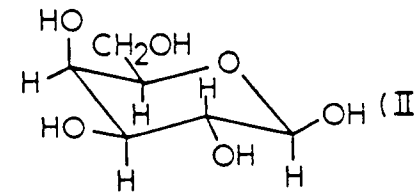
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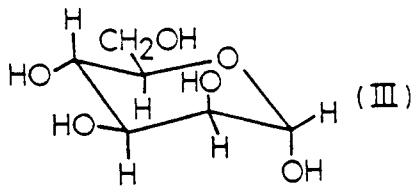
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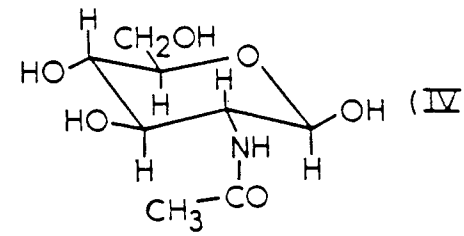
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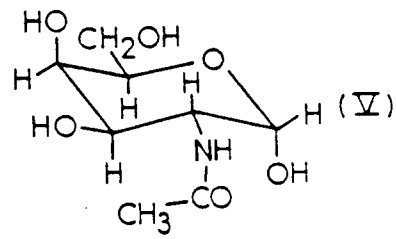
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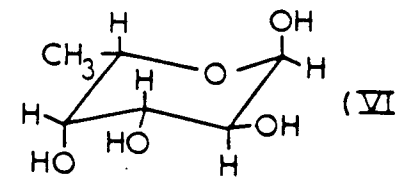
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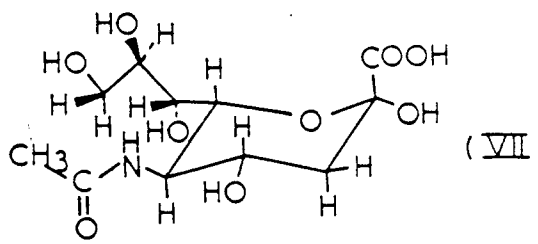
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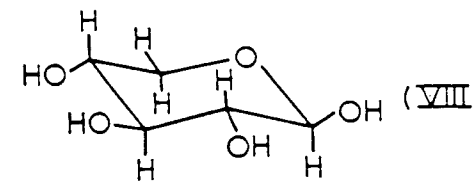

(VIII)

I	D-glucose	II	D-galactose
III	D-mannose	IV	N-acetylglucosamine
V	N-acetylgalactosamine	VI	L-fucose
VII	N-acetylneuraminic acid	VIII	D-xylose

Most lectins interact with the nonreducing terminal glycosyl groups of polysaccharide and glycoprotein chain ends. One exception is Con A, which in addition to its interaction with α-mannopyranosyl and α-glucopyranosyl terminal groups, binds internal 2-O-α-mannopyranosyl residues (Goldstein et al., 1973). Another exception is WGA, which interacts with internal 4-O-substituted glcNAc residues (Allen et al., 1973).

Although some lectins appear to bind only a single glycosyl unit, many have been found to possess extended binding sites and accommodate 2-5 sugar residues. For example, WGA interacts most strongly with ß(1,4)-linked glcNAc oligomers and PNA binds preferentially to galß1,3galNAc units (Wu, 1984).

Lectin-Carbohydrate Interactions

There is a fair amount of controversy about the actual nature of forces responsible for lectin-carbohydrate interactions. Considering the polyhydroxylic and hydrophilic nature of the sugars, it would seem logical that polar interactions (hydrogen bonds and dipole interactions) would play a dominant role in these carbohydrate-protein interactions. Experimental support for this view is given from x-ray crystallographic studies on Con A-methyl-α-mannopyranoside complexes (Becker et al., 1975; Hardman & Ainsworth, 1976). Further support for the involvement of polar interactions comes from chemical modification, spectroscopic, and proton titration studies (Hassing et al., 1971; Hassing and Goldstein, 1972). For example, by studying free energy contributions of H-bonds between the hydroxyl groups of sugars and their specific lectins, the nature of the groups of the lectins involved in hydrogen bonding have been evaluated (Bhattacharyya & Brewer, 1988). These

studies have revealed that depending on the particular lectin, hydroxyl groups on the carbohydrate interact differently with a particular lectin. In the RCA-galactose interaction, the C-2 hydroxyl group forms two weak hydrogen bonds in the capacity of a H-bond acceptor and a donor, the C-3 and C-4 hydroxyl groups forms at least one H-bond each with a charged residue of RCA, and both the C-1 and C-6 hydroxyl groups appear not to be involved with lectin binding. Conversely, the hydrogen bonding between Con A and α-methyl mannoside shows a different pattern: the C-1 and C-2 hydroxyl groups accept H-bonds from uncharged groups on Con A, the C-3 hydroxyl accepts a H-bond from a charged group, and both the C-4 and C-6 hydroxyl groups donate a H-bond to charged groups on Con A.

Some other researchers (Lemieux and his colleagues, 1982) believe that lectin-carbohydrate binding results from hydrophobic interactions. In this view, it is proposed that the lectin-combining site recognizes interactions between surfaces (topographic features) and not sugar units per se (Lemieux, 1982; Hindsgaul et al., 1982; Baker et al., 1983).

A more conservative notion put forth by Roberts & Goldstein (1984) is that both hydrophilic and hydrophobic forces are involved. For example, the interaction between L-fucose and lima bean lectin (LBL) has been studied extensively, and this interaction appears to have a hydrophobic character, as calculated from entropy and enthalpy recordings. Goldstein & Poretz (1986) view that polar interactions between carbohydrate hydroxyl groups and the polar side chains of amino acids (along with some nonpolar contacts) within a lectin's hydrophobic binding site would be an ideal model for specific carbohydrate-protein interactions. For example, looking at the WGA-sialyloligosaccharide system (Kronis & Carver, 1985), dominant forces stabilizing the

associated complex appear to be intermolecular H-bonds and van der Waals forces. In the future, high resolution X-ray crystallographic analysis of lectin-carbohydrate complexes should resolve many of the present uncertainties.

Lectins-Markers

A variety of markers exist for light microscopy (LM) and transmission electron microscopy (TEM). Common markers for LM include peroxidase and fluorescein. Peroxidase has been widely used for TEM as well, however recently particulate markers such as colloidal gold have become more popular. The first use of colloidal gold as a marker for TEM was by Faulk & Taylor (1971) who absorbed antisera to gold particles to use as probes for immunocytochemistry. Colloidal gold was first used in lectin binding studies by Horisberger et al. (1975). The growing interest in this marker system stems from the fact that colloidal gold markers can be reproducibly and easily prepared in a range of sizes, making the system extremely flexible, as well as the fact that gold probes are electron dense, spherical and very easy to identify. In the present study, lectins conjugated to FITC were used for light microscopy, and lectins conjugated to colloidal gold, for transmission electron microscopy.

Lectin Binding Studies of The Basement Membrane: A Review

Lectins have been used not only to map cell surface carbohydrates, but, more recently, to probe for intracellular and extracellular sites using fixed and sectioned material. In this respect, several lectin-binding studies have been carried out on embryonic basement membranes (BMs), and there appears to be a great degree of variation in

the carbohydrate composition of BMs between species as well as between organs within the same species.

Hurle et al. (1988) found that the lectins PNA, RCA, SBA, WGA, and sWGA stained the ectodermal BM of chick limb buds. With all of the lectins, the BM showed an undulating appearance and exhibited a rather uniform staining intensity. However, during (Hamburger & Hamilton) stages 22-25 of growth, a zone of increased staining was observed with PNA at the BM of the apical ectodermal ridge, the area of active morphogenesis. Lectin binding studies on embryonic chick lung bud has also revealed some interesting patterns. Gallagher, (1986b) has shown that the lectins WGA, SBA and RCA stain the BM of epithelium of embryonic chick lung. However, somewhat lesser amounts of these lectins bind to the tips of newly formed buds.

Blottner & Lindner (1987) have studied lectin binding patterns in early odontogenic events in prenatal rats 13-20 days old. They found that Con A labelled dental BM at days 13-15 and labelled inner dental BM and predentin at days 17-19. They found WGA to be specific for the dental BM, and found PNA to strongly label the inner dental BM while labelling the outer dental BM weakly.

Upon comparing lectin binding sites in kidneys of several different animal species, Holthoffer (1983) found differences in the lectin binding properties of BMs among species. For example, the adult GBM of the guinea pig stained with RCA, DBA, and UEA-I, while that of the rabbit stained only with RCA AND UEA-I; the BM of the dog stained with WGA and PNA, while that of the hen stained only with WGA; the BM of the human stained with PNA, RCA and Con A, while that of the mouse, only with PNA. Further studies (Holthofer & Virtanen, 1987) revealed that lectin binding patterns of human fetal glomerular basement membrane

(GBM) differed from those of human mature GBM: Fetal GBM stained with PSA, Con A, WFA, and RCA, whereas in the adult kidney, only RCA, Con A, and PNA were found to label the GBM.

Katow and Solursh (1982), and DeSimone & Spiegel (1986) have revealed developmental stage specific patterns of lectin binding in the early echinoid embryonic BM, which suggests that the regional and temporal expression of glycoproteins and or GAGs are tightly regulated by the developing embryo. They find that Con A binds to the entire basal surface of the epithelium until gastrulation, at which time Con-A binding sites disappear from the vegetal half of the ectoderm, and are localized to the BM of the animal pole region and to the base of the archenteron. Whether or not the disappearance of Con-A binding sites from the vegetal half ectoderm is due to a transformation of binding sites to a masked form is not yet known; however, since this surface alteration also occurs on the migrating primary mesenchyme cells, it might reflect a regulatory mechanism in mesenchymal-ectodermal interactions.

Further studies exploring the phylogenetic polymorphism of lectin binding of BMs was explored by Ribera et al. (1987), in their studies of vertebrate neuromuscular junctions (NMJs). They found that DBA, SBA and PNA bound to synaptic BM in all mammalian, reptilian, and less intensly in amphibians, whereas no staining was seen in the majority of avians and fishes. In contrast, WGA and Con A bound consistently to all species studied.

It appears, from these studies, that BMs from a wide range of tissues and species bind a variety of lectins, including Con A, WGA, sWGA, PNA, RCA, DBA, UEA, and SBA. Although some hint is given to suggest a species specific trend when the data on NMJs is reviewed, in

general, there is substantial overlap in the lectin binding patterns of all BMs studied. As well, no histo-specific trends are notable from the data presented. Of significance are certain developmentally related patterns of lectin-binding, which are seen in branching morphogenesis, gastrulation, and tooth development.

1.7 INVERTEBRATE ECM AND MIGRATION

In invertebrates, cell migration during morphogenesis has also been studied quite extensively (Okazaki, 1960; Gustafson & Wolpert, 1967; Schneider et al., 1978; Heifitz & Lennarz, 1979; Katow & Solursh, 1981; Spiegel & Burger, 1982; Desimone & Spiegel, 1986). Mesenchymal cells migrating through the blastocoele of developing echinoderms has been a favorite system to study cell-ECM interactions because of the transparency of the embryos, and the ease of which enzymes and inhibitors can be placed in the culture medium. An ECM that has an ultrastructural appearance similar to vertebrate ECM (strands of fibers associated with granules) has been described in the blastocoele of echinoids during early development (Endo & Noda, 1977; Katow & Solursh, 1979; Kawabe et al., 1981; Spiegel et al., 1983; Galeleo & Morrill, 1983). It appears to contain GAGs (Karp & Solursh, 1974), proteoglycans (Oguri & Yamagat, 1978) collagen (Pucci-Minafra et al., 1972) and fibronectin and laminin (Spiegel et al., 1980, 1983; Katow et al., 1982; Wessel et al., 1984). In the developing sea urchin, primary mesenchyme cells are in close association with the ECM. However, if the embryos are placed in sulfate-free sea water, the cells do not migrate and the number of granules in the ECM is greatly reduced, suggesting that the granules may represent sulfate-containing macromolecules and may be an important component of the migratory substratum (Katow & Solursh,

1981).

In the developing asteroid, a material with similar EM appearance has been described (Crawford & Chia, 1981; Abed & Crawford, 1986a). Although it is probable that the ECM in the starfish blastocoele contains similar components found in the echinoid ECM, further biochemical work is necessary to confirm this.

1.8 RATIONALE FOR THE CURRENT MODEL SYSTEM

In higher organisms, it is difficult to find a simple model system with which to approach the (complex) problem of morphogenesis. This has been overcome to a certain extent by the use of various cell and tissue culture techniques, which serve to provide a simple and reproducible in vitro system to study tissue and cellular interactions. Several "simple" invertebrate embryo systems such as those of sea urchins, tunicates and annelids have also been used extensively to investigate the phenomema of cell-cell communication during morphogenesis. These often provide a simplified system in which to study development in vivo. In addition, invertebrate embryos are easily maintained in the laboratory at a minimal cost, and can be grown in large numbers in synchronous cultures. Asteroid embryos have several added advantages which, when combined, make them a desirable system in which to study embryology. Firstly, the asteroid adults are very easy to feed and can be kept ripe at least 6 months of the year. Secondly, one adult provides a very large number of gametes, enabling massive amounts of embryos to be cultured with great ease. Thirdly, the asteroid embryos are transparent, which makes possible the continual visualization of cell movement and migration during growth. Fourthly, development is synchronous, allowing large numbers of embryos of the same stage to be

removed if necessary. And fifthly, the embryos do not develop calcium carbonate spicules, as do echinoids, which makes them ideal for EM studies. In addition to the reasons mentioned above it is probable that the basic and most essential mechanisms of development will transcend phyletic differences, as do the basic and essential mechanisms of cell activities. It therefore follows that solving the puzzle of morphogenesis in the asteroid embryo could lead to a greater understanding of development in higher organisms.

1.9 DEVELOPMENT OF THE ESOPHAGUS IN PISASTER OCHRACEUS

During the early development of the asteroid P. ochraceus, mesenchyme cells are formed. These cells leave the tip of the archenteron at the mid gastrula stage and migrate into the blastocoele. Locomotion appears to involve extension and retraction of filopodia in a manner similar to that seen during the migration of secondary mesenchyme cells of echinoids (Gustafson & Wolpert, 1967). Shortly after the egress of the mesenchyme cells the archenteron begins to bend toward one region of the ectoderm, and a blister of BL extends from the tip of the archenteron to spikes in the cells of the presumptive stomodeal ectoderm. Both endodermal and ectodermal cells appear to migrate along this BL template eventually making contact to form a mouth. At approximately the time of mouth formation, the gut becomes segmented into 4 regions, resulting in the formation of the stomodeum, esophagus, stomach and intestine. Some of the mesenchyme cells finally come to rest and attach exclusively to the presumptive esophageal endoderm, where they subsequently develop processes which surround the esophagus and differentiate into muscle cells; the end result is an interlocking of muscle cells forming a smooth muscle sheath around the esophageal

endoderm.

The migration of mesenchymal cells during esophagus formation has been studied in the developing asteriod P. ochraceus, using time lapse videomicrocopy (Crawford, unpublished data): It is interesting to note that the mesenchymal cells, much like the NC cells of vertebrates, can choose more that one route to migrate. Firstly, they can migrate to the esophagus where they further differentiate into muscle. Secondly, they can migrate elsewhere in the blastocoele, where they do not differentiate into muscle, or if so, do so at a much later time. It is therefore possible that, just as was seen in the ECM through which NC cells migrate through, the ECM in the blastocoele of asteroids has a different composition in different regions of the embyro. It is possible that this plays an important role in directing cell migration.

This undifferentiated mesenchymal cell population provides a good system with which to study some of the primary processes of development (cell migration, cell adhesion and cell differentiation) and how they interact with each other. Several questions can be asked regarding this system: For example, "How do the mesenchyme cells "know" where to go? How do they know where and when to stop? What is telling them to differentiate?". In looking for answers to these questions, one inevitably thinks of the role of the ECM as a controller of morphogenetic events. For example, it is possible that the mesenchyme cells are being told where to go simply by the pre-arranged pattern of matrix present in the blastocoele which they are able to identify. In vertebrate systems, it is well documented that filopodia of migrating cells interact with ECM, which is organized into a three-dimensional array of fibrils and interstitial bodies (Trelstad et al., 1974). In echinoids, studies involving inhibition of GAG and glycoprotein

synthesis has suggested that the ECM in the blastocoele may be involved in controlling mesenchyme cell movement during morphogenesis (Katow & Solursh, 1979; Akasaka et al., 1980; Venkatasubramanian & Solursh, 1984). In asteroids, SEM studies have shown that ECM in the blastocoele is located as organized strands in routes of mesenchyme cell migration between the endoderm and ectoderm. It was suggested that these form "roadways" for cell travel and thus form one mechanism for directing mesenchyme cell movement (Crawford & Chia, 1981). This means of control is only relative as the cells do not use these pathways exclusively.

Furthermore, one subpopulation migrates to the esophagus, and only the esophagus, stops and differentiates into muscle cells; this attachment is characterized by intimate contacts with the BL. Following their arrival, contacts with the epithelial cell surface occur in some regions. An obvious question then results: "How do the mesenchymal cells which are destined to form the muscle know 'where to go'?". Since the initial attachment appears to be with the BL, is there something different about the ECM and/or BL in this region that allows the presumptive muscle cells to recognize it? Indeed it has been observed (Crawford, 1988) that the BL in this region appears to have less of the dense alcianophilic material which is normally attached to the fibrous meshwork. One possibility is that the BL in this region is biochemically different, thus providing a recognizable difference for migrating mesenchymal cells, and directing them to this region for further differentiation. This could represent a developmentally regulated degradation of the BL, or alternatively, since the esophageal BL is the last BL to have formed in the developing gut (Abed & Crawford, 1986b), it may simply be "incomplete" at the time of arrival of the presumptive muscle cells, thus facilitating direct epithelial-

mesenchymal interactions.

In the present study, two different techniques (lectin binding and autoradiography) were used to examine the macromolecular nature of both the BL and the ECM during the development of the esophagus in P. ochraceus. The purpose of these studies was to determine whether there were regional differences in the carbohydrate composition of the BL and ECM, and specifically, to visualize differences between the BL underlying the esophagus and that underlying epithelium in other areas of the embryo. Lectins representing the five major saccharide binding groups were chosen, and were tagged to the markers FITC and colloidal gold in order to enable visualization of the binding sites with the light and electron microscope. In addition, autoradiography with ^{3}H-glucosamine, arabinose, mannose and fucose was undertaken in order to reveal, at the light microscopic level, the uptake and distribution of these monosaccharides.

2. MATERIALS AND METHODS

2.1 REARING OF PISASTER OCHRACEUS EMBRYOS

Glass and Plastic Ware

All glass and plastic ware used in the rearing of embryos was exclusively used for this purpose, and was not exposed to detergents or chemicals of any kind. After use, containers were washed first with regular tap water and then with sea water. The sea water was collected from Victoria B.C. rather than Vancouver to avoid fresh water contamination from the Fraser river. Before use, it was filtered through a Whatman #1 filter and aerated. Plastic ware was discarded every 2 weeks to avoid contamination from residual debris left over from the cultures.

Preparation of Gametes

Adult starfish were collected intertidally in May and June of 1986 and 1987 at three locations on the west coast of British Columbia near Vancouver, Victoria, and Bamfield. They were placed in running sea water tanks (9-10^{o}C) at the Department of Zoology, UBC, where they were kept under conditions of constant light in order to prevent spawning of gametes. Ovaries were removed from the starfish by excising 1 arm and extracting the organs with forceps. They were placed in a plastic petri dish, then minced and placed in 0.1mg/ml 1-methyl adenine in sea water for approximately 70-90 min. at 10.5^{o}C, to allow breakdown of the germinal vesicles. The eggs were then washed two times by settlement through 500 mls of sea water after which they were ready for fertilization.

Testes, isolated from the starfish in a similar manner, were

placed in a plastic petri dish and kept "dry" until the eggs were ready for fertilization. At this point, a few drops of "dry" undiluted sperm were placed in 25 ml sea water to make a cloudy suspension, and the sperm suspension was examined to check for sperm motility.

Fertilization

Sufficient washed eggs to cover 1/2-3/4 of the bottom of a 1 liter plastic beaker were placed in 300-400 ml sea water; they were then fertilized by the addition of 5-10 drops of active sperm suspension, and the beakers were aggitated gently for 1 min. to ensure even dispersion of the sperm to the eggs. Once the fertilized eggs had settled on the bottom of the beakers, the sea water was poured off and the eggs were resuspended in 500 ml fresh sea water. Following hatching, sea water containing the embryos was poured into new beakers to separate the now swimming embryos from the debris on the bottom of the beakers. Development of the embryos and larvae was carried out at 10.5^{o}C, and was monitored by microscopic observation, until it was observed that mouth formation was complete, at which time the embryos were harvested for experimental use by low speed centrifugation (120xg for 3 min. in 12 ml conical centrifuge tubes).

2.2 LM AND TEM MORPHOLOGY OF ESOPHAGEAL MUSCLE FORMATION

Embryos at the appropriate developmental stage were harvested and fixed by immersion in 1% glutaraldehyde in 80% sea water, pH 7.0, saturated with alcian blue for 12 hours (see appendix 1). Following two 5 min. rinses in 2.5% sodium bicarbonate buffer, the embryos were post fixed in 2% OsO_4 with 1.25% sodium bicarbonate, pH 7.4, for 1 hr. at room temperature (Wood & Luft, 1965). This was followed by staining en

bloc in 2% aqueous uranyl acetate for 30 min. The embryos were dehydrated through a graded series of ethanols and propylene oxide, and then embedded in Epon 812 (Luft, 1961).

For LM, 0.5μ sections were cut with a glass knife on a Porter-Blum MT-1 ultramicrotome, dried on glass slides and post-stained with Richardson's cationic dye (0.5% methylene blue, 0.5% Azure II and 0.5% borax in distilled water, Richardson et al., 1960). Sections were viewed and photographed on a Zeiss Photo-microscope III with Adox KB17 film at 100 ASA, and the film was developed 5 min. with Kodak D76 full strength, rinsed in tap water and fixed for 4 min. in Kodak rapid fixer.

For TEM, silver/grey sections were picked up on carbon/parlodian coated 100 mesh Copper grids, counterstained with 2% uranyl acetate (aq) 8 min. and with Reynold's lead citrate 5 min. (Reynolds, 1963). They were then examined on a Phillips 301 EM, and photographed using Kodak Eastman fine grain 5302 35 mm film. The film was developed for 5 min. with Kodak D19 full strength, rinsed and fixed as above.

2.3 LECTINS USED FOR THIS STUDY

The lectins used for this study were representatives of the 6 major carbohydrate-binding groups. These include the mannose/glucose-binding lectins (Con A), the N-acetylgalactosamine-binding lectins (Dolichos biflorus lectin, Soybean agglutinin) the galactose-binding lectins (Peanut lectin, Ricinus communis agglutinin), the N-acetyl-glucosamine-binding lectins (Wheat germ agglutinin), the L-fucose-binding lectins (Ulex Europeus agglutinin), and the sialic acid-binding lectins (Limax flavus lectin). These lectins, and some of their properties pertinent to this study are listed in table 2.

2.4 FITC-LECTIN LABELLING OF THE BL & ECM DURING ESOPHAGEAL MUSCLE FORMATION

Fixation and Embedding

Animals in the late gastrula/early bipinnaria stage, in which the mesenchyme cells were just arriving at the esophagus, were harvested as above and fixed by immersion in a variety of fixatives for FITC-lectin labelling (see appendix #2). The best fixative for the labelling studies was determined individually for each lectin. Following fixation, the embryos were dehydrated in a graded series of ethanols as follows: 5 min. in each of 30%, 50%, 70%, 2 changes of 5 min. each in 95%, and then embedded in JB4 hydrophilic plastic resin (JEM Sciences). 1-2μm sections were cut on a dry glass knife, and were floated on distilled water (dH_2O) containing 1-2 drops conc. NH_4OH/100 ml. The sections were dried on glass slides and placed in a 40^oC oven for 30 min. Prior to staining, the sections were rehydrated in PBS for 5 min.

FITC Staining

Sections were incubated in a dark moist chamber (parafilm on moist filter paper in a petri dish) for 1-8 hr. in 40 μl of lectin solutions or lectin/sugar controls (the FITC-lectins, their sources and the conjugate sugars used are listed in table 3). The lectins were dissolved in PBS (see appendix #3) to make a final concentration of 200 μg/ml PBS. The sugars used for controls were dissolved in PBS to make a final concentration of 1.0 M, and then added to the lectin solutions in a 1:1 ratio, after which they were mixed by pipette for 30 min. prior to staining. Following staining, the slides were washed 2 x 5 min. in PBS and 1 x 5 min. in distilled water before mounting coverslips with gelvatol/DABCO anti-fading mounting medium (Johnson et al., 1982; Taylor & Heimer, 1974). For recipe, see appendix 4.

Microscopy

Slides were viewed with a Zeiss Photo-microscope III equipped with epifluorescence; photographs were taken on Ilford HP5 film, developed 17 min. with Ilford Microphen at full strength to give an ASA of 3200, rinsed and fixed as above.

2.5 COLLOIDAL GOLD LECTIN LABELLING OF THE BL & ECM DURING ESOPHAGEAL MUSCLE FORMATION

Fixation and Embedding

Embryos at the appropriate developmental stage (late gastrula/ early bipinnaria) were harvested and fixed by immersion in a variety of fixatives (see appendix #1). Following fixation, the embryos were dehydrated through a graded series of ethanols (1 x 5 min. each in 30%, 50%, 70%, and 2 x 5 min. each in 95%, 100%) and then infiltrated with Lowicryl K4M. Infiltration into the Lowicryl resin was done gradually as follows: 1 x 30 min. in 20%, 40%, 60%, 80% Lowicryl in 100% ethanol at room temperature, and then 2 x 2 hr. in pure Lowicryl at $-2^{o}C$. Polymeriztion was performed in gelatin capsules with UV radiation at $-2^{o}C$ for 24 hr. The polymerization chamber consisted of 1-15 watt fluorescent tube placed 20 cm from the gelatin capsules in a box lined with aluminum foil. Following polymerization, the capsules were U.V. cured a further 2 days at room temperature. Silver/gray sections were picked up on carbon/parlodion coated 100 mesh copper or nickel grids.

Preparation of Colloidal Gold

Colloidal gold was prepared using the citrate method characterized by Frens (1973), which involved the reduction of chloroauric acid (Sigma) with a sodium citrate (EM Sciences) solution to give monodisperse sols having a mean diameter of ≃25nm (Au_{25}). For details, see appendix #5.

Preparation of Lectin-Gold Conjugates

Lectin-gold conjugates were prepared using a method adapated from Morris et al. (1986). This involved an initial assay to determine the appropriate concentration of each lectin to add to the gold sol to ensure stabilization followed by the conjugation itself. After the conjugation step, each conjugate was washed twice in 5 mM phosphate buffer and then dialysed to bring the proteins back up to their physiological strength. Detailed procedures for both the assay and the conjugation steps can be found in appendices #6 and #7, and lectin concentrations used for the conjugation are listed in table 4.

Sugar controls were prepared by adding 0.1 ml of 1 M inhibitory sugar solutions (see table 2) to 0.9 ml of lectin-gold conjugate; this was agitated by pipetting for 1 min, allowed to stand 1 hr., and then used to stain as above.

Lectin-Gold Labelling

Prior to actual staining, the grids were preincubated in a moist chamber on drops of tris bufferred saline (TBS), which was adjusted to the pH of the isoelectric point (pI) of the particular lectin or control sugar/conjugate solution for 5 min. at room temperature (see table 2). They were then placed on drops of lectin-gold conjugates or sugar

control conjugates for 30 min. Following this, the grids were washed with a steady stream of distilled water for 10 sec, and counter stained with 2% uranyl acetate (aq) for 5 min, followed by lead citrate for 1 min.

Microscopy

Stained grids were observed and recorded using a Philips 301 TEM with Kodak Eastman fine grain 5302 35 mm film, which was processed as above.

Morphometry

Morphometric analysis was done on prints from electron micrographs, which were taken at a magnification of x2,000. For measurement of both the length of basal lamina and counting of gold particles, negatives were printed at an enlargement of x 15,000. Counts were recorded in 4 - 8 random areas of specified length; areas were considered random, as low magnification photographs in which gold particles were not within resolving power were taken. The total linear length of BL measured for each region was $\simeq$ 0.5 μm.

2.6 AUTORADIOGRAPHY OF ^{3}H-SUGARS INCORPORATED INTO LATE GASTRULA EMBRYOS

Labelling of Embryos

0.4 ml of concentrated embryos were labelled in 150 ml plastic urine specimen cups, in 12 ml sea water containing 250μCi of one of ^{3}H-glucosamine, ^{3}H-fucose, ^{3}H-arabinose or ^{3}H-mannose at 12^{o}C. The beakers were rotated on a shaker at 12^{o}C for 6 hr. The embryos were then centrifuged, washed 2 times in fresh filtered sea water, resuspended in larger volumes of sea water in new plastic beakers, and rotated at 12^{o}C as above for chase times of 3, 9 or 18 hr. Following this, the embryos were again isolated by centrifugation, fixed by immersion in 1% glutaraldehyde in 80% sea water, pH 7.0, saturated with alcian blue for 10 hr. at room temperature, followed by dehydration and embedding in JB4 or Epon 812 as above.

High Speed Scintillation Autoradiography.

Slide Preparation

Prior to use, slides were cleaned in a mixture of 35 ml saturated sodium bichromate and 1 liter concentrated sulfuric acid for 2 hr, followed by an overnight wash in tap water. They were then washed twice for 1 hr. in distilled water with constant stirring, covered, and put in a 40^{o} oven to dry. From this point on, the slides were handled with gloves.

Sectioning

0.5-2.0 μm sections of JB4 embedded material were prepared as above.

Emulsion Preparation/Dipping

The emulsion (Ilford K.2) was melted in complete darkness in a constant temperature water bath set at 40°C, which had been allowed to equilibrate for 1 hr. before using. Only enough emulsion was melted to make about 25 ml. During melting, the emulsion was stirred gently with a glass rod. After 45 min, a clean slide was dipped in it to test for completeness of melting and for air bubbles. Once the emulsion was ready, the slides were placed 2 at a time in an automatic dipping machine (V. Avarlaid), and were dipped for 5 s. After dipping, they were dried upright with the emulsion end facing up for 1-2 hr. at room temperature. For high speed autoradiography the dried emulsion coated slides were dipped in Aquasol for 5 min. (Baserga et al., 1969; Durie et al., 1975; Goldgefter et al., 1976; and Wolfe 1976) before being stored in light tight boxes containing a small amount of Drierite. The boxes were wrapped with aluminum foil and stored in a -70°C freezer, to ensure low background. Test slides were developed at 3 day intervals to determine the correct exposure time.

Developing

In complete darkness, and at room temperature, slides were placed in glass staining holders for 100 ml coplin jars, and the scintillation fluor was removed by soaking them in a decreasing ethanol series (5 min. each in 95%, 80%, 70%, and 50%). The slides were then developed for 5 min. in Kodak D19 full strength with occassional agitation, rinsed briefly in water and fixed in Kodak Rapid Fix, diluted 1:1 with tap water, for 5 min. Following this, the slides were rinsed twice for 5 min. in distilled water, and stained immediately.

Staining and Microscopy

The slides were stained for 30 sec. with Richardson's stain (Richardson et al., 1960) at room temperature, washed for 10 min. in 2 changes of distilled water, and then coverslipped. They were then viewed and photographed as above on a Zeiss Photo-microscope III.

TABLE 2: LECTINS AND THEIR PROPERTIES

LECTIN	SOURCE	ABBREV	MW*	pI	SUGARS WHICH INHIBIT AGGLUTINATION*	CONTROL SUGARS USED [1 M] (mg/ml)
Concanavalin A (Canavalis ensiformis)	Sigma	Con A	106,000	7.1 (Agrawal & Goldstein'67)	man-α1,2man-α1,2man> α-man>α-glc	mannose 180
Wheat germ (Triticum vulgare)	Sigma	WGA	43,200	8.5 (Monsigny et al.'79)	glcNAcß1,4glcNAcß1,4glcNAc> glcNAcß1,4glcNAc>glcNAc	glcNAc 220
Soybean (Glycine max)	Sigma	SBA	120,000	5.8 (Lotan et al '74)	α-galNAc=ß-galNAc>α-gal	galNAc 220
Castor bean (Ricinus communis)	Sigma	RCA	120,000	7.8 (Wei & Koh'78)	gal>galNAc	galactose 180
Horse gram (Dolichos biflorus)	Vector	DBA	120,000	4.5 (Etzler & Kabat'70)	galNAc-α1,3galNAc> α-gal NAc	
Peanut (Arachis hypogaea)	Vector	PNA	111,000	6.7 (Metler'83)	galß1,3galNAc>galNH$_2$>gal	galactose 180
Slug (Limax flavus)	Sigma	LFA	44,000	9.0-9.5(*)	Neu5Ac>Neu5Gc	Neu5Ac 310
Gorse or Furze Seed (Ulex Europaeus-I)	Sigma	UEA-I	60,000-68,000		α-L-Fucose	L-Fucose 164

*Goldstein & Poretz, 1986

TABLE 3: FITC-LECTINS AND SPECIFIC INHIBITORS USED IN THIS STUDY

LECTIN	SOURCE	BLOCKING SUGAR
FITC-WGA	EY LAB	N-acetyl-D-glucosamine
FITC-SBA	Sigma	N-acetyl-D-galactosamine
FITC-Con A	Sigma	Methyl-D-mannnoside
FITC-RCA	Sigma	ß-D-Galactose
FITC-UEA-I	Sigma	L-fucose

TABLE 4: LECTIN CONCENTRATIONS FOR CONJUGATION TO GOLD

Lectin	Amount of protein needed to stabilize 10 mls Au_{25} (µg)
Con A	200
WGA	100
SBA*	100
PNA*	130
DBA	200
LFA	200

*Roth, 1983

Appendix #1: Fixatives Used For TEM and Colloidal-Gold Labelling

* All fixatives should have a final pH of 7.0-7.5, and a final osmolarity of 900-1000 mOs.

1. 1% Glutaraldehyde, alcian blue in sea water. (Crawford & Abed, 1986)

25% glutaraldehyde	0.4 ml
1% alcian blue in sea water (saturated)[1]	7.8 ml
distilled water	1.8 ml

[1]Stir alcian blue in sea water 2-8 hr, and filter before use.

2. Dunlaps Phosphate buffered glutaraldehyde. (Dunlap, 1965; Cloney & Florey, 1968)

Stock A:	25% glutaraldehyde	5 ml
	0.34 M NaCl	20 ml
Stock B:	0.4 M Millonigs phosphate buffer	25 ml

Mix stock A and B 1:1.

3. 2% Paraformaldehyde, 0.1% glutaraldehyde in PBS.

8% paraformaldehyde[2]	1 part
25% glutaraldehyde	0.16 parts
PBS	3 parts

[2]To make up paraformaldehyde solution, dissolve dry paraformaldehyde in water at 60°C and add 1 N NaOH drop by drop until clear.

Appendix #2: Fixatives Used For FITC-Lectin Labelling

* All fixatives should have a final pH of 7.0-7.5, and a final osmolarity of 900-1000 mOs.

1. Paraformaldehyde, cetylpyridinium chloride (CPC) in sea water

8% paraformaldehyde[1]	1 part
0.04% CPC	1 part
sea water	2 parts

[1]To make up paraformaldehyde solution, dissolve dry paraformaldehyde in water at 60°C and add 1 N NaOH drop by drop until clear.

2. Paraformaldehyde, alcian blue in sea water

1% alcian blue in sea water (saturated)[2]	7 parts
8% paraformaldehyde	3 parts

[2]Stir alcian blue in sea water 2-8 hr; filter before use.

3. Paraformaldehyde, CPC in Millonig's

8% paraformaldehyde	1 part
0.04% CPC	1 part
Millonig's phosphate buffer (0.2M)	2 parts

4. Paraformaldehyde in sea water with 2° fixation of paraformaldehyde/CPC in sea water

1° Fix:	8% paraformaldehyde	2 parts
	sea water	2 parts
2° Fix:	8% paraformaldehyde	1 part
	0.04% CPC	1 part
	sea water	2 parts

5. Bouin's

saturated (aq) picric acid	15 parts
formalin (40% formaldehyde)	5 parts
glacial acetic acid	1 part

6. 95% Ethanol

Appendix #3: Buffers

1. Phosphate buffered saline (Crawford, 1972)

Stock Solutions:

Saline G Stock V		Saline G Stock VI	
NaCl	160.0g	$MgSO_4.7H_2O$	15.4g
KCl	8.0g	$CaCl_2.2H_2O$	1.6g
KH_2PO_4	3.0g	or $CaCl_2.6H_2O$	2.4g
$Na_2HPO_4.7H_2O$	5.8g		
Dissolve in dH_2O to 1000 ml		Dissolve separately in dH_2O; total volume = 1000 ml.	

To make a working solution, dissolve:

Saline G Stock V	50 ml
Saline G Stock VI	50 ml

in dH_2O to 1000ml; adjust pH to 7.4.

2. Millonig's phosphate buffer, 0.2M (Millonig, 1961)

$NaH_2PO_4.H_2O$	11.08g
NaOH	2.85g

Dissolve in dH_2O to make 400 ml

3. Sorensen's phosphate buffer (Humason, 1962)

Na_2HPO_4	5.68g
KH_2PO_4	1.35g

Dissolve salts separately; add dH_2O up to make 250 ml.

4. Tris Buffered Saline (TBS)

0.5 M Tris Stock, pH 7.6:

Trizma (Sigma)	60.57g
dH_2O	500 ml
1 N HCl	370 ml

Dissolve Trisma in dH_2O; add HCl to pH 7.6, and fill to 1000 ml with dH_2O.

To make TBS, dissolve:

NaCl	8.0g
KCl	0.4g
Saline G Stock VI (Ca, Mg)	50 ml
in 0.5 M Tris stock, pH 7.6	100 ml

5. Sodium Bicarbonate 2.5% (Wood & Luft, 1963)

$NaHCO_3$	2.5g
dH_2O	100ml

Adjust pH to 7.2 prior to use.

Appendix # 4: Gelvatol/DABCO "Anti fading" mounting medium for fluorescence microscopy. (Taylor & Heimer, 1974; Johnson et al., 1982,).

1. Dissolve 20g gelvatol 20/30 (Monsanto) in 80 ml 0.2 M Tris (2.42 g Trisma/100 ml, pH 8.6) by stirring overnight.

2. Dissolve 3.75 g 1,4 Diazobicyclo [2.2.2] octane, DABCO, (Aldrich) in 40 ml glycerol at 50-60^{o}C.

3. Add glycerol to gelvatol mixture and stir.

4. Remove undissolved gelvatol by centrifugation (5000 x g for 20 min) or decantation.

5. Aliquot and store at -20^{o}C.

Appendix #5: Preparation of Colloidal Gold (Slot & Geuze, 1985)

To make 100 mls:

1. Stock Solutions:

   Solution I: 1 ml of 1% $HAuCl_4$ in 79 mls ddH_2O.
   Solution II: Reducing mixture
   4 ml 1% tri-sodium citrate $2H_2O$
   0-5 ml 1% tannic acid
   25mM K_2CO_3
   ddH_2O to bring volume to 20 ml

2. Bring both I & II to 60°C separately.

3. Add reducing mixture to solution I quickly while stirring; keep at 60° to avoid a heterodisperse sol.

4. After sol has turned red, heat to boiling and store at 277K.
   NB: reaction time will increase α to particle size of gold.

5. The particle size can be controlled by adding different amounts of tannic acid as follows:

Volume 1% Tannic Acid	Volume 25mM K_2CO_3	Particle Size
2.0 ml	2 ml	4nm
0.5 ml	/	6nm
0.125 ml	/	10-15nm
0.03 ml	/	20-25nm
0.0 ml	/	20-25nm

Appendix #6: MICROTITRATION ASSAY FOR DETERMINATION OF OPTIMAL [PROTEIN] REQUIRED TO STABILIZE COLLOIDAL GOLD.
(Horisberger, 1981)

1. Add 100 µl dH_2O to microtiter well.

2. Add 100 µl of 1 mg/ml protein in dH_2O to first well.

3. Serially dilute to 9^{th} well; leave 10^{th} well protein free.

4. Adjust gold sol to appropriate pH for protein using 0.2 M K_2CO_3.*

5. Add 500µl gold sol to each well and mix by pipetting up and down several times. Allow to stand for 15 min.

6. To assess the resistance of the mixture to salt-induced flocculation, add 100µls of 10% NaCl and let stand for 5 min. The NaCl solution will coagulate the unstabilized gold particles, i.e. those particles not sufficiently coated with protein.

7. Coagulation is judged visually by the colour changing from red to violet and finally to light blue. The last well which remains red represents the diluted endpoint, i.e. that concentration of protein which is just able to stabilize the colloidal gold.**

8. The final concentration of protein used for conjugation should be double the amount determined in step 7 to ensure sufficient protein to bind up all available sites on the gold colloid.

* The pH value of the colloidal gold is a crucial parameter when assessing the success of conjuation. Strong adsorption of macromolecules resulting in stable probes occurs at values close to or slightly basic to the isoelectric point (pI) of a given protein, because at these pH values, the zwitterion form of the protein is dominant and the interfacial tension is maxiamal.

** If tannic acid is used in colloidal gold prep, color change may be slow, because of the masking effect of polymers formed by the excess TA. The excess TA can be broken down by adding 0.1 - 0.2% H_2O_2 to the sols after which salt induced flocculation is fast and clear.

Appendix #7: PREPARATION OF LECTIN-GOLD CONJUGATES.
(Morris & Saelinger, 1986; De May, 1983)

1. At room temperature, adjust pH of 10 mls gold sol at or just to the basic side of the pI for the lectin to be conjugated (See table 2).

2. Optional: Centrifuge gold sol 5 min. x 7000g to remove large aggregates.

3. Prepare the lectin solutions by dissolving salt-free lectins in dH_2O immediately prior to use. If the lectins are already in solution, they should be dialyzed first against water or against very low molarity NaCl, in order to prevent salts from interfering with adsorption to the gold colloid. For concentrations used in this study, see table 2.

4. Add 0.5 ml of lectin solution all at once to 10 ml gold sol and stir 30 min. at room temperature. Addition of a stabilizer (1% PEG 20,000 MW to give final concentration of 0.5mg/ml) at this point is optional and if possible should be put off until after centrifugation, as the PEG causes aggregate formation.

5. Centrifuge 30 min. x 15,000g at 277K (for Au_{20}), or until supernatent is no longer red. NB: smaller gold particle size will require longer centrifugation time.

6. Carefully aspirate the supernatant containing free protein. Then, resuspend the loose dark red coloured pellet in 5mM phosphate buffer at appropriate pH. Tightly pelleted material should not be resuspended as this introduces large aggregates into the conjugate solution.

7. Repeat steps 4 & 5 twice, to ensure removal of free protein and gold particles which have not been fully stabilized.

8. Add 1% PEG (MW 20,000) to give final concentration of 0.5 mg/ml (approx. 75µl/1.5mls). This stabilizer minimizes aggregation and blocks remaining adsorption sites.

9. Dialyze conjugate against buffers* of increasing strength over a 24 hour period (4°C) to bring it up to physiological strength:

   a) 50 mM Tris
   b) 50 mM Tris with 50 mM NaCl
   c) 50 mM Tris with 100 mM NaCl
   d) 50 mM Tris with 150 mM NaCl

   Found to be successful for all lectins accept PNA, which was dialysed only to 50 mM Tris with 100 mM NaCl.
   * all buffers adjusted to appropriate pH.

10. Store at 4°C and use within 1 - 4 weeks.

11. Just prior to use, centrifuge conjugates for 5 min. x 7000g (277K) to remove large aggregates.

3. RESULTS

3.1 FORMATION OF THE ESOPHAGEAL MUSCULATURE

The cells which form the esophageal musculature are derived from a population of undifferentiated mesenchymal cells, which bud off from the tip of the archenteron and form coeloms at about 3 1/2 days of development. These mesenchymal cells migrate through the ECM-rich blastocoel, while the development of the alimentary canal continues. After 5 1/2 days of development, the endoderm has become segmented to form the stomodeum, esophagus, stomach and intestine (Fig.1). At this time, some of the mesenchymal cells come to rest exclusively on the presumptive esophageal endoderm (Fig.2). With continued differentiation, the mesenchymal cells begin to exhibit muscle fibers and attain a more intimate contact with the esophageal BL via evaginations (Fig.3). These evaginations appear to break through the BL in some areas (Fig.4). By 15 days of growth, the esophageal muscle wall is complete, and is characterized by an interlocking of muscle cells forming a smooth muscle sheath around the esophageal endoderm (Fig.5).

Fig. 1: A 1.0μ saggital section of a glutaraldehyde/alcian blue-fixed 5 1/2 day embryo, showing the segmented alimentary canal consisting of the stomodeum (St), esophagus (Es), stomach (S), and intestine (In). Note the mesenchymal cells (arrows) settling on the esophagus. x300

1

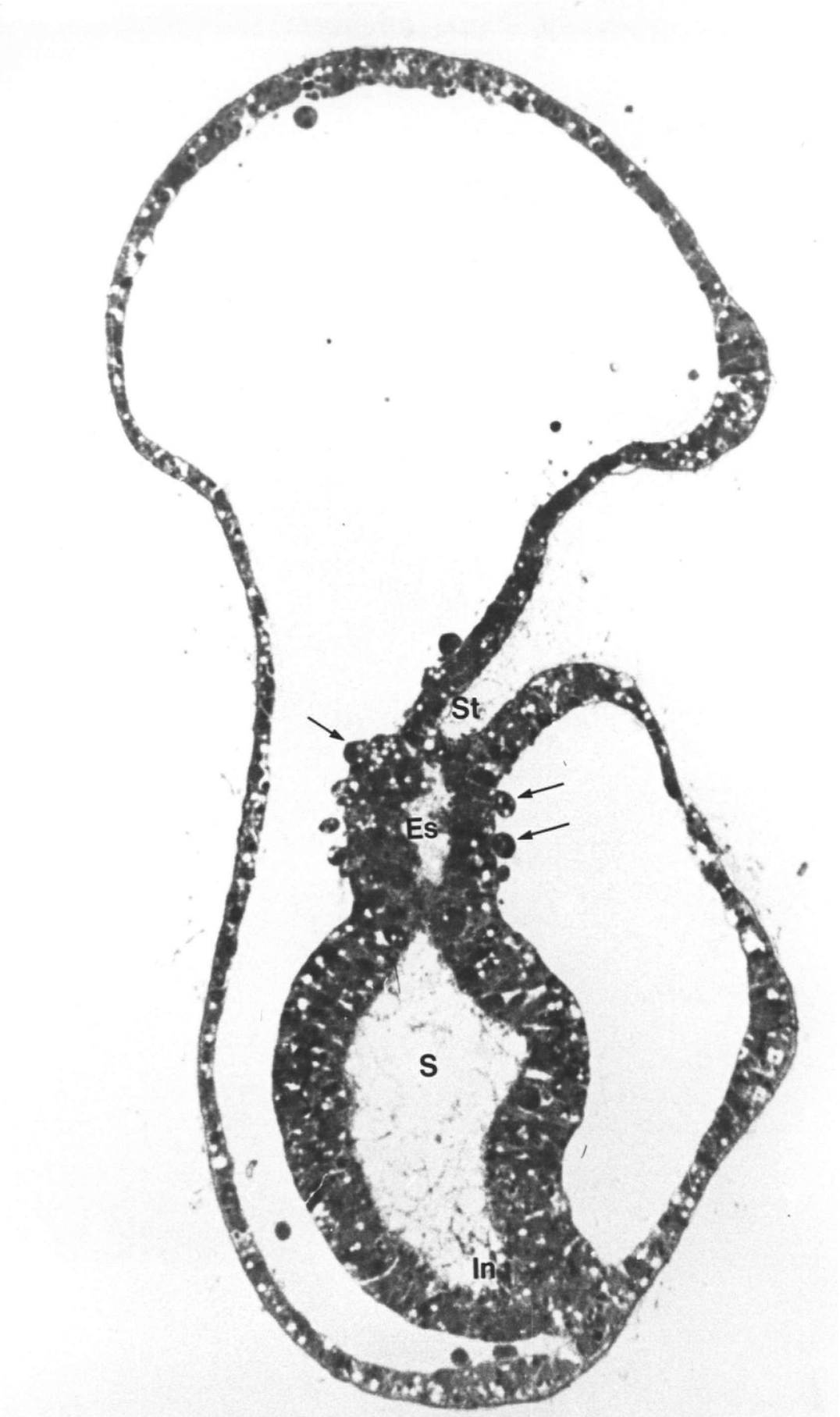
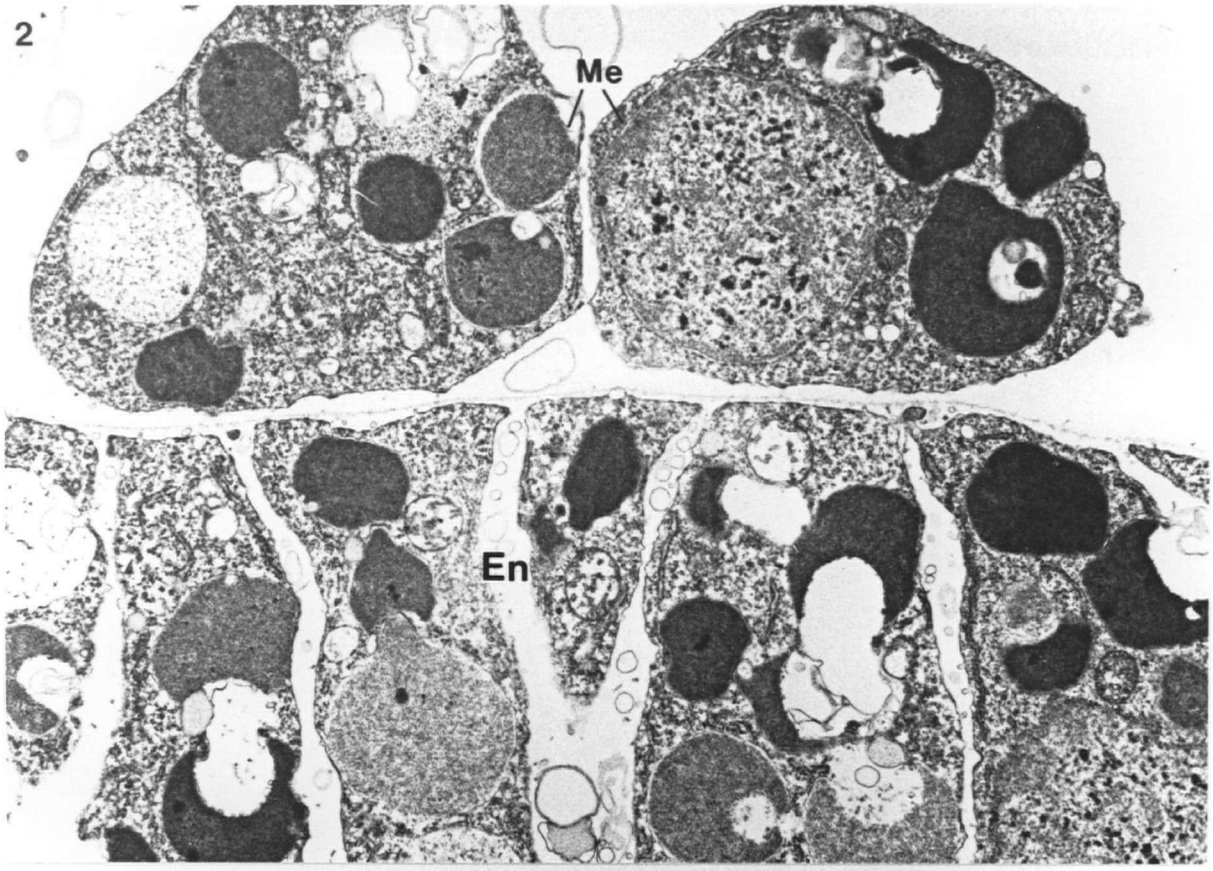


Fig. 2: A TEM through the esophagus of a 5 day embryo fixed with glutaraldehyde/alcian blue, showing two mesenchymal cells (Me) settling on the esophageal endoderm (En). x12,000

Fig. 3: A TEM through the esophagus of a 6 day embryo fixed with glutaraldehyde/alcian blue, showing the differentiating mesenchymal cells (Me) attaching to the esophagus (Es). Of interest are the evaginations of the mesenchymal cells, some of which contact the esophageal BL (arrowheads). Muscle fibers are beginning to appear in the mesenchymal cells, and are seen here in X-section (square). x23,100


2
Me
En

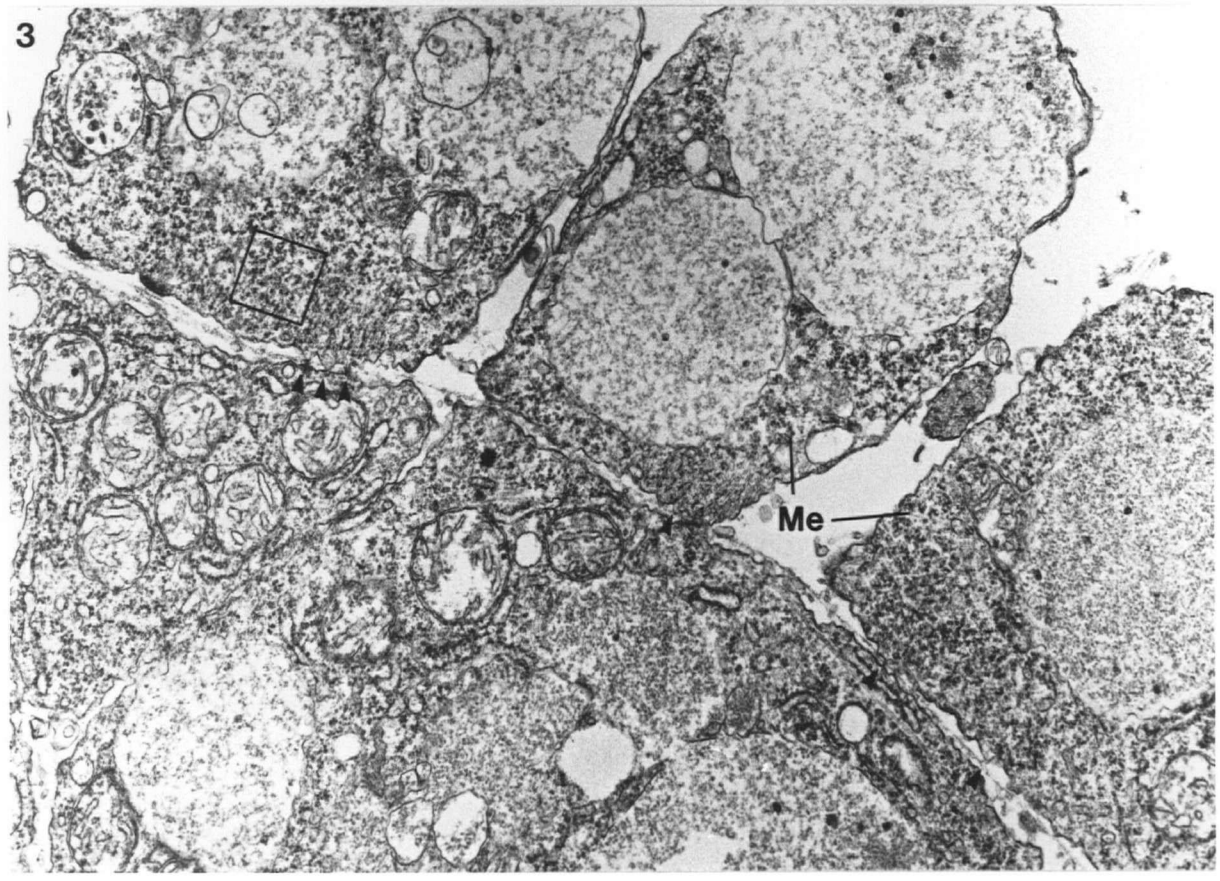

3
Me

Fig. 4: A TEM through the esophagus of a 6 day embryo fixed with glutaraldehyde/alcian blue, showing the interactions of two mesenchymal cells (Me) with the esophagus (Es). Of interest are the mesenchymal evaginations which in some cases appear to traverse the BL (arrows) and make contact with the esophageal endoderm. x57,600

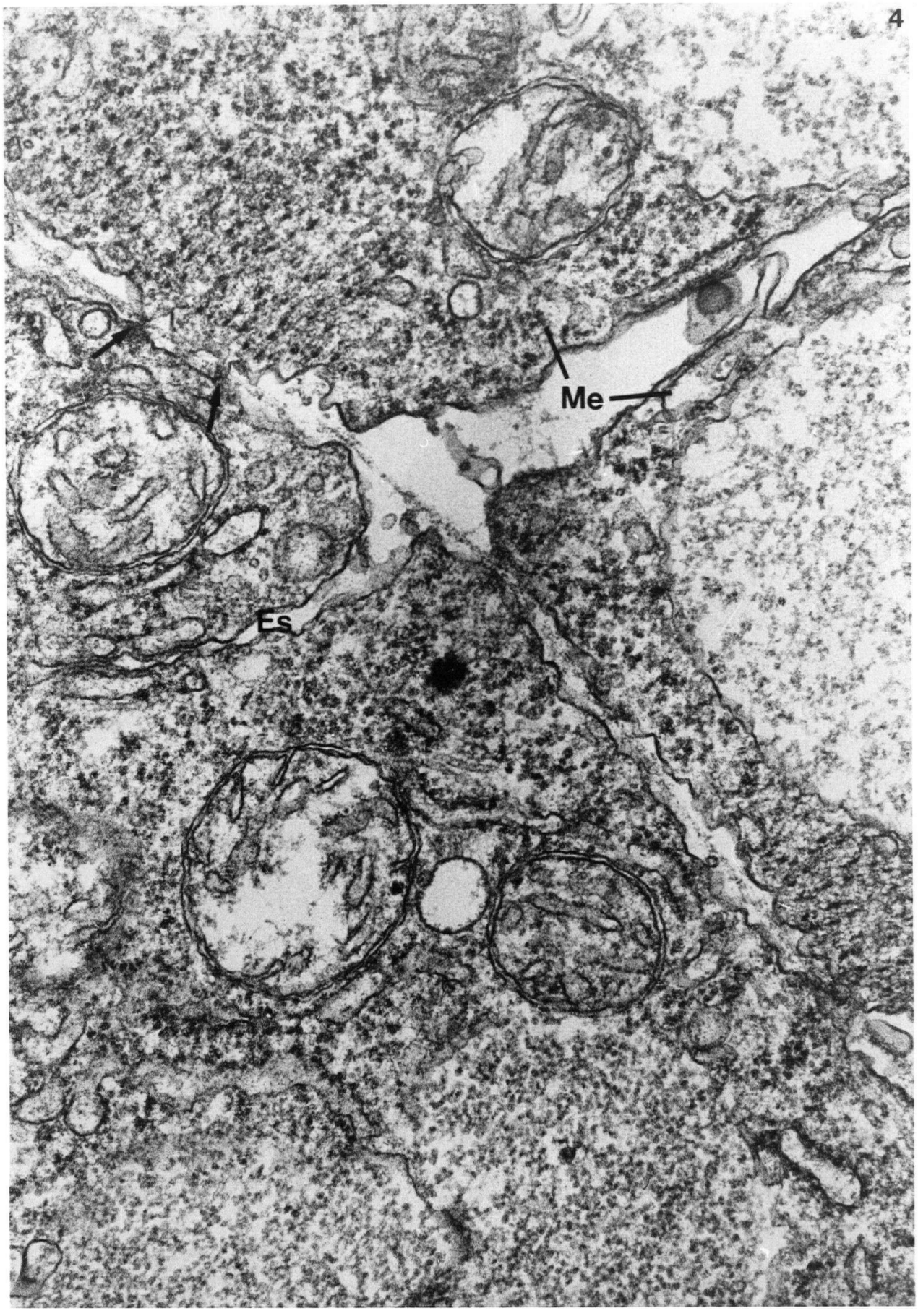
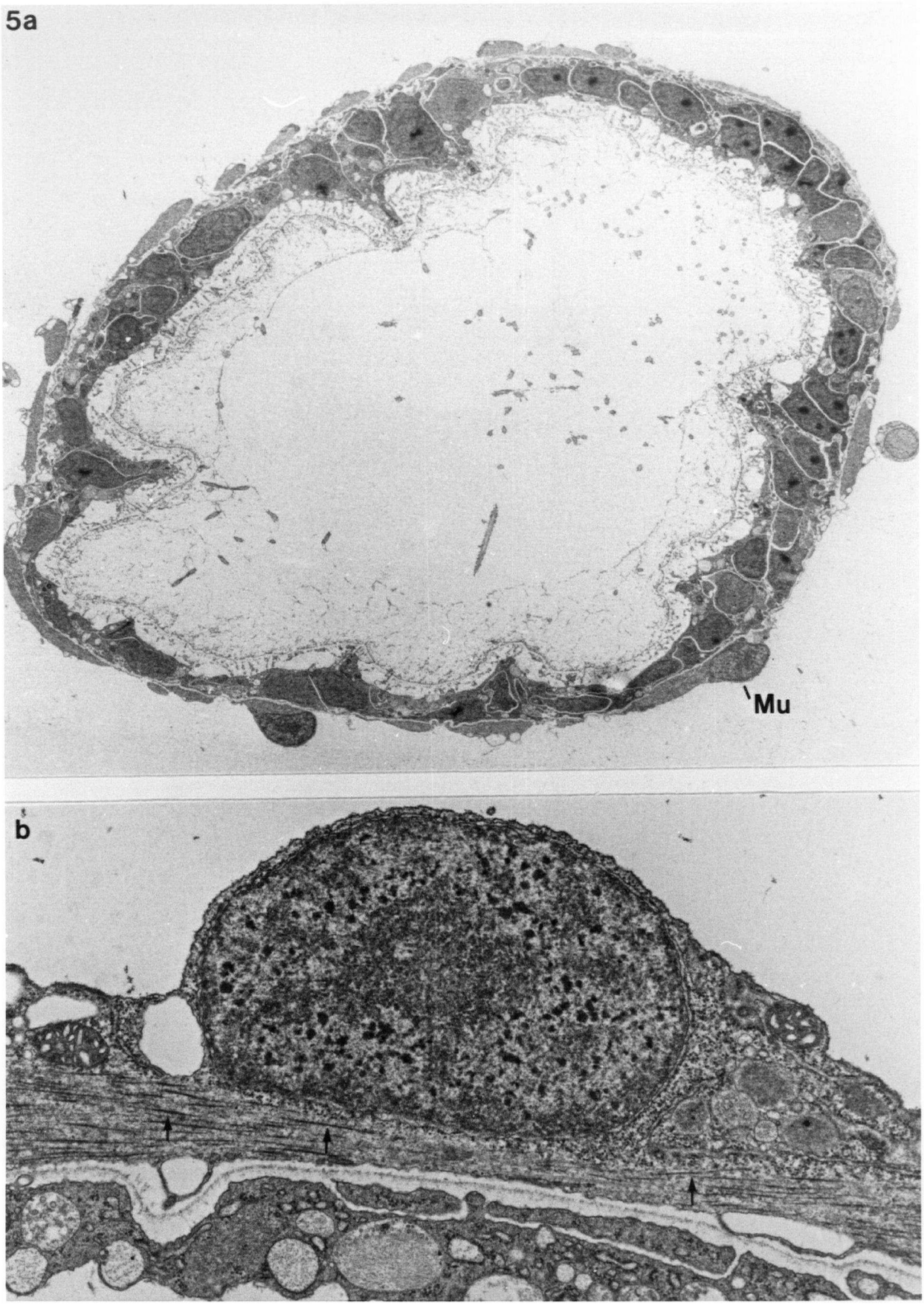

4
Me
Es

Fig. 5a: A TEM of a cross-section through the esophagus of a 15 day embryo fixed with glutaraldehyde/alcian blue, showing the esophageal endodermal cells on the inside of the tube, and the muscle cells (Mu) wrapping around the outside of the tube. x2300

Fig. 5b: A higher magnification of the above, showing a muscle cell in X-section. The muscle fibers are readily visible (arrows). x19,800


5a
Mu
b

3.2 FIXATION

Light Microscopy

Several fixations were attempted in order to achieve a balance between optimal cellular preservation and maximal carbohydrate precipitation. Fixatives containing glutaraldehyde were not used in the study because glutaraldehyde creates an induced fluorescence in tissues as a result of its interactions with arylethylamines, which interfered with observation of FITC-lectin labelling.

The 3 fixatives, 95% ethanol, paraformaldehyde in sea water, and paraformaldehyde/CPC in sea water were all satisfactory, but each one had its strengths and weaknesses. Although 95% ethanol gave very poor cell preservation, including poor BM and ECM fixation, it preserved the hyaline layer, an outer ECM surrounding the embryo, very well. The hyaline layer of tissue fixed with 95% ethanol showed intense fluorescence after staining with SBA, RCA and Con A, but virtually no staining with WGA was noted.

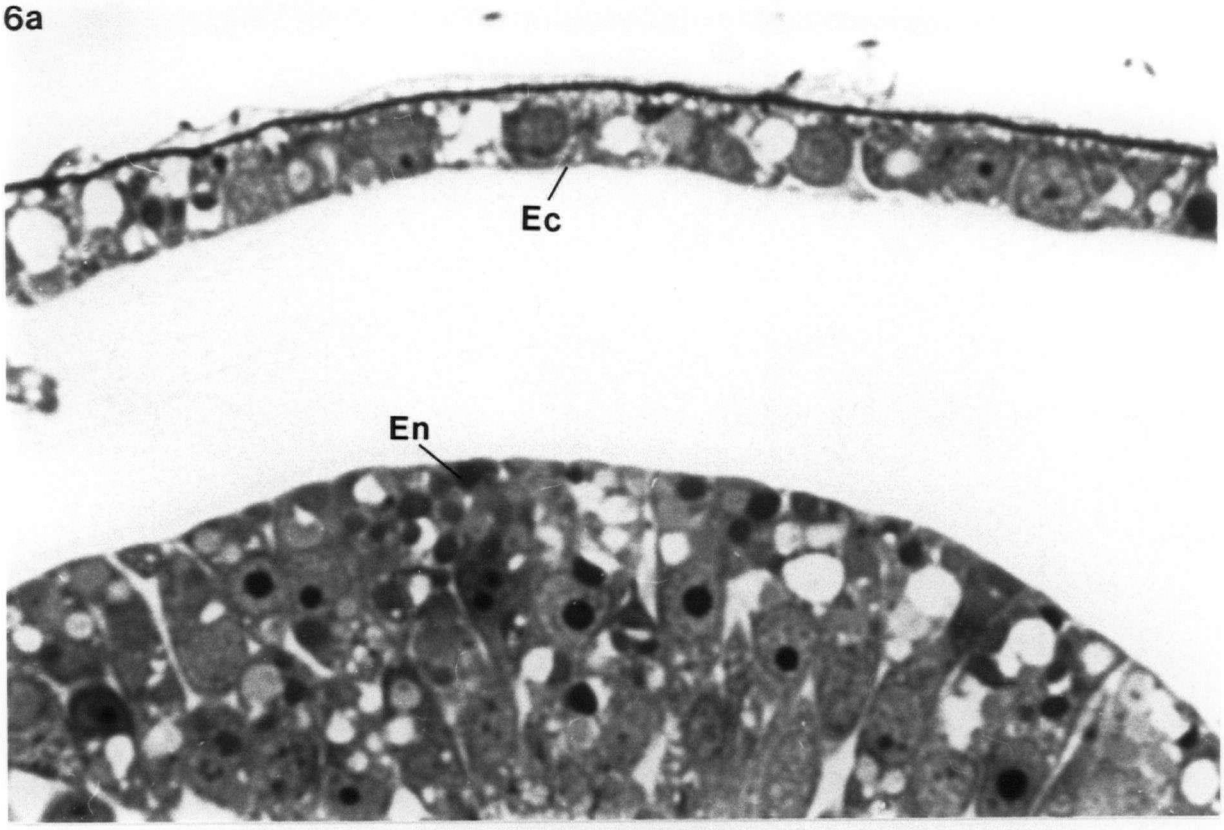
Freshly prepared paraformaldehyde in either sea water or Millonig's phosphate buffer gave better cellular preservation than did the ethanol. There was little or no difference in tissue preservation between the fixative buffered in sea water and that in Millonig's, but labelling was more intense in tissues fixed with the former, and it therefore became the buffer of choice. Addition of CPC to the this fixative gave better preservation of the BM and the ECM (Figs.6a,6b) than an either paraformaldehyde or glutaraldehyde alone, although the cellular preservation was poor. Con A and WGA labelling was observed following this fixation, however SBA labelled very poorly and RCA did not label at all.

The use of paraformaldehyde with 1% alcian blue in 80% sea water gave excellent preservation of cells and ECM, but tissue preserved in

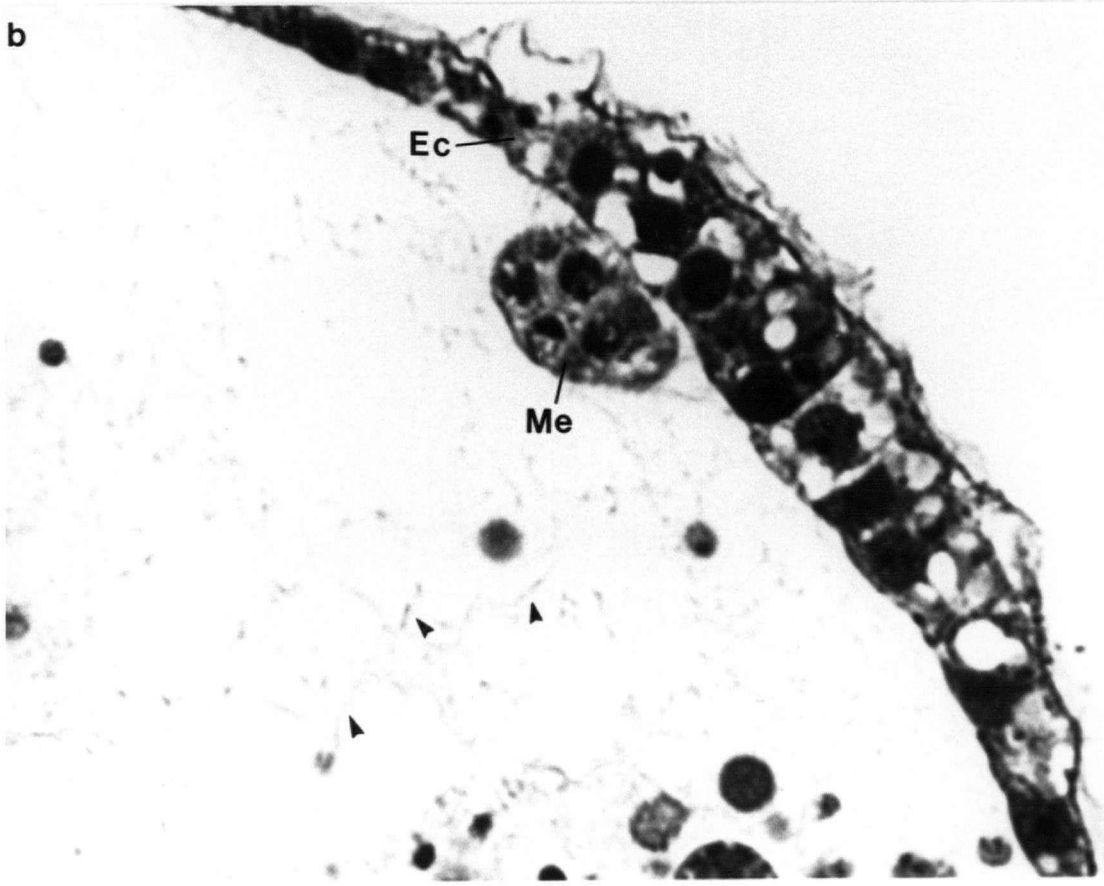
Fig. 6a: A 1.0μ section of a glutaraldehyde/alcian blue-fixed embryo showing the ectoderm (Ec), the stomach endoderm (En), and some hint of ECM in the blastocoel. Note that the BM is not readily visualized in this preparation. x2000

Fig. 6b: A 1.0μ section of a paraformaldehyde/CPC-fixed embryo, showing the ectoderm (Ec), and a mesenchymal cell (Me). Note that in this preparation, well-defined strands of ECM can be visualized in the blastocoel (arrowheads). x2200

6a



b



this manner did not label with any of the FITC-lectins.

Tissue fixed in Bouin's was generally not very receptive to lectin binding; however, some granules in the cells labelled intensely with both Con A and WGA. The general tissue morphology was not as good as that of tissues fixed in paraformaldehyde.

The effect of each fixative on the cell preservation and lectin binding are summarized in table 5.

TABLE 5: THE EFFECT OF VARIOUS FIXATIVES ON FITC-LECTIN BINDING SITES

FIXATIVES	COMMENTS
(1) Paraformaldehyde/CPC in sea water	-poor preservation of cells (due to CPC) -good staining of BL and ECM with WGA & Con A -poor staining of HL accept with WGA -moderate staining of granules with WGA only
(2) Paraformaldehyde/alcian blue in sea water	-good preservation of cells, BL, ECM & HL -no staining with any lectins
(3) Paraformaldehyde/CPC in Millonigs	-similar to (1), but decreased staining of BL & ECM with WGA & Con A
(4) 1°: Paraformaldehyde in sea water 2°: Paraformaldehyde/CPC in sea water	-staining similar to (1), with no improvement of cellular preservation.
(5) Bouins	-poor cell preservation -excellent staining of granules for WGA & Con A -poor staining of HL, ECM & BM for WGA, Con A, SBA & RCA
(6) 95% Ethanol	-very poor cell preservation -excellent staining of HL with RCA, SBA, & Con A -moderate staining of HL with WGA -poor staining of BM with Con A & WGA -moderate staining of ECM with Con A & WGA

Fixation-TEM

Since glutaraldehyde-related induced fluorescence was not a concern with the TEM studies, glutaraldehyde formed the basis for the two fixations used.

The standard fixative quoted in the literature for use when processing tissue in Lowicryl K4M resin is 0.1% glutaraldehyde, 2% paraformaldehyde in PBS. This fixative was tried initially, but was not used for the majority of the lectin-binding studies because there was no difference in lectin-binding between this fixation and those described below, and the latter fixatives gave better ultrastructural preservation of this material.

The 2 fixations which were used for the study were Dunlap's phosphate buffered glutaraldehyde, and glutaraldehyde with alcian blue in sea water. In general, Dunlap's fixative preserved the tissue very well, especially the BL and ECM. Fixation of the hyaline layer was very poor, however, and consisted of a thin band of material, indicating that quite a bit was lost during the processing. All lectin-binding sites were preserved well with this fixative, and in fact, labelling of the basal lamina and ECM appeared to be slighty more intense with Con A, DBA and LFA after this fixation than after fixation in glutaraldehyde/alcian blue.

The combined glutaraldehyde and alcian blue fixative was selected for use as it gave better ultrastructural preservation of the tissue in general and of the hyaline layer, basal lamina and ECM in particular. Good labelling was achieved using tissue preserved with this fixative suggesting that the alcian blue did not interfere significantly with the lectin-binding sites. In addition, it was found that this fixative gave better ultrastructural preservation of the hyaline layer, than did

Dunlap's fixative.

3.3 FITC-LECTIN LABELLING

Lectin Labelling Patterns of the Basement Membrane and ECM

Although the structure of the basement membrane (BM) in this tissue is too thin to be satisfactorally resolved with the LM after staining with Richardson's, a structure located in the region of the BM is readily visualized after staining with FITC labelled reagents. Some difficulty arises, however, when differentiating labelling of the BM from that of the dense ECM associated with it, and therefore no attempt will be made to separate the two in the description.

Labelling with both Con A and WGA was observed in PF/CPC-fixed tissue. Con A densely labelled the ectodermal and endodermal BM in all regions of the embryo, except for the esophagus (both dorsal and ventral) and the posterior aspect of the stomach (Fig.7a). Very intense labelling of the BM and associated ECM was present along the dorsal ectoderm (Fig.8a), which was quite different than the staining pattern along the stomodeum and esophagus. Along the stomodeum, labelling of the BM and ECM exhibited a patchy appearance (Fig.8c), while little or no labelling was present along the BM of the esophagus (Fig.8b). At higher magnification, labelling of the ECM resembled granules or beads arranged along an "invisible string" (Fig.8d).

The majority of the BM and associated ECM also labelled densely with WGA, again with the exception of the dorsal and ventral esophageal regions and the region posterior to the stomach (Fig.9a). As with Con A, dense labelling of the BM/ECM underlying the dorsal ectoderm was observed (Fig.10a). As well, labelling continued around to the area of the stomodeum, where it exhibited a linear appearance rather than the

patchy one seen with Con A. In the region of the esophagus, the BM exhibited little or no staining, as with Con A. Little staining was also noted beneath the endoderm of the posterior stomach (Fig. 10c). The staining of the ECM in all regions of the embryo appeared filamentous and not granular as was the staining with Con A. In the region between the esophagus and ectoderm, there was intense labelling of a "web" of matrix both in the dorsal and ventral region.

No labelling of the BM or associated ECM was observed after staining with either SBA or RCA.

Controls

In all cases the lectins did not bind specifically to any tissue in the embryos when sections were incubated with the control stains (0.1M inhibitory sugar solution incubated with FITC-lectin conjugate).

Fig. 7a: A saggital section of a 6 day embryo, fixed with PF/CPC, and stained with FITC-Con A. The regions immediately beneath the dorsal ectoderm and dorsal stomach (large arrowheads) label densely, while moderate labelling is present beneath the epithelia in the other areas. Of interest is the esophageal region, which in this case shows little or no labelling, both dorsally and ventrally (small arrowheads), where the BM is located. x480

Fig. 7b: An adjacent saggital section of the above, stained with the control sugar/conjugate solution, mannose/FITC-Con A. x480

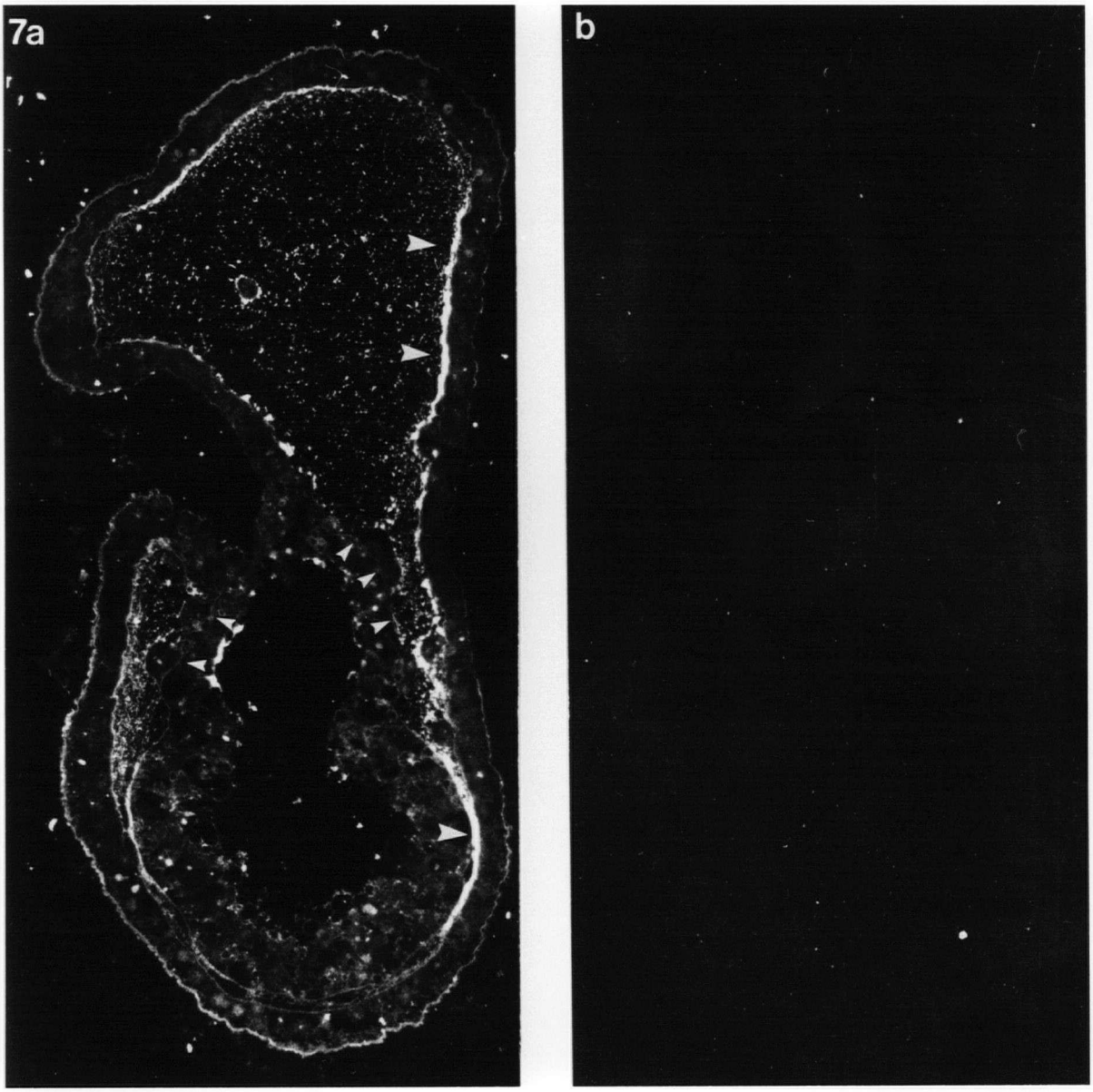

7a
b

Fig. 8a-d are all sections fixed with PF/CPC as above, and stained with FITC-Con A

Fig. 8a: The dorsal ectoderm, showing the densely labelled BM and its associated ECM (arrowheads). x1120

Fig. 8b: A region of the alimentary canal, showing the esophagus (Es), with mesenchymal cells (Me) attached to it, and the stomach (S), which is devoid of mesenchymal cells. Of interest is the faintly labelled esophageal BM (arrows), which differs markedly from the heavily labelled ECM found between the stomach epithelium and the dorsal ectoderm (arrowheads). x1120

Fig. 8c: A region of the stomodeum (St), showing the patchy staining of the BM and associated ECM (arrowheads). This is in contrast to the linear-type staining of the ECM associated with the ectoderm (Ec) in other regions of the embyro (Fig.8a). x1120

Fig. 8d: The region between the ventral esophagus (Es) and ectoderm (Ec) showing the web-like arrangement of ECM consisting of amorphous regions aligned into organized linear patterns. x2000

Fig. 8e: A region of the dorsal ectoderm, stained with the control sugar conjugate, mannose/FITC-Con A. x1120

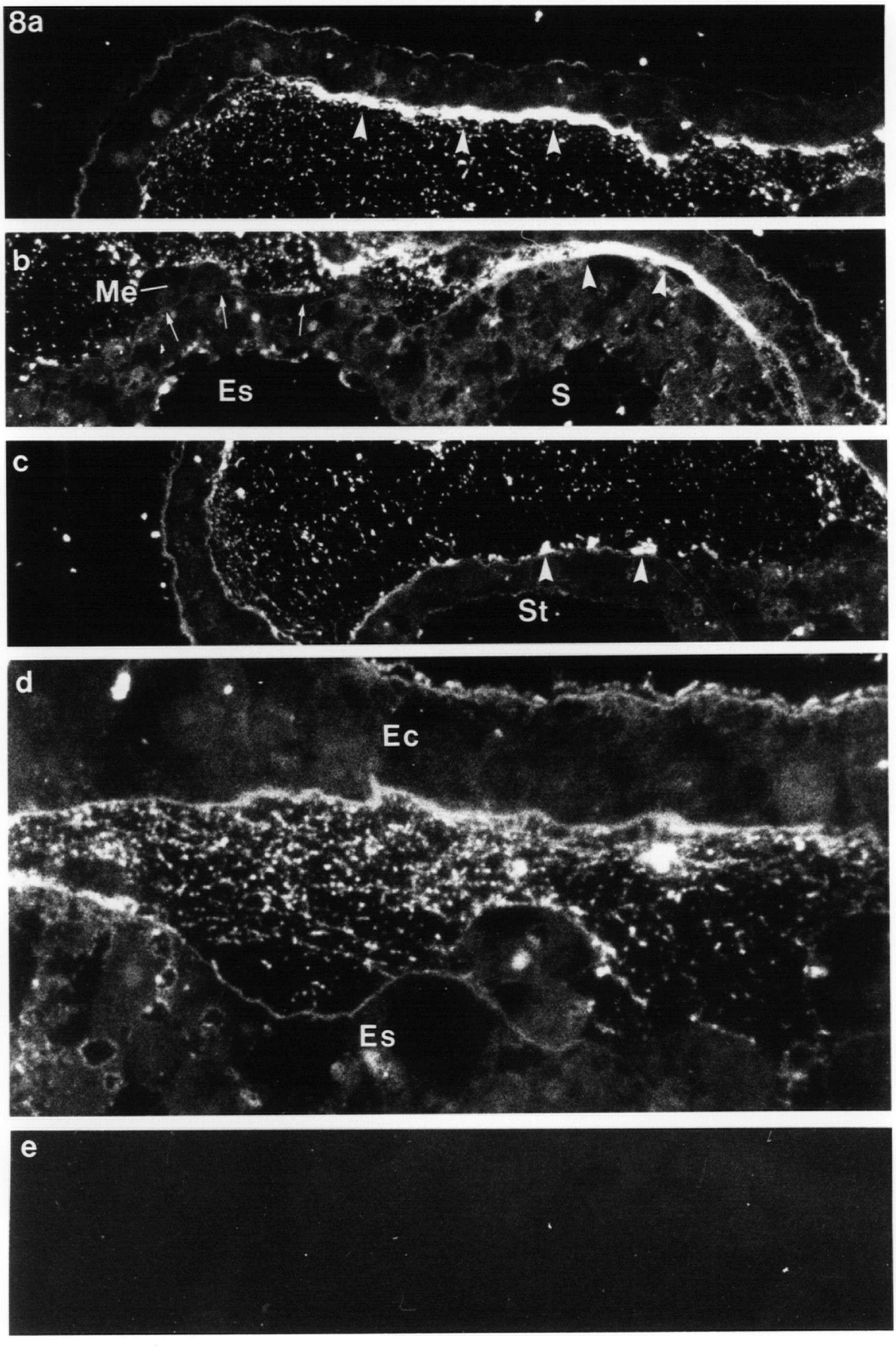

8a
b
Me
Es
S
c
St
d
Ec
Es
e

Fig. 9a: A saggital section of a 6 day embryo, fixed with PF/CPC, and stained with FITC-WGA. The BM region is very densely labelled in most areas of the embryo (arrows), with the exception of the esophagus (arrowheads) and distal area of the stomach, which exhibit very weak staining. x480

Fig. 9b: An adjacent saggital section of the above, stained with the control sugar conjugate, galNAc/FITC-WGA. x480

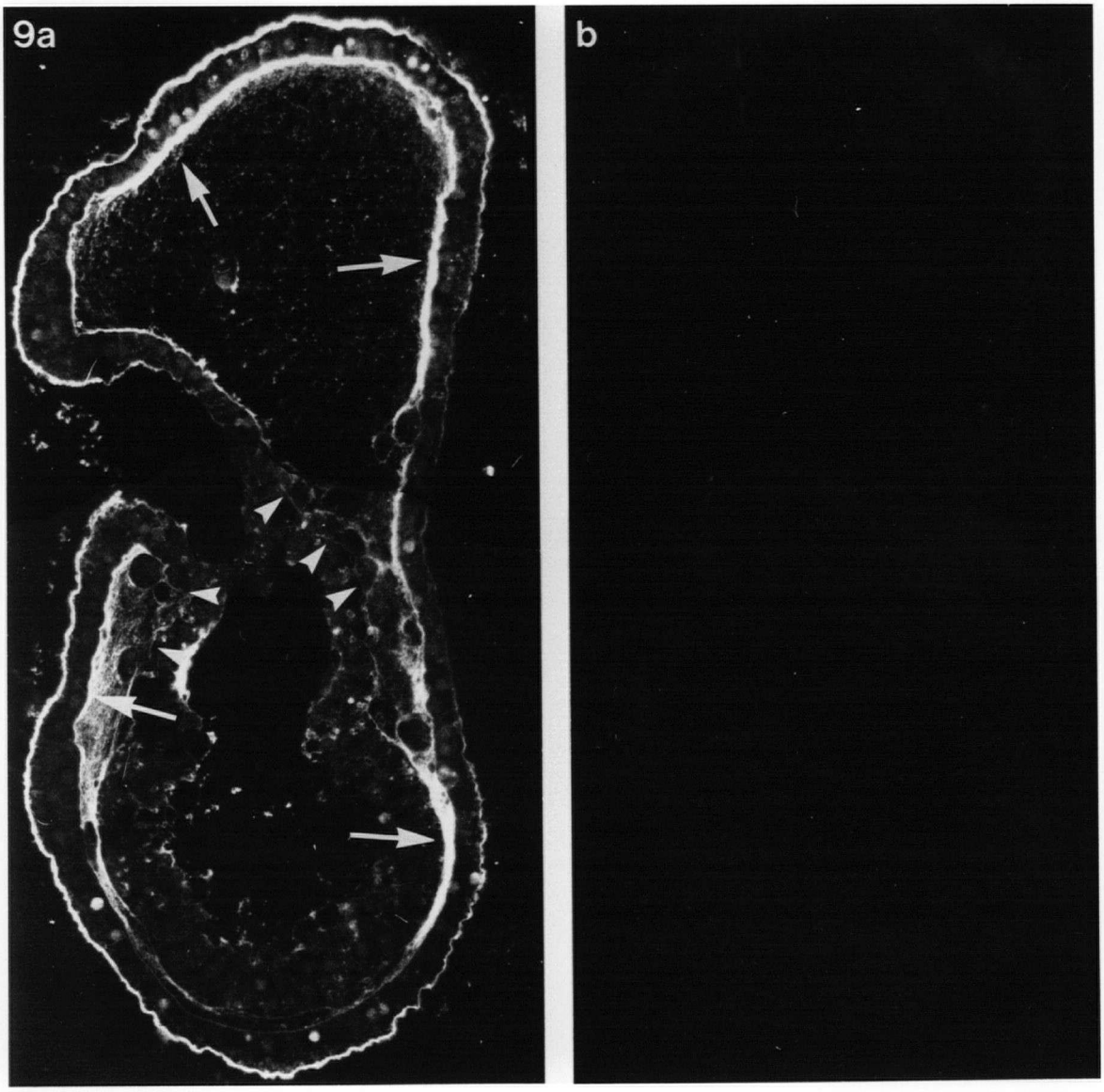

9a
b

Fig. 10a-10d are higher magnifications of different embryonic regions, fixed as above, and stained with FITC-WGA.

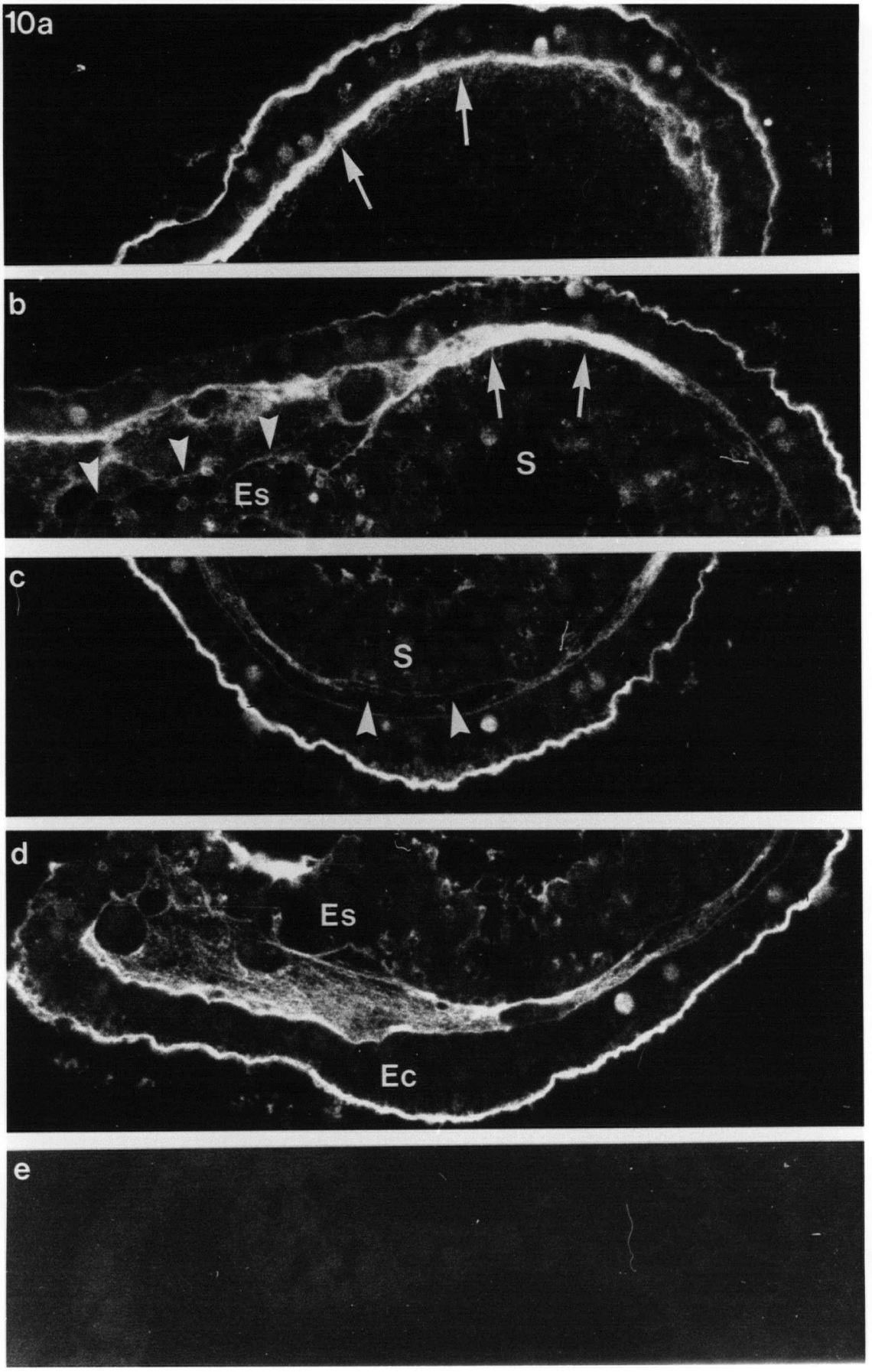
Fig. 10a: The anterior ectoderm, showing the densely staining BM region and associated ECM (arrows) underlying the epithelium. x1120

Fig. 10b: A region of the alimentary canal, showing the esophageal endoderm (Es), the faintly labelling esophageal BM (arrowheads), and the characteristic mesenchymal cells settling on it. Shown also is a part of the dorsal stomach (S), and the dense BM/ECM associated with it (arrows). x1120

Fig. 10c: A region of the posterior end of the embryo, showing the stomach epithelium (S) with its faintly staining BM (arrowheads). x1120

Fig. 10d: A region of the ventral esophagus (Es), showing the web-like appearance of ECM located between the esophagus and ectoderm (Ec). x1120

Fig. 10e: A region of the ventral esophagus, stained with the sugar control for the above, (glcNAc/FITC-WGA). x1120


10a
b
S
Es
c
S
d
Es
Ec
e

3.4 TEM-LECTIN LABELLING STUDIES

Effect of Embedding Media on Labelling

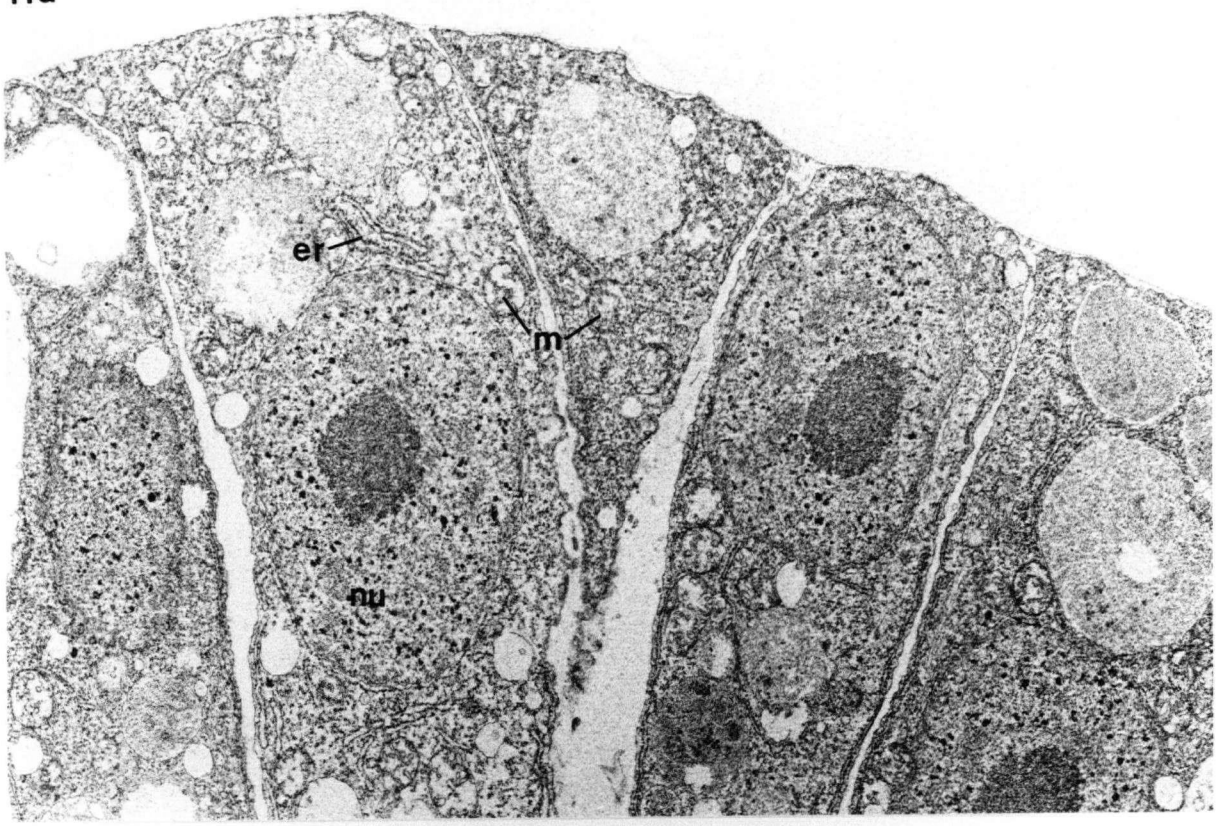
Although conventional embedding in Epon resulted in good preservation of cell ultrastructure, sections of Epon-embedded material did not label with lectin-gold preparations probably because Epon is highly crosslinked and hydrophobic in nature. Material embedded in Epon could be used if it was exposed to the lectin-gold conjugate prior to embedding; however the stain did not penetrate well into the tissue. While the stain could be admitted by cutting the embryo open, it did not appear to diffuse well through the blastocoel, meaning that the exposure to the stain could easily be different in different parts of the embryo. Although embedding in Lowicryl resulted in poor cell preservation, it was adequate for the ultrastructural preservation of the celluar and extracellular components (Fig.11a,11b). In contrast to Epon, Lowicryl undergoes only secondary crosslinking during polymerization, and is polar in nature. Because of this, sections of Lowicryl-embedded material could be stained directly, which meant that all regions of the tissue were exposed to the lectin. In addition, with this technique, different lectins could be tested on adjacent serial sections of tissue. Since the object was to compare the labelling pattern of lectins in different regions of the embryo, material embedded in Lowicryl was used for the majority of the work.

The basal lamina of Dunlap's fixed-Lowicryl processed tissue consisted of a lamina densa, approximately 80nm in width, and an electron lucent area of about the same width, the lamina lucida (Fig.12). In some areas, strands of ECM were associated with the lamina densa. The ECM consisted of strands approximately 40nm in width, with irregularly shaped amorphous regions arranged along the strands. These

Fig. 11a: A TEM through the stomach of a 6 day embryo, fixed with glut/alcian blue and embedded in Epon. Preservation of cell ultrastructure is good, and cell organelles such as endoplasmic reticulum (er), nuclei (nu), and mitochondria (m) are readily visualized. x12,000

Fig. 11b: A TEM through the stomach of a 6 day embryo, fixed as above, but embedded in Lowicryl K4M. Ultrastructure in this tissue is not as readily apparent, as in the conventionally prepared tissue. The BL, nuclei (nu), and intracellular granules (G) are, however, readily visualized. x14,000

11a



b

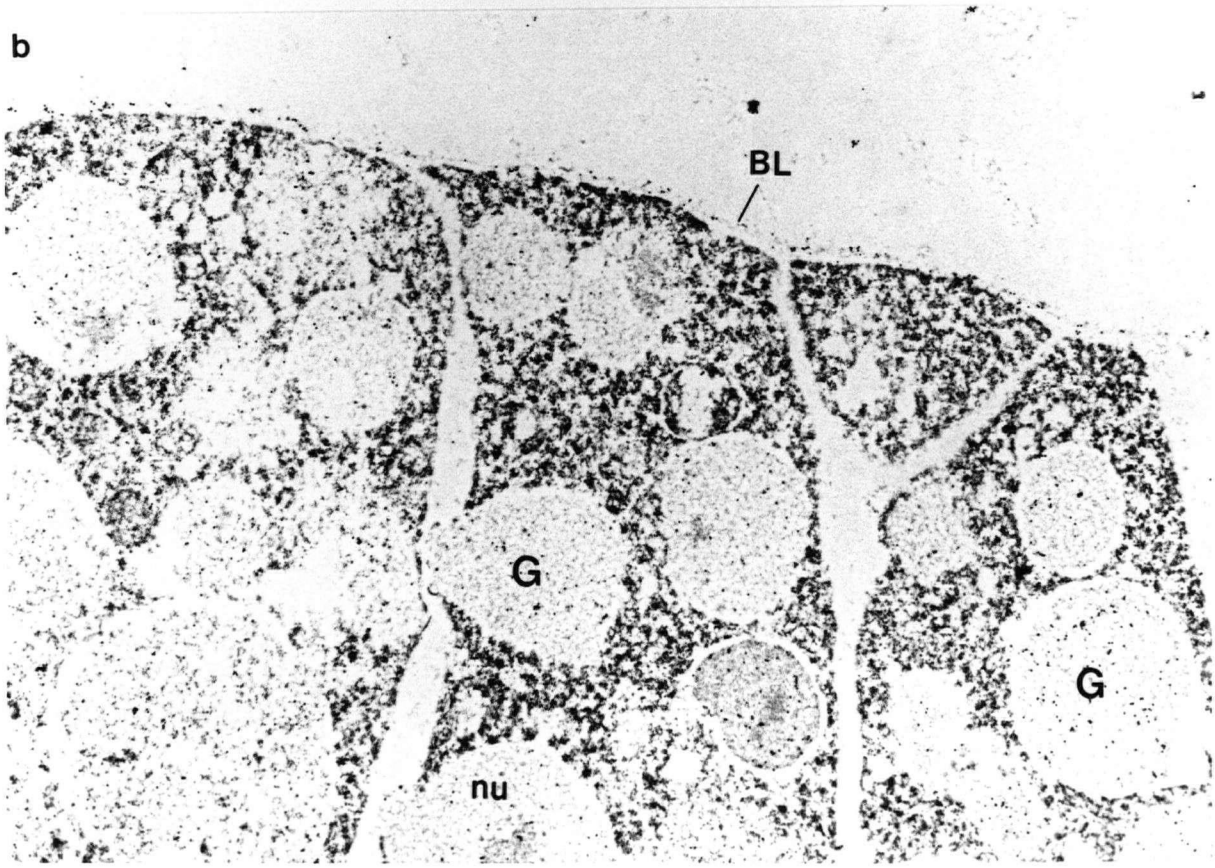
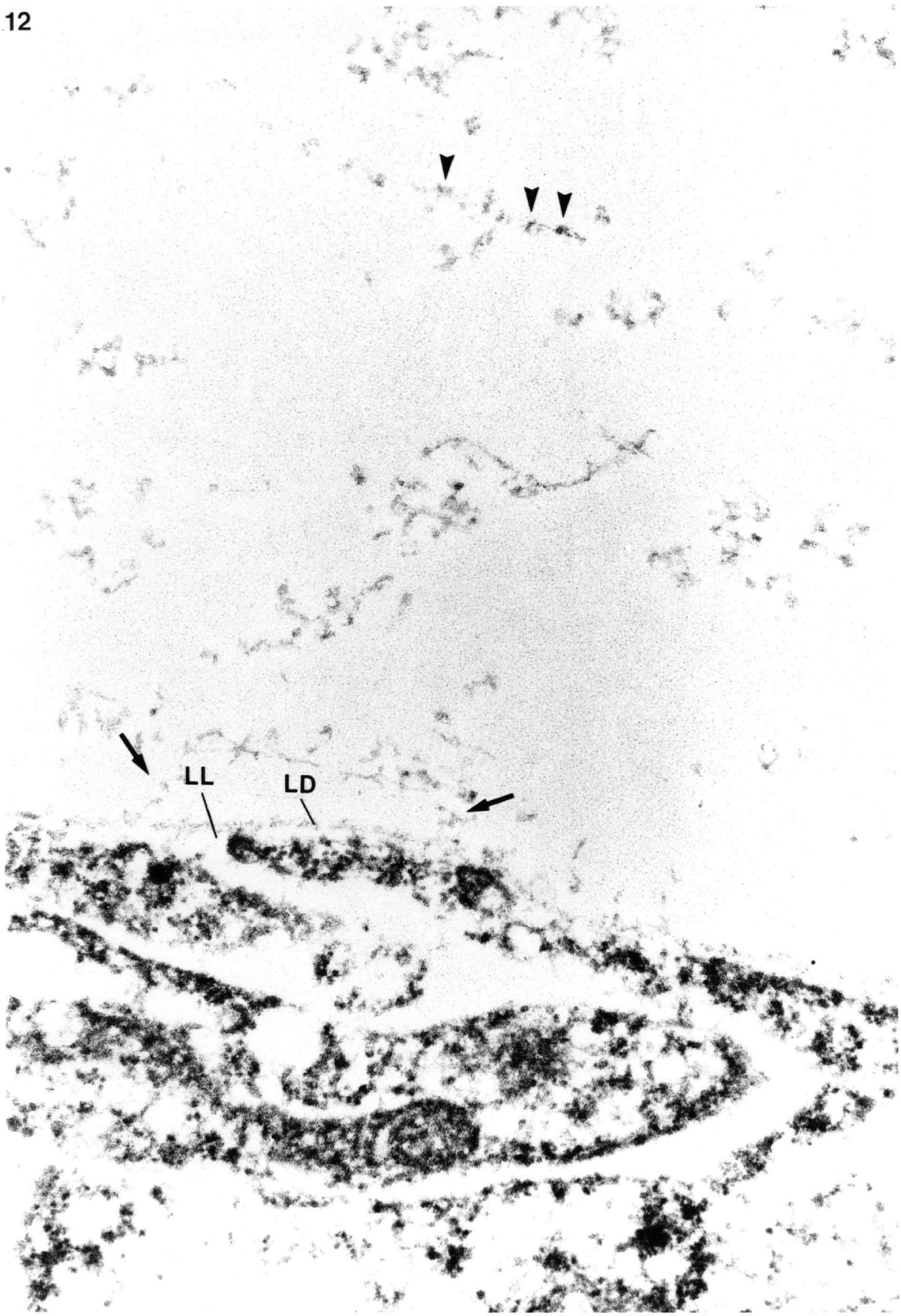


Fig. 12: A TEM through the stomach of a 6 day embryo, fixed with glutaraldehyde/alcian blue and embedded in Lowicryl K4M. At this higher magnification, two components of the BL are evident: an electron dense lamina densa (LD), and an electron lucent lamina lucida (LL). The ECM is composed of strands ≃40nm in width with irregularly shaped amorphous regions arranged along their length (arrowheads). These amorphous regions range from 50–200 nm in diameter. In some areas, the ECM is associated and continuous with the lamina densa of the BL (arrows). x74,300

12



amorphous regions ranged from 50 to 200 nm in diameter (Fig.12).

Au_{25}-Lectin Labelling Patterns of the Basal Lamina and ECM

Labelling of both the basal lamina and ECM was observed with the lectin-gold conjugates Au_{25}-Con A, Au_{25}-LFA and Au_{25}-DBA (Fig.13,14,15), while labelling of ECM alone was observed with Au_{25}-WGA and Au_{25}-SBA (Fig.16,17). There was substantial variation in both the density of the label as well as in the ultrastructural localization of the label among the different lectins. Of the 3 lectins, labelling over the BL was heaviest with DBA, a galNAc-α1,3-galNAc-binding lectin: The gold particles were arranged in clusters containing from 3 - 12 particles per cluster (Fig.13), and were located over the entire width of the basal lamina, including the lamina lucida, the lamina densa and the dense fibers of ECM associated with the lamina densa. Labelling of the ECM occurred primarily over the amorphous regions, and not over the thin strands (Fig.13).

Another lectin which labelled both the basal lamina and ECM was Au_{25}-Con A, a galactose/ mannose binding lectin. In this case, label was predominantly found over the lamina densa, with only an occasional gold particle located over the lamina lucida. The particles formed small clusters in some areas, but were primarily present as single units (Fig.14a). There was some labelling of the amorphous regions of the ECM as well (Fig.14), although it was not as dense as that seen with DBA.

Au_{25}-LFA, a sialic acid-binding lectin, was the 3rd lectin which labelled the basal lamina and ECM specifically. Unlike the previous 2 lectin-gold conjugates, there appeared to be more labelling over the lamina lucida, as well as some over the lamina densa (Fig.15a). The gold particles were most frequently visualized in large clusters

containing 10 - 20 particles per cluster. Labelling of the ECM was distributed over both the thin fibers and the larger amorphous regions (Fig.15a).

The lectin-gold conjugates Au_{25}-WGA, Au_{25}-SBA and Au_{25}-PNA showed no specific labelling of the basal lamina, however there was some scattered labelling of the ECM with WGA and SBA. While the SBA labelling appeared specifically over the amorphous regions of the ECM rather than along the strands (Fig.16a), such a distinction could not be made with WGA, which was distributed more evenly over both elements of the ECM (Fig.17a). With both lectins, the gold particles were present mostly as single units or small clusters of 2 - 3.

Controls

Sections were incubated with control stains, which consisted of 0.1M inhibitory sugar conjugated with the Au_{25}-lectin. Controls were run for all lectins accept Au_{25}-DBA; this lectin binds to the terminal disaccharide, galNAcα1,3-galNAc, which was not available for the study. The monosaccharide galNAc was attempted as a control for DBA, but this sugar did not significantly reduce the amount of labelling. The controls for the lectins Con A, LFA, WGA, and SBA showed a very minimal amount of background labelling. This is documented in Figures 14b,15b,16b,& 17b.

Fig. 13 - 17 are all ultrathin sections of 6 day embryos, which have been fixed with glutaraldehyde/alcian blue and embedded in Lowicryl K4M.

Fig. 13: A TEM through the ectoderm stained with Au_{25}-DBA, showing heavy labelling of the BL and ECM. Labelling of the BL occurs predominantly over the lamina densa (LD), while labelling of the ECM appears to be concentrated over the amorphous regions (arrows). The gold particles are frequently seen as clusters, averaging 8 particles. x33,600

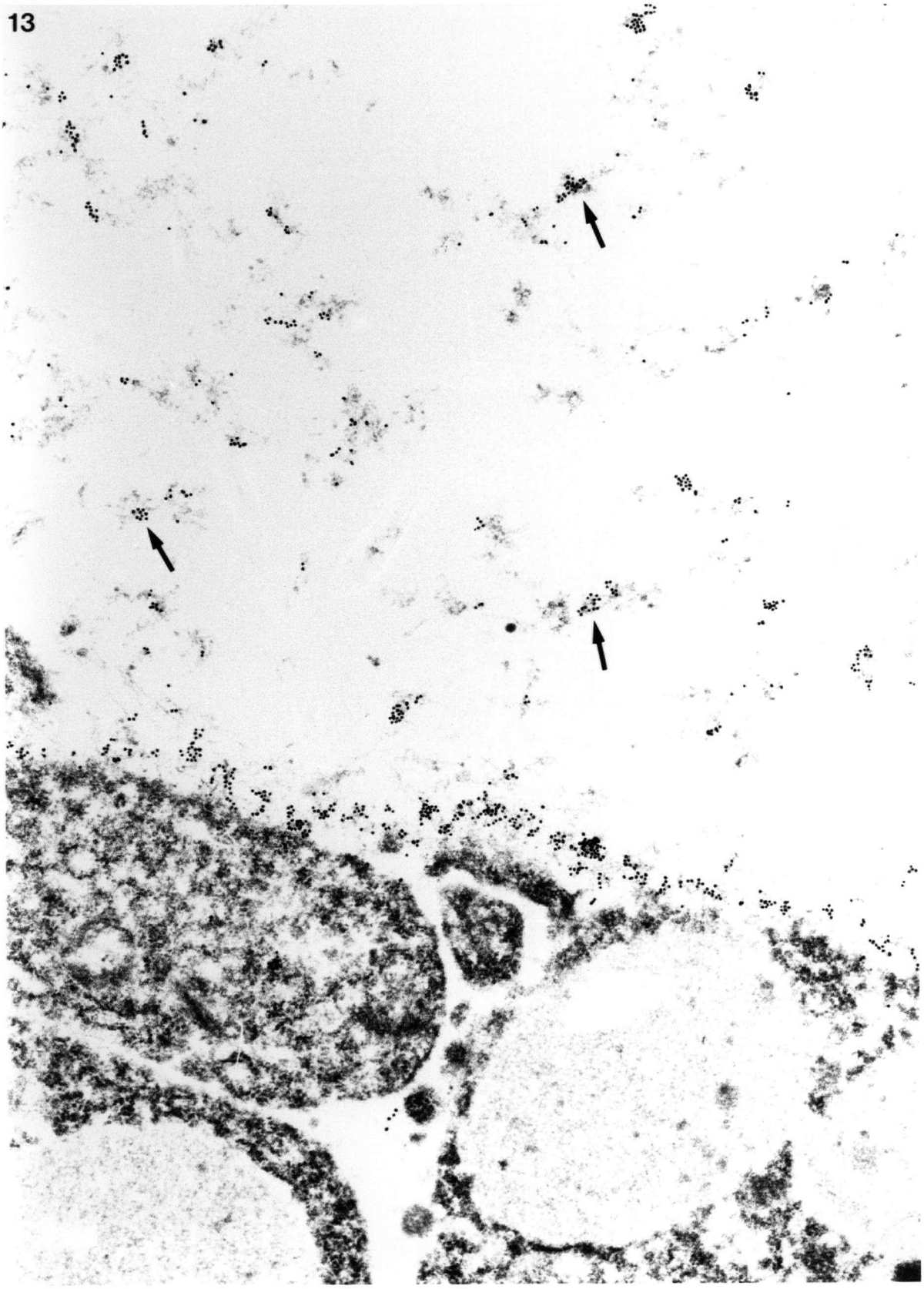
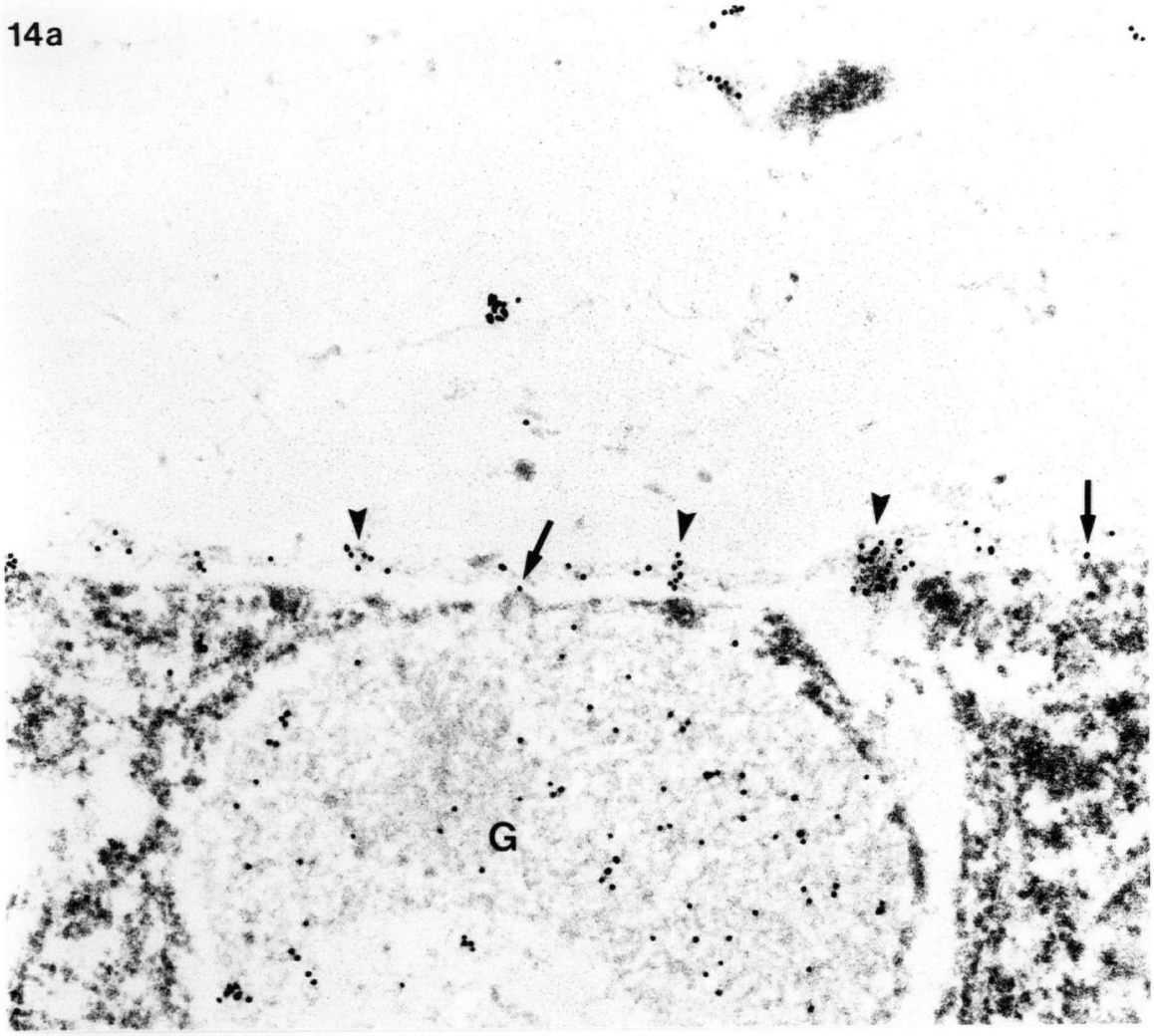

13

Fig. 14a: A TEM through the ectoderm stained with Au_{25}-Con A, showing labelling of the BL and ECM. Labelling occurs predominantly over the lamina densa (arrowheads), although an occasional particle is seen over the lamina lucida (arrow). Very scattered labelling of the ECM is observed. Specific labelling of the intracellular granules (G) is also present with Au_{25}-Con A. x49,500

Fig. 14b: A TEM through the ectoderm, stained with the control sugar/conjugate solution of the above, 0.1M mannose/ Au_{25}-Con A. x42,000

14a



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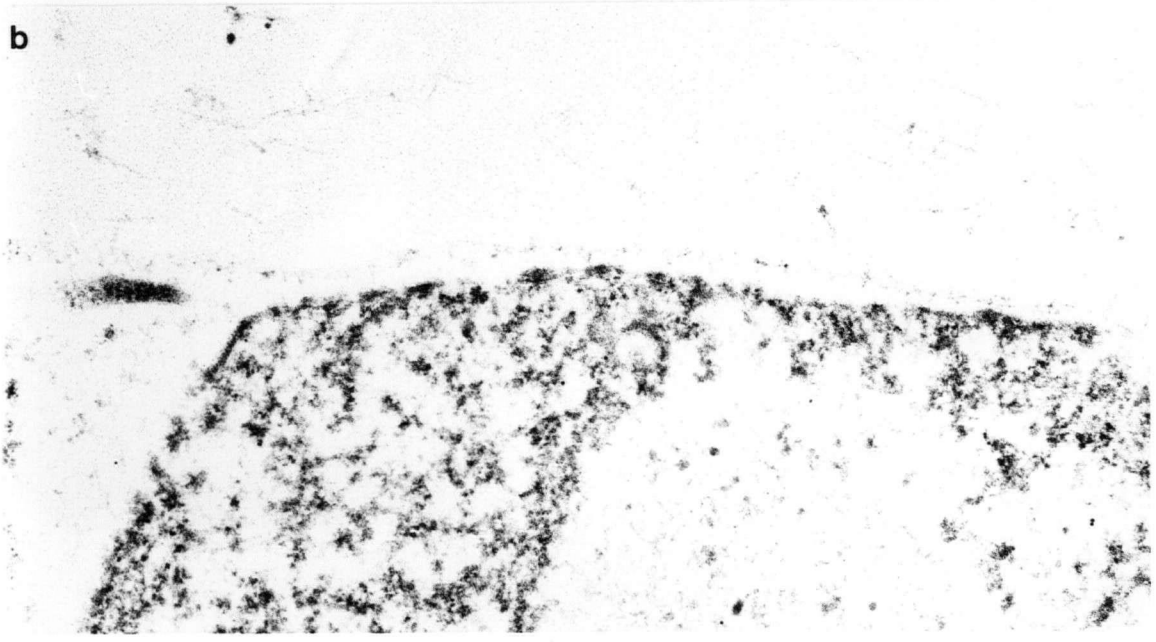
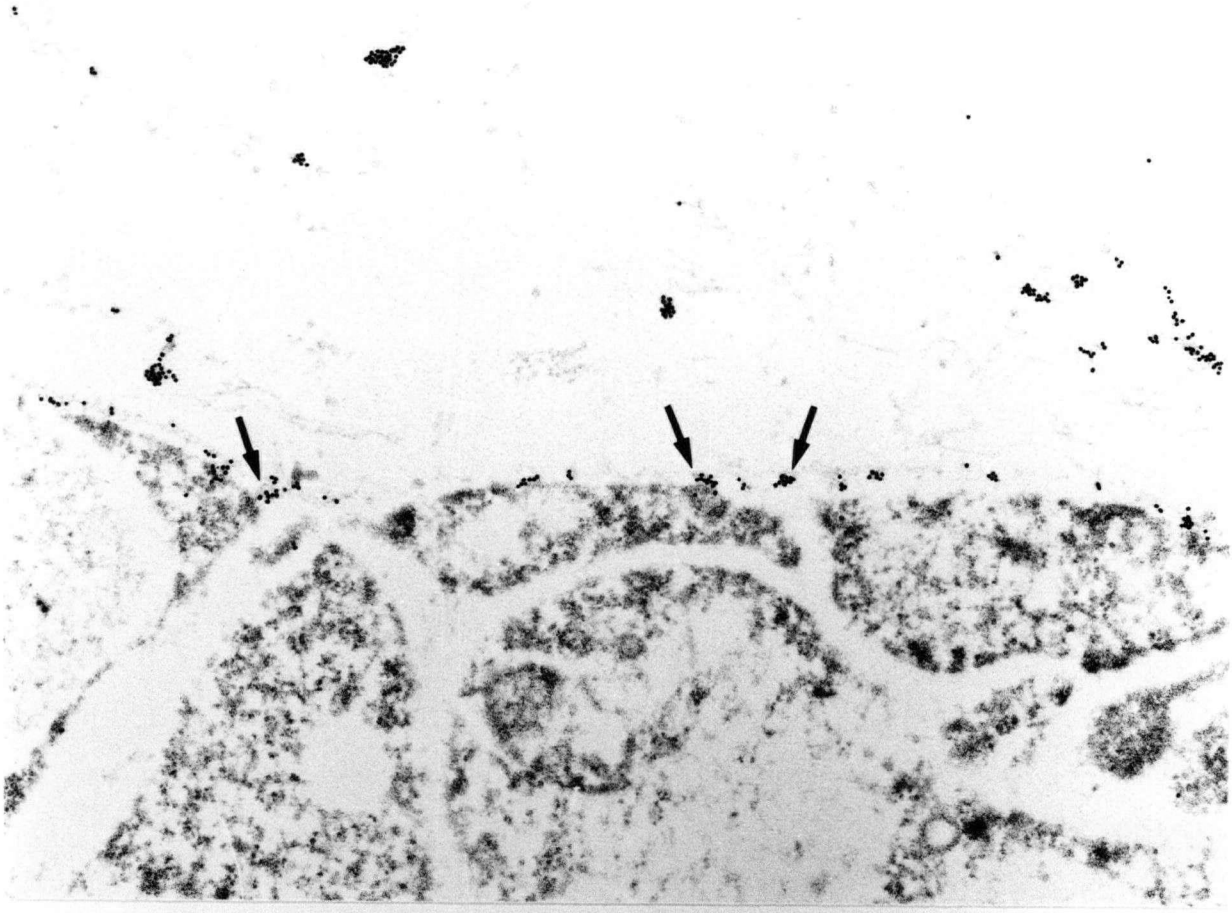


Fig. 15a: A TEM through the stomach region stained with Au_{25}-LFA, showing specific labelling over the BL and ECM. Labelling of the BL occurs predominantly over the lamina lucida (arrows); in the ECM, gold particles are over both the strands and amorphous regions. x37,800

Fig. 15b: A TEM through the esophagus stained with the control sugar/ conjugate solution of the above, 0.1M sialic acid/ Au_{25}-LFA. x35,700

15a



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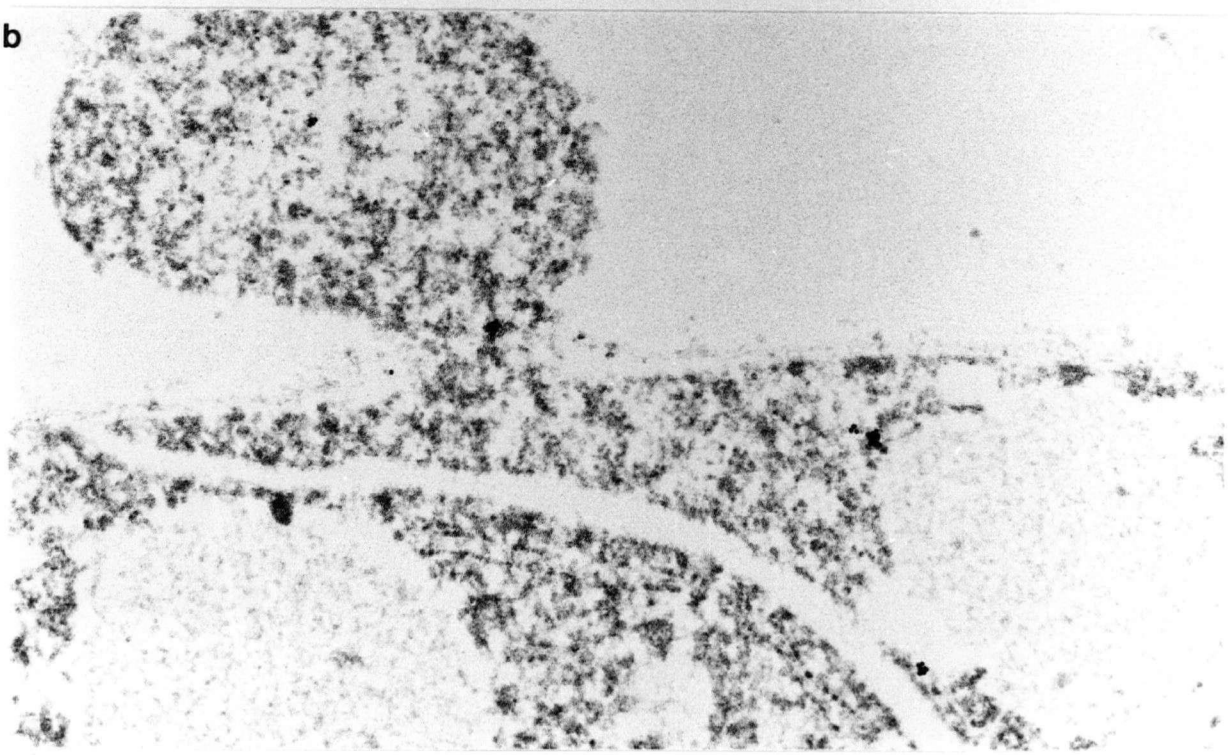
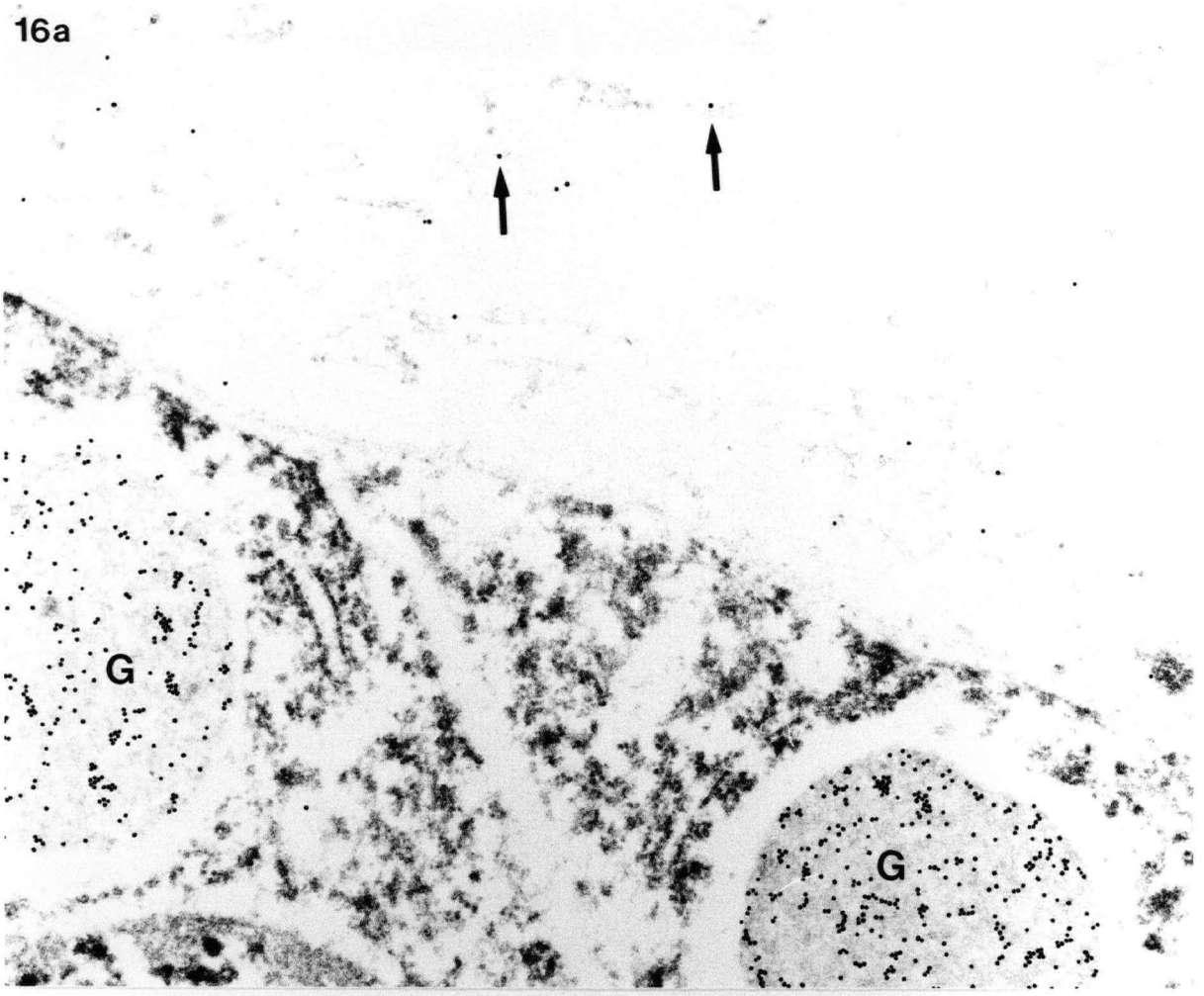


Fig. 16a: A TEM through the ectoderm stained with Au_{25}-WGA, showing scattered yet specific labelling of the ECM (arrows). Labelling is also observed over the two intracellular granules (G) shown in this section. Although the granules appear to differ in their electron density, they are both heavily labelled. x37,800

Fig. 16b: A TEM through the ectoderm stained with the control sugar/conjugate solution of the above, 0.1M glcNAc/Au_{25}-WGA. x38,500

16a



b

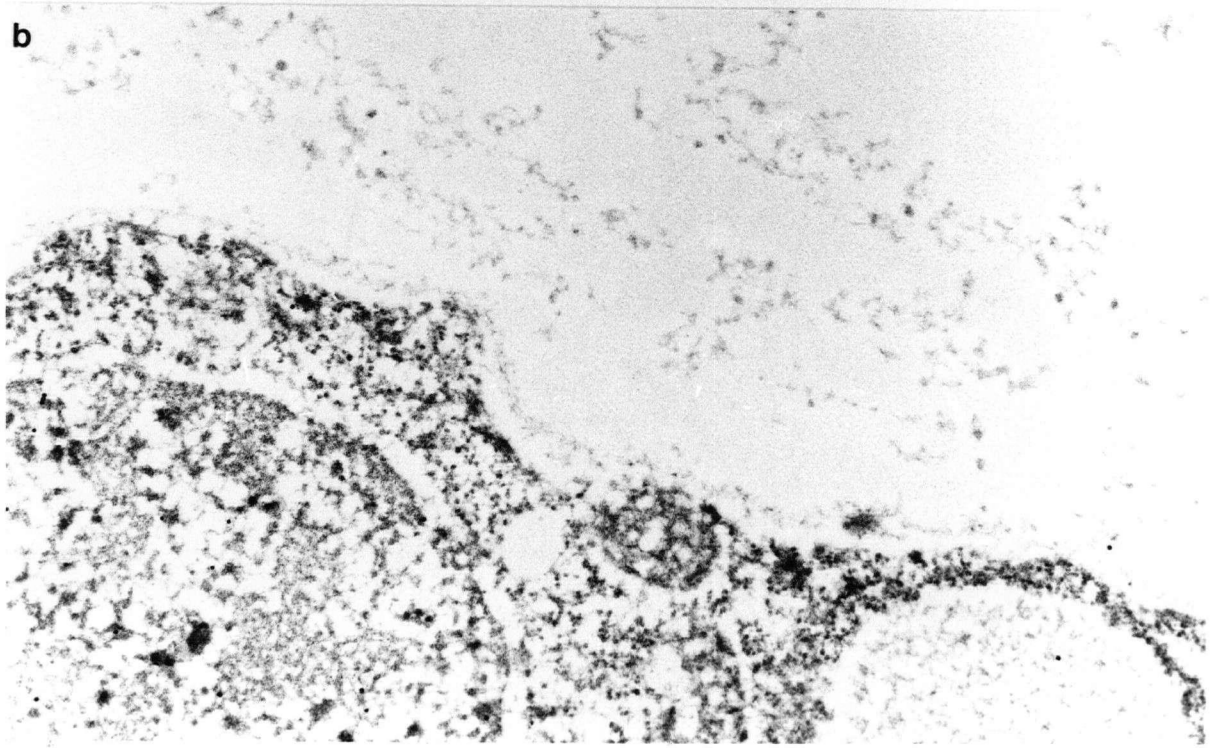
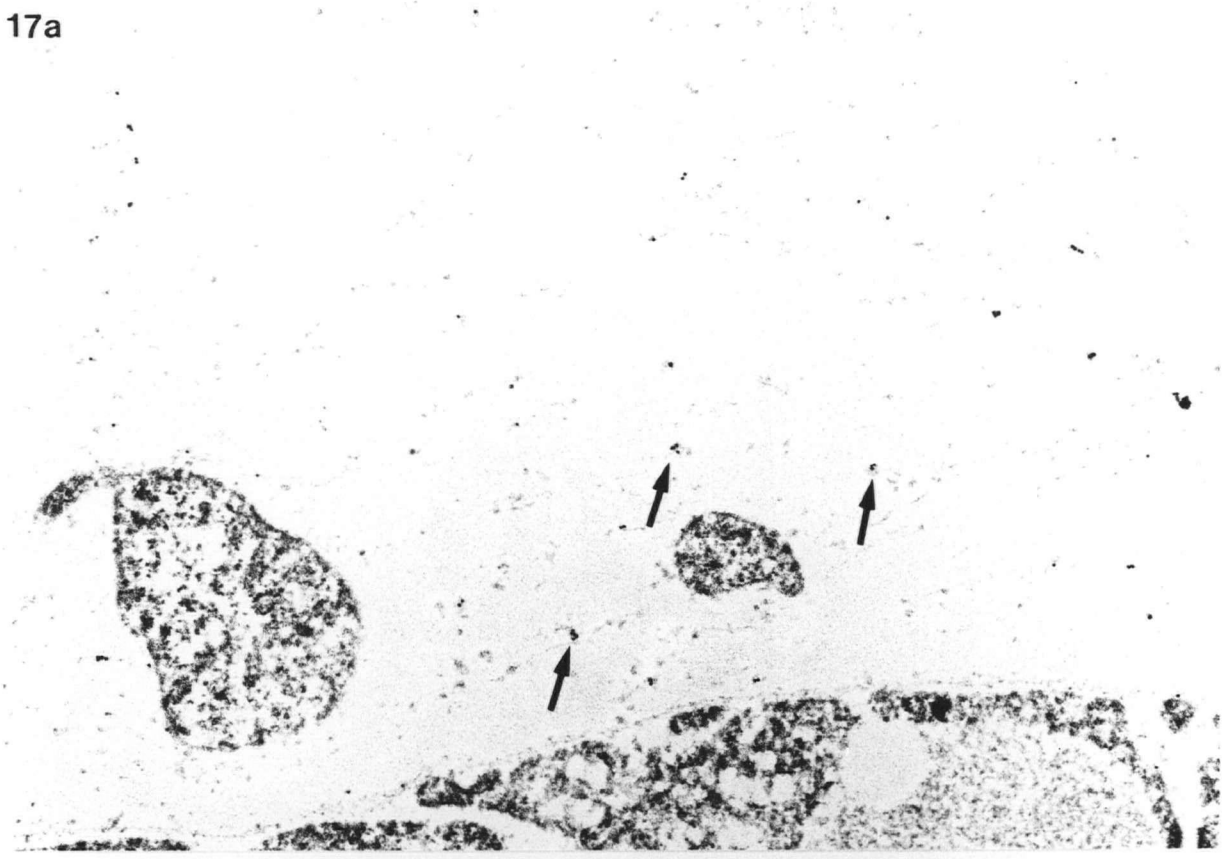


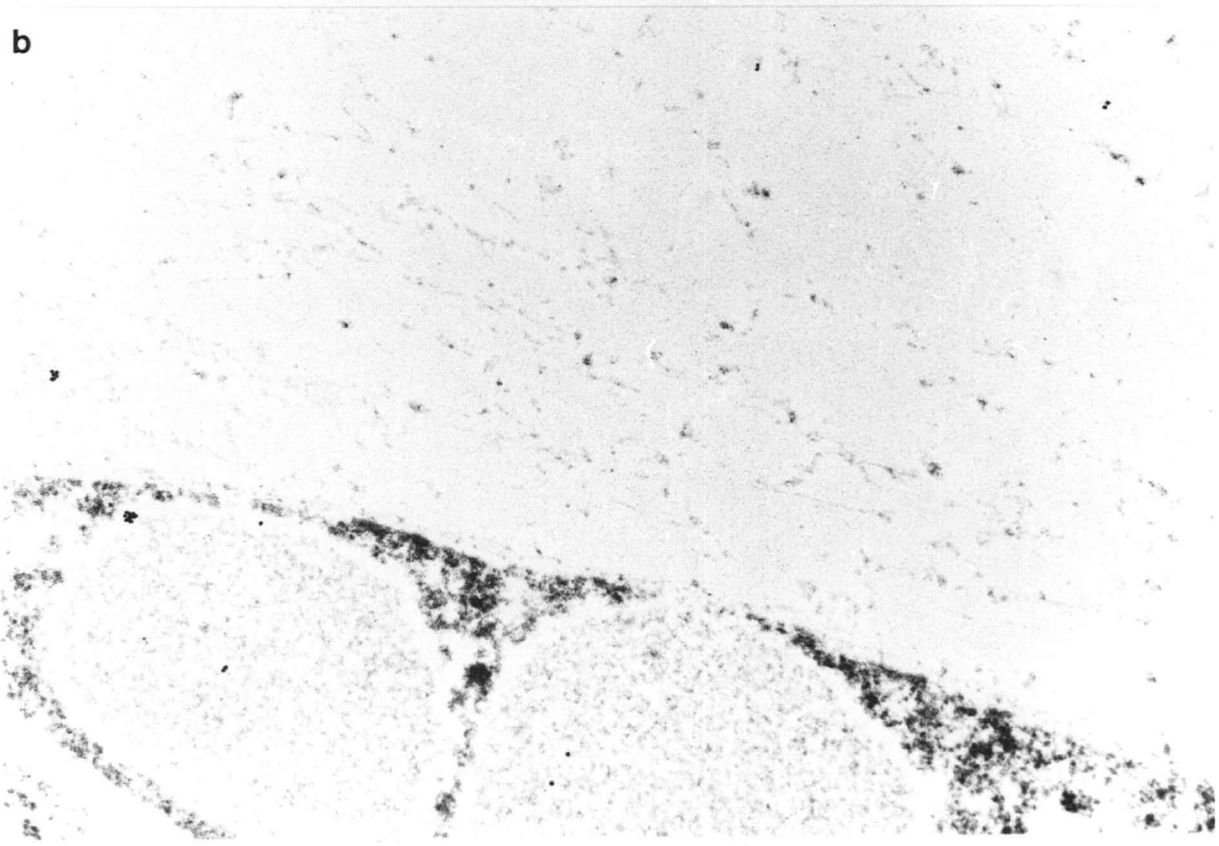
Fig. 17a: A TEM through the esophagus stained with Au_{25}-SBA, showing specific labelling over the amorphous regions of the ECM (arrows). x29,700

Fig. 17b: A TEM through the ectoderm stained with the control sugar/conjugate solution of the above, 0.1M galNAc/Au_{25}-SBA. x32,000

17a



b



A Regional Comparison of Lectin Labelling Over the Basal Lamina

A morphometric analysis of labelling over the basal lamina revealed that with 2 of the lectins, there were significant regional differences in the amount of labelling present over the basal lamina. With the lectins Con A and LFA, there was a reduction in the amount of label over the esophageal basal lamina when compared to the basal lamina underlying the stomach, intestine or ectoderm. This was determined by performing a 1-way analysis of variance test on the data obtained. With Con A, the total number of particles over the basal lamina of the esophagus, which averaged 45.5 particles/6.67μm linear BL, was significantly less ($p<0.001$) than the amount over the stomach, which averaged 80.8 particles/6.6μm linear BL, the intestine, which averaged 75 particles/6.6μm linear BL, and the ectoderm, which averaged 77 particles/6.6μm linear BL. These results are summarized in table 6.

TABLE 6: NUMBER OF GOLD PARTICLES BOUND/LENGTH OF BASAL LAMINA AFTER LABELLING WITH Au_{25}-Con A.
(Data are X ± MSE, with n in parenthesis)

REGION	GOLD PARTICLES/6.67mm BL
stomach	80.8 ± 1.68 (5)
→esophagus	→45.5 ± 3.32 (8)
ectoderm	77.0 ± 4.29 (4)
intestine	75.2 ± 1.60 (4)

A similar result was noted with LFA. In this case a morphometric analysis revealed that labelling over the esophageal basal lamina (169.0/3.3μm) was significantly less ($p<0.05$) than labelling over the ectodermal basal lamina (253.5/3.3μm). Although the amount of labelling over the basal lamina of the stomach (221.67/3.3μm) and intestinal (229.0/3.3μm) endoderm was less than that in the region of

the ectoderm, it did not differ significantly ($P<0.05$) from it. These results are listed in table 7.

TABLE 7: NUMBER OF GOLD PARTICLES BOUND/LENGTH OF BASAL LAMINA AFTER LABELLING WITH LFA.
(Data are X ± SE, with n in parenthesis)

REGION	GOLD PARTICLES/3.33mm BL
stomach	221.67 ± 7.77 (6)
→esophagus	→169.00 ± 11.88 (7)
ectoderm	253.50 ± 22.23 (6)
intestine	229.00 ± 14.66 (5)

After DBA labelling of the basal lamina, the total number of particles averaged ≃151/6.67μm linear BL. There was no significant difference ($p<0.05$) in average particle counts over the basal lamina of the esophagus, stomach, intestine and ectoderm with this lectin. These results are listed in table 8.

TABLE 8: NUMBER OF GOLD PARTICLES BOUND/LENGTH OF BASAL LAMINA AFTER LABELLING WITH DBA.
(Data are X ± SE, with n in parenthesis)

REGION	GOLD PARTICLES/6.67mm BL
stomach	157.89 ± 5.13 (9)
esophagus	161.78 ± 4.20 (9)
ectoderm	145.88 ± 6.89 (8)
intestine	140 00 ± 10.20 (4)

3.5 INTRACELLULAR GRANULES

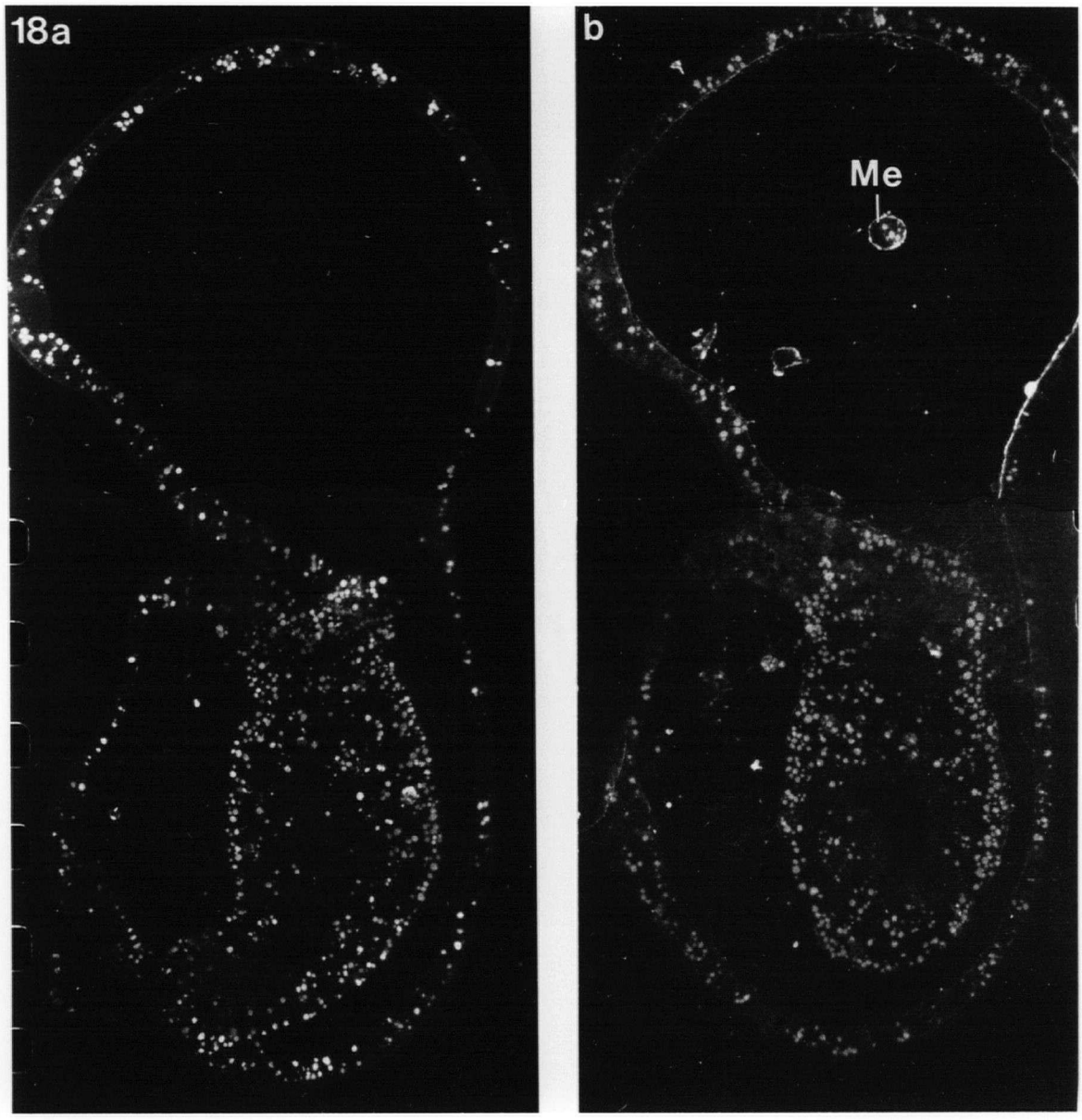
At least two different populations of granules were seen in cells fixed in glutaraldehyde with alcian blue. Under the TEM, one population stained more densely (Fig.14,16). Both the darker and lighter-staining granules appeared predominantly as spherical structures ranging in size from 0.5-3.0 μm in diameter. The two populations of granules were present in approximately equal numbers, and were distributed in all cell types of the embryo, including the epithelial cells of the ectoderm and endoderm, as well as the undifferentiated mesenchyme cells. However the cells of the endoderm appeared to have a greater number of granules that the ectodermal cells.

At both the LM and TEM level, only the lectins WGA and Con A, labelled the granules. At the LM level, Bouin's-fixed material gave optimal preservation of the granules, which were visualized as brightly-labelled structures in the cells of the ectoderm, endoderm and mesenchyme (Fig.18). FITC-WGA appeared to label the granules to a greater degree than did FITC-Con A, but aside from the intensity of the labelling, the pattern appeared to be similar with both lectins. The greatest number of labelled granules was seen in the endodermal cells of the esophagus, stomach and intestine. There were fewer granules in the cells of the ectoderm, and a greatly reduced number in the cells of the stomodeum.

Similarly, under the TEM, only the two lectins Con A (Fig.14) and WGA (Fig.16) labelled the granules in the cells of the ectoderm, endoderm and mesenchyme (Fig.18b). Gold particles were seen over both light and dark-staining granules, although labelling appeared to be heavier over the dark-staining granules.

Fig. 18a: A thin saggital section through a 6 day embryo fixed with Bouin's, and stained with FITC-WGA. Of particular interest is the specific labelling of the intracellular granules, which are distributed in the epithelium throughout the embryo. x480

Fig. 18b: A thin saggital section, as above, fixed with Bouin's, stained with FITC-Con A. The labelling of the intracellular granules is also noted, although it is not as intense as the WGA labelling. Note also the granules in the mesenchymal cell (Me). x480


18a
b
Me

3.6 AUTORADIOGRAPHY

^{3}H-Sugars Which Were Taken Up By The Embryo

Of the four ^{3}H-sugars placed in the sea water incubation medium (glucosamine, mannose, fucose, arabinose), only glucosamine was taken up by the embryos. The grain counts over cells of the embryos which were incubated in mannose, fucose, or arabinose did not exceed the background level, and it was thus concluded that the embryos did not take up these exogenous sugars.

Uptake by Glucosamine

After a 3 hour chase in "cold" sea water, most of the grains were located in the region of the stomach (Fig.19a). All of the stomach epithelial cells had grains over them, averaging 4-6 grains/cell (Fig.19b). This was not the case for other epithelial cells of the alimentary canal however; intestinal and esophageal cells had scattered labelling (Fig.19c,19d), some cells containing up to 2 grains/cell, but most others not having any. The material inside the alimentary canal, which is continuous with the hyaline layer, was also labelled (Fig.19b), whereas few, if any, grains were found over the hyaline layer. The ectodermal epithelium was similar to the intestine and esophagus, showing only a few scattered grains. Virtually no grains were observed over areas of ECM (Fig.19d). The control section showed a very low background level (Fig.19e).

Over the course of a 9 and 18 hour chase in cold sea water, there was little change observed in the distribution of glucosamine. The only change of any significance was in the intestinal epithelium, where an increase in the number of grains was observed, so that at 18 hours, most cells, as apposed to only some cells, had 1-2 grains over them.

These results are summarized in table 9.

TABLE 9: DISTRIBUTION OF ^{3}H-GLUCOSAMINE AFTER A 6 HR LABEL IN THE LATE GASTRULA EMBRYO OF THE STARFISH PISASTER OCHRACEUS

	EPITHELIAL REGIONS WHERE GRAINS COUNTED*				
CHASE TIME (HR)	ECTODERM	ESOPHAGUS	STOMACH	INTESTINE	ECM
3	+	+	+++	+	0
9	+	+	+++	+++	0
18	+	+	+++	++	0

*LEGEND:

+	=	1-2 grains/cell in some cells
++	=	2-4 grains/cell in most cells
+++	=	4-6 grains/cell in most cells
0	=	no grains

Fig. 19a: A saggital section of a 6 day embryo, processed for autoradiography. This embryo was incubated with ^{3}H-glucosamine for 4 hours, followed by a chase in cold sea water for 3 hours. At this low magnification, it is evident that the majority of grains are located in the stomach endothelial cells (S). Es=esophagus, In=intestine, Ec=ectoderm. x450

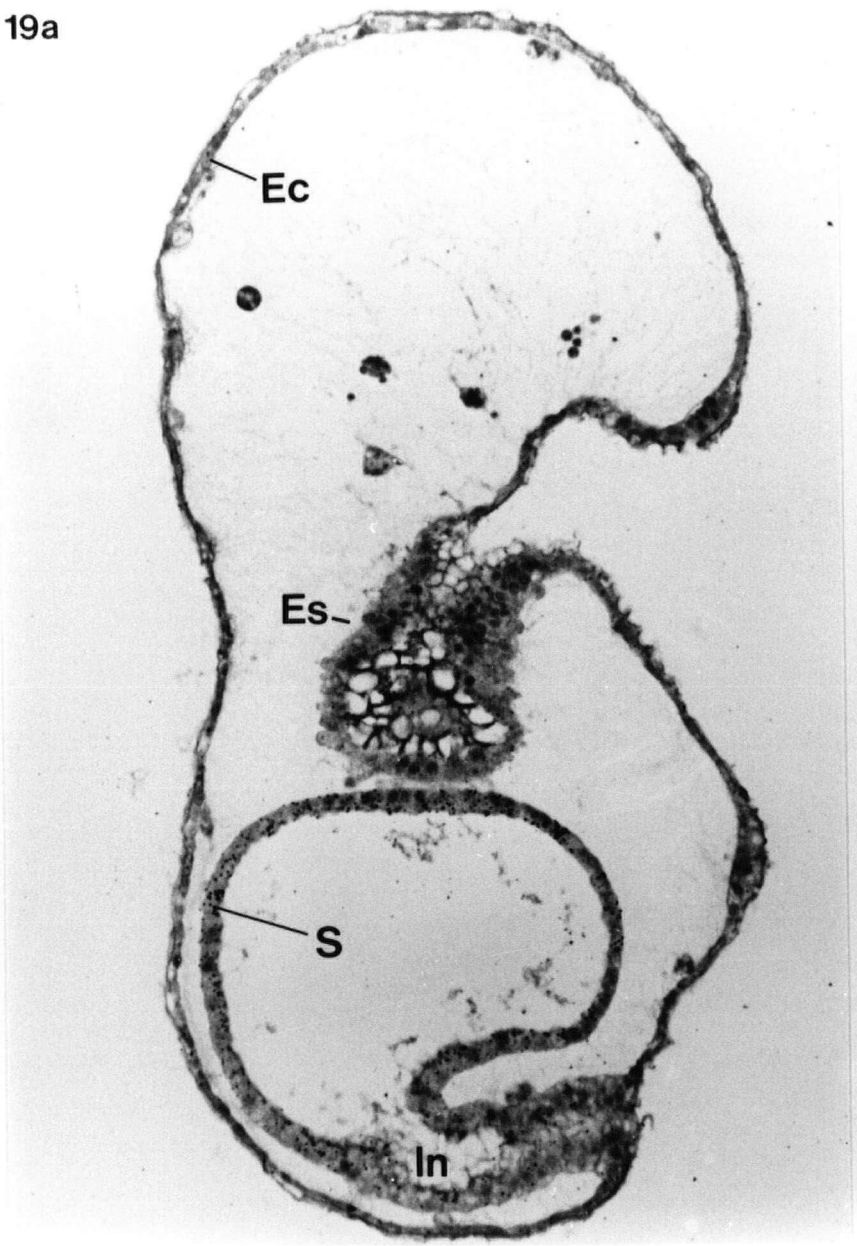
Fig. 19b: A higher magnification of Fig.19a, showing the stomach endothelial cells which are heavily labelled with grains (arrows), averaging 4-6 grains/cell. Grains are also seen over the material inside the lumen of the stomach (arrowheads). x1200

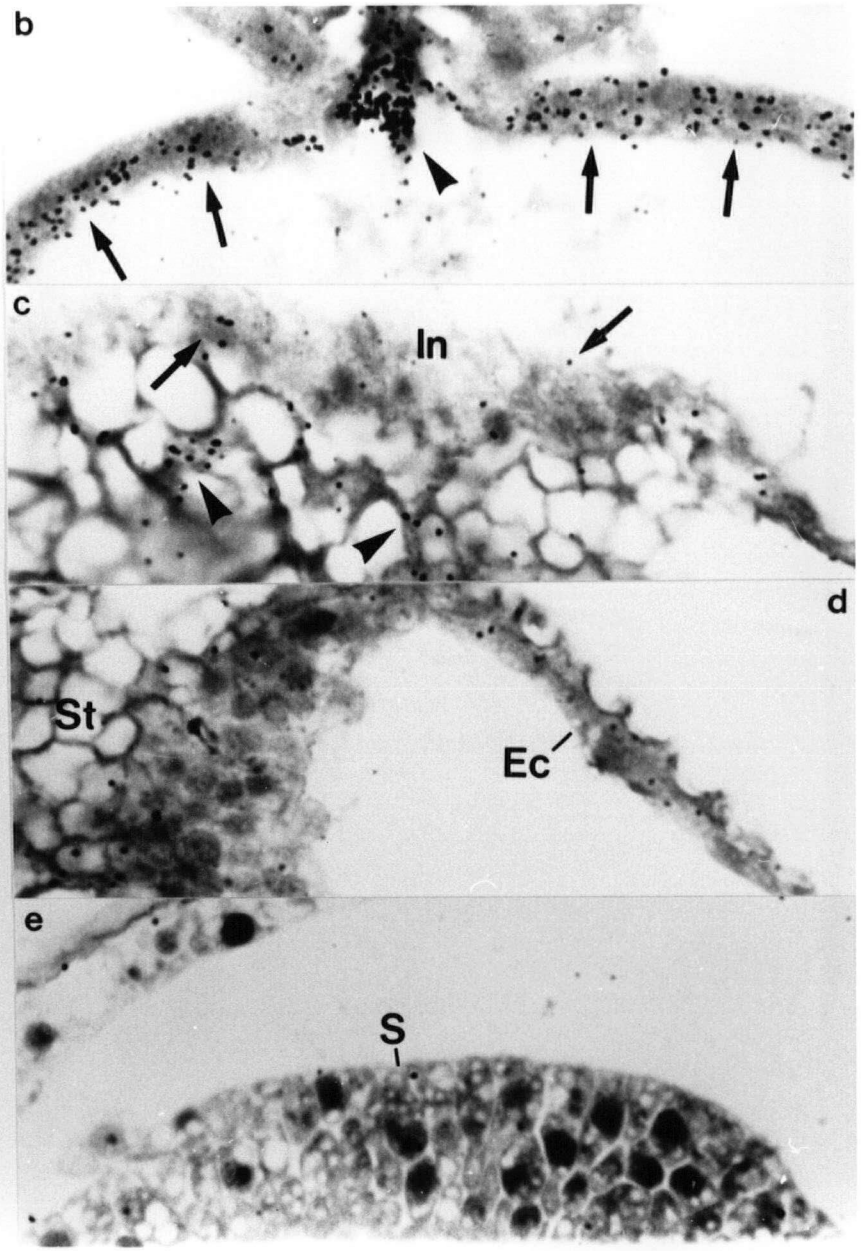
Fig. 19c: A higher magnification of Fig.19a, showing the intestinal (In) region of the embryo. Some scattered labelling of the cells is present (arrows), and as with the stomach lumen, the material inside this lumen shows substantial labelling (arrowheads). x1200

Fig. 19d: A higher manification of Fig.19a, showing a region of the stomodeum (St) and ventral ectoderm (Ec). Minimal labelling is observed over the cells, with only the occasional cell showing 1-2 grains. x1200

Fig. 19e: A control section through the stomach (S), showing very minimal background labelling. Control embryos were not incubated with ^{3}H-glucosamine, but were, thereafter, processed the same as were the experimental embyros. x1200

19a





4. CONCLUSIONS AND DISCUSSION

4.1 OPENING REMARKS

Before discussing in detail the specific labeling patterns of the lectins, a few general comments regarding lectin binding studies can be made. Some researchers approach the general area of lectin histochemistry with a great deal of skepticism, and unjustly so, for it is not the technical aspect of lectin histochemistry, but rather the interpretation of the results obtained with this technique, which is often at fault. As with any histochemical procedure, there is the danger that results may be misrepresented and/or misinterpreted. To avoid this, several important points must be kept in mind when obtaining information about tissue carbohydrates using lectin histochemistry. First, although a positive result implies the presence of a sugar, a negative result does not necessarily mean that the sugar is not there, since the sugar could simply be inaccessible to staining (i.e. as sugar located internally within a polysaccharide chain very often will not be detected by lectin histochemistry). Secondly, the appropriate controls must be run just as in any other histochemical procedure. The nonspecific interactions of lectins must be distinguished by sugar inhibition tests; for example, addition of galactose to the FITC-RCA conjugate should render the conjugate ineffective for staining, whereas the addition of mannose to the same conjugate should not change its labelling properties. Thirdly, of great importance is the problem of fixation-induced changes in the molecular arrangement of tissue glycoconjugates. As discussed below, some lectin-binding sites are consistently altered during fixation. However the use of several different fixations in the experimental protocol can serve to reduce

experimental error due to this variable.

In the present study, the above three points were taken into consideration before the data was interpreted: Controls in all cases were negative, suggesting that the reactions were relatively specific, and the use of a range of fixations ensured that misinterpretations of the results due to fixation-induced changes of tissue carbohydrates were minimized .

4.2 FIXATION

The mechanism responsible for the fixation of lectin receptor sites is very likely similar to that of carbohydrates in general, which is achieved through the cross-linking of protein components of complex glycoconjugates, rather than via the sugars themselves (Allison, 1987). Fixatives differ greatly in the way in which they cross-link proteins, and because of this, the binding of lectins is influenced strongly by the fixation method used. While some fixatives may cause configurational changes in the protein part of the glycoconjugate, making certain sugar residues no longer accessible to the lectins, others may expose new sugar residues for lectin binding. Although lectin-binding is influenced by the fixation method used, some lectins are more sensitive to the mode of fixation than others. For example, in the present study, RCA and SBA labelled only those tissues fixed with ethanol and glutaraldehyde, whereas Con A and WGA were less sensitive to the fixation method used, and labelled tissues fixed in ethanol, Bouin's, glutaraldehyde and paraformaldehyde.

Ethanol is considered to be among the best of fixatives for LM lectin binding studies. It is thought to crosslink proteins loosely, which probably leaves more sites exposed in the glycoconjugates to

accomodate binding, particularly by those lectins with multiple binding sites (Rittman & Mackenzie, 1983; Allison, 1987). These predictions were bourne out by the present study, where fixation with ethanol gave the best staining results for FITC-RCA, FITC-SBA and FITC-Con A. There is a problem in using ethanol as a fixative, however, in that cell morphology is poorly preserved. Characteristically, the cytoplasm shrinks and may pile up against one side of the cell opposite the side at which the fixative enters; nuclei may also be misshapen. This became a problem in the present study in that the BM and ECM were displaced to the center of the embyro after fixaton with ethanol. However, the hyaline layer (the ECM on the outer surface of the ectoderm), appeared to be preserved optimally with this fixation.

Although it is not true for all lectins, the decreased staining of tissue structures with several lectins after fixation in paraformaldehyde is a common observation (Allison, 1987; Malmi & Soderstrom, 1988). Formaldehyde reacts with proteins in several different ways, the most important being the reaction with uncharged amino groups in amino acids, which condense with amide or other groups to yield methylene cross-bridges between proteins (Pearse, 1960). The cross bridging is thought to cause conformational changes in the sugar- containing molecules which could block access to the FITC-conjugated lectins and thus reduce or elimate staining. While embryonic asteroid tissues fixed in 95% ethanol exhibit strong labelling patterns with the lectin conjugates FITC-RCA and FITC-SBA, labelling is weak or non-existant on tissue fixed in paraformaldehyde. The decreased labelling is probably due to formaldehyde induced configurational changes of the glycoproteins of the type described above, which mask the terminal sugar residues.

CPC selectively precipitates polyanionic molecules, and when added to paraformaldehyde, it improves the retention of carbohydrate containing compounds (Williams & Jackson, 1956). CPC, while effective with the LM, has not found widespread use at the ultrastructural level, since it results in poor ultrastructural preservation and tends to leave contaminating deposits (Sanders, 1986a). Work on asteroid embryos confirmed these observations. After addition of the detergent compound CPC to paraformaldehyde, a tremendous increase in labelling is observed with the lectins FITC-Con A and FITC-WGA. Unfortunately however, as with vertebrate tissues described above (Sanders, 1986a), ultrastructural preservation is very poor. Attempts to overcome this by adding CPC to the post fixative are unsuccessfull, as no appreciable difference between material fixed in this manner from that in which CPC is used in the primary fixative is observed.

Another fixative used in these studies was Bouin's fixative, which gives better morphological preservation than ethanol. Although the mechanism of fixation by Bouin's is not entirely understood, one of the main components, picric acid, is known to react with proteins to form picrates. In this study, Bouin's fixed material demonstrated strong labelling of the intracellular granules with FITC-Con A and FITC-WGA, granules which do not label clearly in paraformaldehyde/CPC-fixed tissue. This difference in fixation is probably due to the fact that Bouin's, like ethanol, does not change the tertiary configuration of the molecules as extensively as does paraformaldehyde.

Glutaraldehyde is a 5-carbon straight chain dialdehyde with a molecular weight of 100. It is used extensively as a fixative in electron microscopy because of its ability to preserve ultrastructure. The mechanism of glutaraldehyde fixation appears to be very similar to

that of formaldehyde (Chambers et al., 1968). At pH 7.0 or above, the main reaction product is an imine which forms between a free aldehyde group and an amino group (such as the ε-amino group of a lysine). Alcian blue was first used as a fixative with glutaraldehyde by Leak (1967) to preserve carbohydrates. Although the mechanism of alcian blue/glutaraldehyde fixation is not yet established, it is highly probable that the alcian blue, a cationic dye derived from copper pthalocyanin, reacts with various negatively charged acidic carbohydrates (Mowry, 1963), and probably forms a salt linkage (Scott et al., 1964). The mechanism of formation of the electron-dense labelling is completely unknown. When osmium tetroxide is not used in the fixative, it is thought that the copper moiety of the cationic dye is implicated in the electron opacity (Rothman, 1969). When osmium is used in the fixation, it has been suggested that an osmiophilic dye-mucosubstance complex is formed, which accounts for the electron opacity (Behnke and Zelander, 1970). Glutaraldehyde/alcian blue is the fixative of choice in the present TEM studies because it gives moderately good ultrastructural preservation of the tissue coupled with excellent preservation of the basal lamina (BL) and ECM, while still allowing the gold-conjugated lectins to bind to them.

Although tissues preserved in the presence of alcian blue allow labelling with gold-conjugated lectins at the ultrastructural level, FITC-conjugated lectins do not label with material preserved in alcian blue/paraformaldehyde. The reason for this is presently unknown, but it is probably due to a combination of factors, including paraformaldehyde induced changes in the tertiary structure of glycoconjugates discussed above, as well as the blocking of terminal sugars with alcian blue, leading to steric hindrance of the FITC-conjugated lectins.

4.3 THE NATURE OF LECTIN-BINDING SITES

In contrast to antibody-antigen histochemistry, where binding of a certain antibody immediately uncovers the location of a specific molecule, the results of lectin histochemistry are much more difficult to interpret because of the diverse nature of glycoconjugates in tissues. Two major kinds of glycoconjugates present in the extracellular matrix are glycoproteins and proteoglycans. The carbohydrate containing component of proteoglycans are the glycosaminoglycans (GAGs), of which there are four major classes: hyaluronic acid (HA), chondroitin sulfate (CS), keratin sulfate (KS) and heparan sulfate (HS). Chondroitin sulfate is divided into chondroitin-4-sulfate (CS4), chondroitin-6-sulfate (CS6), and dermatan sulfate (DS); the latter contains iduronate residues in place of D-glucuronic acid. In general, the structure of GAGs includes repeating disaccharides units, with a characteristic oligosaccharide linkage region that contains glucuronic acid, galactose and xylose, where the GAGs are attached to the core protein (Hascall & Kimura, 1982).

GAGs represent the most probable lectin binding sites in tissues since their carbohydrate content is so high, due to the repeating sugar units intrinsic in their structure. WGA, a glcNAc-binding lectin, therefore tends to bind to HA because HA has in its structure, repeating units of glcNAc. Likewise, SBA, a galNAc-binding lectin, would bind to CS and DS, since they both contain alternating units of galNAc. On the other hand, PNA and RCA are galactose-binding lectins, and should therefore bind strongly to keratan sulphate which contains this sugar. Although, to the best of my knowledge, no one has yet tested the binding capaities of PNA and RCA for these GAGs, Gallager (1986a) has indeed shown that commercially available HA and CS bind WGA and SBA

respectively. The proposed lectin binding specificities to GAGs are summarized below in tabular form.

TABLE 10: LECTIN-BINDING SPECIFICITIES FOR GAGs

LECTIN	SUGAR	GAG
WGA	glucuronate-ß1,3-glcNAcß1,4	Hyaluronic acid
SBA	glucuronate-ß1,3-galNAc-ß1,4	Chondroitin sulfate
SBA	iduronate-ß1,3-galNAc-ß1,4	Dermatan sulfate
PNA,RCA	glcNAc-ß1,3gal-ß1,4	Keratan sulfate

While GAGs provide prospective binding sites for the glcNAc-binding, galactose and galNAc-binding lectins, they do not represent potential binding sites for the mannose/glucose-binding lectins, (eg. Con A), nor the lectins which bind the sialic acids (eg. LFA). There are other glycoconjugates, however, which may be likely candidates for lectin binding sites. Studies have shown that lectins will also bind to the sugar residues located in glycoproteins, such as fibronectin and laminin, as well as to collagen IV (Kefalides, 1970; Yamada, 1981; Timpl & Martin, 1982). Laminin, a large glycoprotein of MW 1,000,000, has the configuration of an asymmetric "cross", with one long arm (77nm), and 3 identicle short arms (37nm) containing globular regions on the end of each arm when viewed under the electron microscope, (Rao et al., 1983). It has been hypothesized that the 4-arm structure of laminin is of biological importance enabling the molecules to "reach out" and interact with multiple cellular and matrix components in different directions (Hay, 1981). Lectins which have been shown to bind laminin isolated from mouse Engelbreth-Holm-Swarm (EHS) tumor

include RCA, WGA and Con A. RCA binds to a galactose residue on the end globular domains located on the short arms of laminin, while WGA and Con A bind to glcNAc and α-D-mannopyranosyl residues thought to be located on the arms themselves (Rao, et al; 1983). Of the latter two, Con A binds more strongly to the arms.

Collagen is another glycoprotein which contains possible lectin binding sites. The lectins Con A, WGA, sWGA (succinylated WGA, which has a specificity for glcNAc only, whereas WGA binds to both glcNAc and sialic acid, Monsigny, 1979), and GSA-II (a lectin specific for CH_3-α-D-galactose) have been shown to bind to Collagen type I isolated from human skin or uterine ligaments (Soderstrom, 1987). The density of sugars in glycoproteins is relatively low when compared to that found in proteoglycans, and therefore lectins would be expected to bind to the glycoproteins (i.e. laminin, fibronectin, collagen) much more weakly that to GAGs. It does appear likely, however, that Con A is binding some variety of glycoprotein, since no known GAG contains mannose/glucose residues.

Limax flavus agglutinin (LFA) is a sialic acid-binding lectin which binds to N-acetyl-neuraminic acid (Neu5Ac), as well as to the hydroxylated form, N-glycoloyl-neuraminic acid (Neu5Gc), although binding to the former is stronger (Miller et al., 1982). It is known that glycoproteins and glycolipids of cell surfaces are often sialated, with most of the sialic acid residues occupying terminal positions of oligosaccharide chains (Corfield & Schauer, 1982). However, it is not known what the manner of integration of these sialic acids into the BL and ECM is.

It is worth mentioning here a note regarding the binding characteristics of Dolichos biflorus agglutinin. Although DBA is

another galNAc-binding lectin (Holthofer, 1983; Ribera et al., 1987), Baker et al. (1983) have shown that DBA binds 20 times more strongly to the oligosaccharide containing two galNAc-linked units (galNAc-α1,-3galNAc), than to the monosaccharide with only one galNAc unit. Since in the present study, DBA did not label the same sites as the galactose or galNAc-binding lectins, but instead exhibited a strong labelling pattern which was unique, it is suggested that DBA bound oligosaccharides containing two galNAc-linked units, and that it did not bind oligosaccharides with only a single terminal galNAc unit, as did the lectin, SBA.

The following table summarizes the most probable lectin binding sites based on the above-mentioned evidence.

TABLE 11: PROPOSED LECTIN BINDING SITES

LECTIN	LECTIN BINDING SITE
Con A	oligosaccharides containing mannose/glucose, possible including 1^{o} laminin and 2^{o} collagen
LFA	1^{o} Neu5Ac, 2^{o} Neu5Gc
DBA	galNAcα1,3galNAc-containing oligosaccharide
WGA	1^{o} HA, 2^{o} collagen
PNA,RCA	1^{o} KS, 2^{o} laminin, collagen
SBA	1^{o} CS, DS

4.4 LECTIN BINDING TO THE BASAL LAMINA AND ECM OF P. OCHRACEUS

Description of the BL & ECM

The embryonic asteroid BL as described by Crawford (1988) is similar to that described in vertebrate embyros (Hay, 1978; Sanders, 1986a). It consists of a lamina densa which is separated from the basal cellular membrane by a lamina lucida. The use of alcian blue in the fixative reveals that the lamina densa consists of a fine felt-work of intermediately stained fibers with a coarse meshwork of thick, densely stained and thinner, intermediately stained strands embedded in the inner aspect (the side adjacent to the blastocoel). The latter material is identical in appearance with and connects to the ECM located in the blastocoel. Tubule-like structures which originate in the dense material associated with the lamina densa, cross this structure and the adjacent lamina lucida and attach to the basal plasmalemma of the epithelial cells, thus anchoring the ECM of the blastocoel to them. The ECM consists of strands approximately 40nm in width, with irregularly shaped amorphous regions arranged along the strands. These amorphous regions range from 50 to 200 nm in diameter. Extensive biochemical and immunohistochemical studies have not been carried out on the embryonic asteroid BL to date. However studies on embryonic BL of the echinoids, another echinoderm group, have demonstrated the presence of collagen, fibronectin, laminin and HSPG (Spiegel, et al., 1983; Wessel, et al., 1984; Wessel & McClay, 1987), suggesting that these proteoglycans and glycoproteins may also be present in the asteroid basal lamina.

Distribution of Carbohydrates in the BL & ECM

Based on the lectin binding studies of the BL, we now have an understanding of the types of sugar moities that are present in the BL and ECM. We also can gain an appreciation of the distribution of these sugars throughout various regions of the embryo. We can, however, only speculate on the nature of the glycoconjugates in which these saccharides are incorporated.

To begin with, both the FITC-lectin and the Au_{25}-lectin binding studies revealed binding of Con A, WGA, LFA, and DBA to the embryonic asteroid BL, suggesting it is rich in glycoconjugates containing mannose and/or glucose moities, sialic acids (Neu5Ac, Neu5Gc), and oligosaccharides having two galNAc-linked units (galNAc-α1,3galNAc). The distribution of the labelling in the BL is not entirely uniform. The majority of Con A labelling is found over the lamina densa; although there is some over lamina lucida, no labelling is found over the associated ECM fibers. Staining with DBA shows a similar pattern, accept that some labelling is present over the associated ECM fibers. The staining pattern with LFA is somewhat different, in that the majority of labelling is over the lamina lucida, and not the lamina densa. There is a lesser degree of labelling over the lamina densa, and no labelling over the associated ECM fibers.

Lectin-binding studies of the ECM in this study have revealed that the ECM labels with Con A, DBA, SBA WGA and LFA. Con A, DBA and SBA label the irregularly shaped amorphous regions exclusively, while WGA and LFA label both these amorphous regions as well as the strands of ECM. While Con A and SBA give moderate labelling of the amorphous regions indicating the presence of mannose/glucose and galNAc residues, very strong labelling over the amorphous regions is seen with DBA

indicating a large amount of the disaccharide galNAc-galNAc is present in these regions. The labelling observed with LFA is similar to that of WGA, in that gold particles are distributed over both amorphous areas and the strands of ECM, indicating the presence of a glycoconjugates containing sialic acids and glcNAc in these regions.

As discussed in section 4.3 of this chapter, the most probable glycoconjugates representing the lectin binding sites include: laminin and collagen (Con A), Neu5Ac and Neu5Gc (LFA), hyaluronic acid and collagen (WGA), and galNAcα1,3galNAc-containing oligosaccharides (DBA). Immunohistochemical work on echinoid ECM has demonstrated the presence of collagen, laminin, fibronectin, and proteoglycans in the BL and ECM (Pucci-Minafra et al., 1972; Oguri & Yamagat, 1978; Spiegel et al., 1980, 1983; Katow et al., 1982; Wessel et al., 1984). Since this species is so closely related to asteroidia, it would seem probable that glycoconjugates such as those present in the echinoid are present also in the developing asteroid.

Although ultrastructural studies of this nature are rare, LM studies using FITC labelled Con A and WGA have been reported with echinoid embryos (DeSimone & Spiegel, 1986). In this case, both Con A and WGA were found to bind to the ECM in the blastocoel. In addition, some of these lectin-binding proteins have been isolated from the ECM in the sea urchin embryos, and have been characterized to some extent. Desimone & Spiegel (1986) have found one WGA-binding protein of MW 125,000, as well as three Con A-binding proteins of MW 29,000, 34,000, and 37,000. In these studies, the expression of the above appeared to be related to developmental stages, and as well, are sensitive to agents known to interfere with the synthesis of GAGs and glycoproteins.

Whereas studies utilizing ^{3}H-glucosamine can provide information

concerning the distribution and accumulation of newly synthesized GAGs (Bernfield & Banerjee, 1972), in this study, the ^{3}H-glucosamine which was taken up by the cells of the GI tract was not used as precursers for glycoconjugates in the ECM or BL, and remained predominanly in the cells. There was, however, some labelling of the extracellular material lining the GI tract.

The labelling of the storage granules appears to indicate the presence of glcNAc and mannose/glucose residues. It is unclear whether these moieties function as simple metabolites, i.e., as building blocks for complex glycoconjugates, or if they are already part of complex glycoconjugates. Both of these options are possible, since, for example, galactose, mannose, glcNAc and galNAc can all be converted to glucose for metabolism, but at the same time, can also be incorporated directly/indirectly into glycoconjugates.

4.5 IMPLICATIONS OF THE RESULTS AS THEY RELATE TO DEVELOPMENT

The distribution of lectin binding sites in the BL throughout different regions of the embryo, as revealed by the ultrastructural labelling study (Au_{25}-lectin) are particularly interesting in light of the morphogenetic event being studied. While both Con A and WGA bind to the ectodermal and endodermal BM rather uniformly at the LM level, TEM studies followed by a morphometric study confirm that there is less labelling over the area of the esophagus with two of the lectins, Con A and LFA. The total number of Con A-bound gold particles over the BL of the esophagus which averaged 45.5 particles/6.67μm linear BL, is significantly less ($p<0.001$) than that over the stomach, which averaged 80.8 particles/6.6μm linear BL, the intestine, which averaged 75 particles/6.6μm linear BL, and the ectoderm, which averaged 77

particles/6.6μm linear BL.

Similarly, Au_{25}-LFA was found to bind less intensely to the esophageal BL than to the ectodermal BL. The total number of particles over the BL of the ectoderm averaged 253.5/3.3μm, while that over the esophagus averaged 169.0/3.3μm. Although the amount of labelling over the BL of the stomach (221.67/3.3μm) and intestinal (229.0/3.3μm) endoderm was less than that in the region of the ectoderm, it did not differ significantly ($P<0.05$) from it.

These results suggest that the BL underlying the esophageal endoderm has a decreased quantity of carbohydrates, as revealed by the staining patterns of Con A and LFA. As mentioned previously, molecules bound by the lectins are unknown. However, based on the extrapolations of molecules found in vertebrate ECM's, it seems possible that this could represent a decrease in the amount of laminin, collagen and mucopolysaccharides containing sialic acids. These lectin binding studies appear to complement previous observations by Crawford (1988) that the endodermal BL underlying the esophagus is less alcianophilic and therefore "thinner" in structure than the BL of the ectoderm. Gallagher (1986b) noted a similar phenomena during the branching morphogenesis of the chick lung. In her study, the lectins WGA, SBA and RCA all stained the BM of the developing chick lungs, and furthermore, thinning of the BM at the tips of newly formed bronchi was visualized with all three lectins, and was particularly evident with SBA. Using ruthenium red and tannic acid to stain the BL for TEM studies, she observed a thinning and sometimes discontinuous BL at the tips of the buds as compared with the more substantial BL in the interbud areas. She hypothesized that the decreased amount of labelling with lectins and cationic dyes at the active site of growth represented a necessary

condition for epithelio-mesenchymal interactions to occur.

In the present study, the difference in the properties of the esophageal BL as compared to the BL in other regions of the embryo could represent a developmentally significant event. During this stage in P. ocraceus development, mesenchyme cells are gathering at the esophagus prior to differentiating into muscle. Although there are mesenchymal cells in other areas of the embryo at this time, only the ones which settle at the esophagus and only on the esophagus differentiate into muscle cells. The end result is an interlocking of muscle cells forming a smooth muscle sheath around the esophageal endoderm.

The distinct localization of one population of the mesenchyme cells to this region suggests that there is some signal to the cells telling them "stop here". Since the initial interaction of the mesenchyme cells appears to be with the esophageal BL, a BL which appears to differ both morphologically and biochemically from those around it, it is possible that the stop signal is localized in the esophagel BL. Indeed, the restriction of movement of these cells to the esophageal BL may be due to the decrease in the molecule(s) associated with the mannose and sialic acid residues.

One further interesting observation stems from the fact that following migration but prior to overt expression of differentiation, the mesenchymal cells in this area develop processes which appear to break through the BL and make contact with the epithelial cells of the esophagus. It is possible that the unique nature of the BL underlying the esophageal endoderm is a necessary prerequisite, first for this interaction between undifferentiated mesenchymal cells and esophageal endodermal cells to occur, and secondly, for subsequent muscle differentiation to occur.

Although there appears to be a logical sequence of events surrounding the thinning of the esophageal BL, several questions still remain unanswered: Firstly, does the BL in this region loose carbohydrates as a genetically preprogrammed event, or is it deficient in these and perhaps other molecules because it is the last area of the BL to form and therefore may not have yet incorportated its full complement of glyconjugates into the BL? Secondly, is this relative lack of certain sugar containing molecules a requirement in order to restrict the presumptive muscle cells to the esophagus? Thirdly, is interaction between the mesenchymal cells and the esophageal endoderm an absolute requirement for muscle differention? And, fourthly, is continued contact between the mesenchymal and epithelial cells required for their complete differentiation, or is one contact enough?

Further work must be done to examine not only the molecular nature of the BL and ECM, but the distribution of the different lectin binding sites in the ECM throughout various regions of the embyro, as was done with the BL. In addition, a more detailed analysis of the components in the BL which are present in reduced quantities could be attempted by isolating the lectin binding fractions, further characterizing them, and perhaps raising monoclonal antibodies to purified fractions. By isolating lectin binding glycoproteins and GAGs, and plating mesenchymal cells on them, the various fractions can be examined for their ability to promote migration. Following this, characterization of the active fractions could eventually bring to light some of the factors involved in regulating cell migration during morphogenesis.

5. THE HYALINE LAYER: ANOTHER EXTRACELLULAR MATRIX

5.1 INTRODUCTION

The hyaline layer (HL), is an extracellular layer of material which surrounds the embryos and larvae of several classes of echinoderms (eg, asteroids, holothuroids, ophiuroids and echinoids Crawford & Abed, 1986) from fertilization to metamorphosis, and is thought to be the indispensable substratum for the overall stereotypic organization of the developing embryo (Dan, 1960; Schroeder, 1988). The HL is formed when, at fertilization, the cortical granules fuse with the plasma membrane of the egg and release their contents (Kane & Hersh, 1959; Endo, 1961; Runnstrom, 1966; Anderson, 1968). The surface of the egg is, thereafter, firmly attached to the HL by means of cell surface microvilli (Dan, 1960; Wolpert & Mercer, 1963). Its function appears to be associated with maintaining cell-cell interactions. Herbst (1900) found that in the absence of Ca^{2+}, the sea urchin HL was sluffed off, and subsequently, the embryo was easily dissociable. More recently, the HL has been described as an adhesive substrate for cells (Dan, 1960) or as an ECM (Spiegel & Spiegel, 1971) providing an attachment site for cells during morphogenesis (Gustafson & Wolpert 1967). Dan (1960) has commented on the mutual arrangement of the blastomeres at the blastula stage which is maintained by their attachment to the HL, and suggests that the HL is similar in function to an epithelial basement membrane. Wolpert and Mercer (1963) have confirmed Dans's observations and have suggested that the attachment of cells to the HL via plasma membrane microvilli maintains a radial polarity in the developing embryo until blastula formation. In this instance, it appears that cell attachment to the shell-like HL fixes the relative positions of blastomeres by

indirectly interlinking them, which forces the old surface (i.e. the only surface with microvilli remaining) to face outwards, thereby defining the cell's apical poles (Schroeder, 1988). This association between microvilli and the HL thus integrates the entire embryo, at least during the early stages of development before the appearance of intercellular juctions, by automatically aligning the blastomeres with the radial axes of the whole embryo.

In sea urchin embryos, the HL has been extensively characterized biochemically. The major component of the HL is the glycoprotein hyalin, a Ca^{2+} requiring protein (Yazaki, 1968; Kane & Stephens, 1969) ranging in MW from 95,000 to 300,000 in different species (Hylander & Summers, 1982). Immunofluorescent studies of sea urchin embryos have revealed that the protein hyalin is confined to the outer regions of the HL. Several other proteins have been isolated from the HL in addition to hyalin. These include two glycoproteins with molecular weights of 175,000 and 145,000, which Hall & Vacquier (1982) have isolated and characterized, and three other major proteins with molecular weights of 110,000, 70,000, and 50,000, which McCarthy & Spiegel (1983) have isolated. More recently, Alliegro et al. (1988) have isolated the glycoprotein echinonectin from the HL, which they refer to as an embryonic substrate adhesion protein, because of its adhesive properties with dissociated embryonic cells.

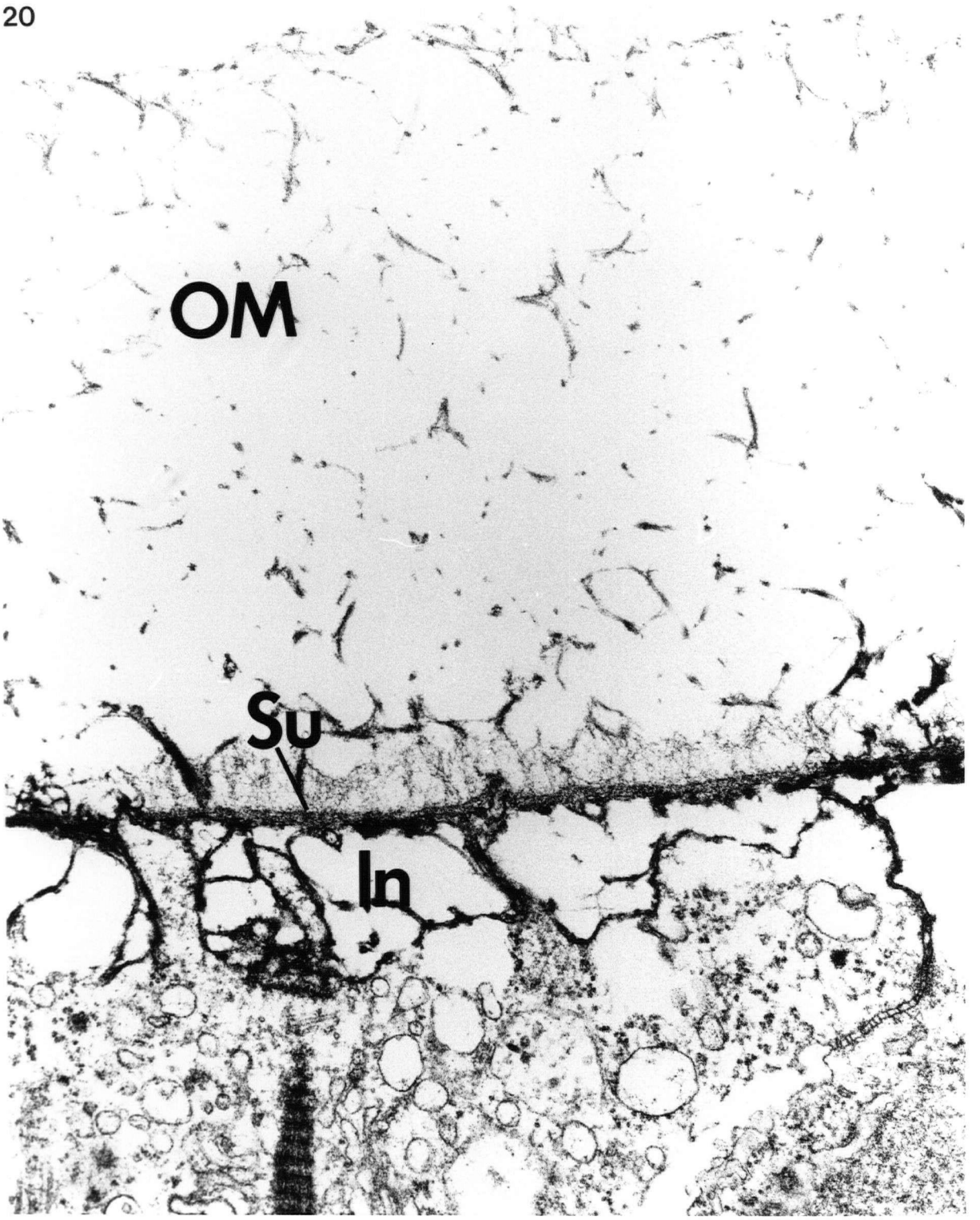
In addition to these proteins isolated from the HL, antibodies to several vertebrate antigens including collagen, fibronectin, laminin and HSPG have cross-reacted with antigens in the HL (Speigel & Speigel, 1979; Spiegel et al., 1979; Wessel et al., 1984)).

The HL in asteroids has not been extensively investigated biochemically; however it has been described morphologically, and

appears to closely resemble the echinoid HL (Crawford & Abed, 1986). After fixation in the presence of the anionic dye alcian blue, it is seen to consist of at least three layers: an intervillus layer located between the microvilli, a supporting layer, attached to the microvillus tips, which is equivalent to the supporting layer described in echinoids (Wolpert & Mercer, 1963; Lundgren, 1973), and an outer boundary layer composed of a coarse meshwork of ECM (Fig.20). At present, it is unclear what role these different morphological regions have in the proposed function of the HL as a substrate for cell attachment, a kind of pseudo BL.

Fig. 20: A TEM of an ultrathin section through the ectodermal epithelium of a 4 day embryo fixed with glutaraldehyde/ alcian blue, and processed for conventional TEM. The three regions of the HL are shown, including the intervillus layer (In), the supporting layer (Su), and the course outer meshwork (OM). x41,200.

20



The objective of this study was to examine the macromolecular nature of the HL and its different regions using lectins as probes for carbohydrates at both the LM and the TEM level. Lectin labelling at the electron microscopic level was essential in order to show the labelling patterns in the different regions of the HL which were visualized after fixation in the presence of Alcian blue, namely the intervillus layer, the supporting layer and the coarse outer meshwork. In this way, it could be determined if these morphological differences represent biochemical (macromolecular) ones. Such information should prove useful in elucidating the functions of the HL and may give clues to the functions of each of these morphologically different regions.

The materials and methods used in this study were the same as those used for the lectin histochemistry of the BL and ECM, which have already been described in chapter 2.

5.2 RESULTS

FITC-Lectin Labelling of the Hyaline Layer

The HL labelled with lectins from 4 out of 6 of the major monosaccharide-binding groups, including the glucose/mannose-binding group (Con A), the galNAc-binding group (SBA), the galactose-binding group (RCA, PNA), and the glcNAc-binding group (WGA). UEA, a lectin representing the fucose-binding group, did not bind, nor did LFA, representing the sialic acid-binding group. Most of the lectin-binding sites appeared to be preserved best with ethanol; however WGA-binding sites were most evident after fixation with PF/CPC. Because of the location of the HL on the outer-most side of the embryo, it was subjected to a degree of mechanical manipulation during the processing of the tissue. This meant that in some areas of the embryo, part of the

HL may have been torn away and lost, which may have accounted for the slight variations in labelling intensities of the HL in different regions of the embyro. Alternately, this may have been due to different planes of section in sections of embryos that were not exactly sagittal. A result of this type is seen in Fig.21a, where the HL showed strong labelling with FITC-Con A in most parts of the embryo, but weaker labelling in other parts. The ECM material equivalent to the HL which lines the alimentary canal gave patchy staining with Con A in the stomach and intestine of the ethanol fixed material, while staining was intense in the esophageal region (Fig.21a).

After exposure of paraformaldehyde/CPC-fixed tissue, HL labelled very intensely with FITC-WGA (Fig.22a). As with the Con A, there was some variability in the intensity of labelling over different regions of the embryo. There was almost no labelling of the ECM lining the stomach or intestine, although some regions of the ECM lining the esophagus labelled heavily (Fig.22a).

The following figures (21a-24b) are fluorescence micrographs showing the FITC-lectin-staining patterns of the HL in 6 day embryos. The embryos were embedded in JB4, and sectioned (1μ) prior to staining.

Fig. 21a: A sagittal section through an ethanol-fixed embyro stained with FITC-Con A, showing the heavily labelled HL (arrows) directly apposed to the ectodermal epithelium. Of interest also is the heavy labelling of the ECM material lining the esophagus (Es), and to a lesser degree the stomach (S) and intestine (In) (arrowheads). x480

Fig. 21b: A serial section of the above stained with the control sugar/conjugate solution (mannose/FITC-Con A); faint labelling of the HL is observed in some areas but it is drastically reduced from that seen in Fig. 21a. x480

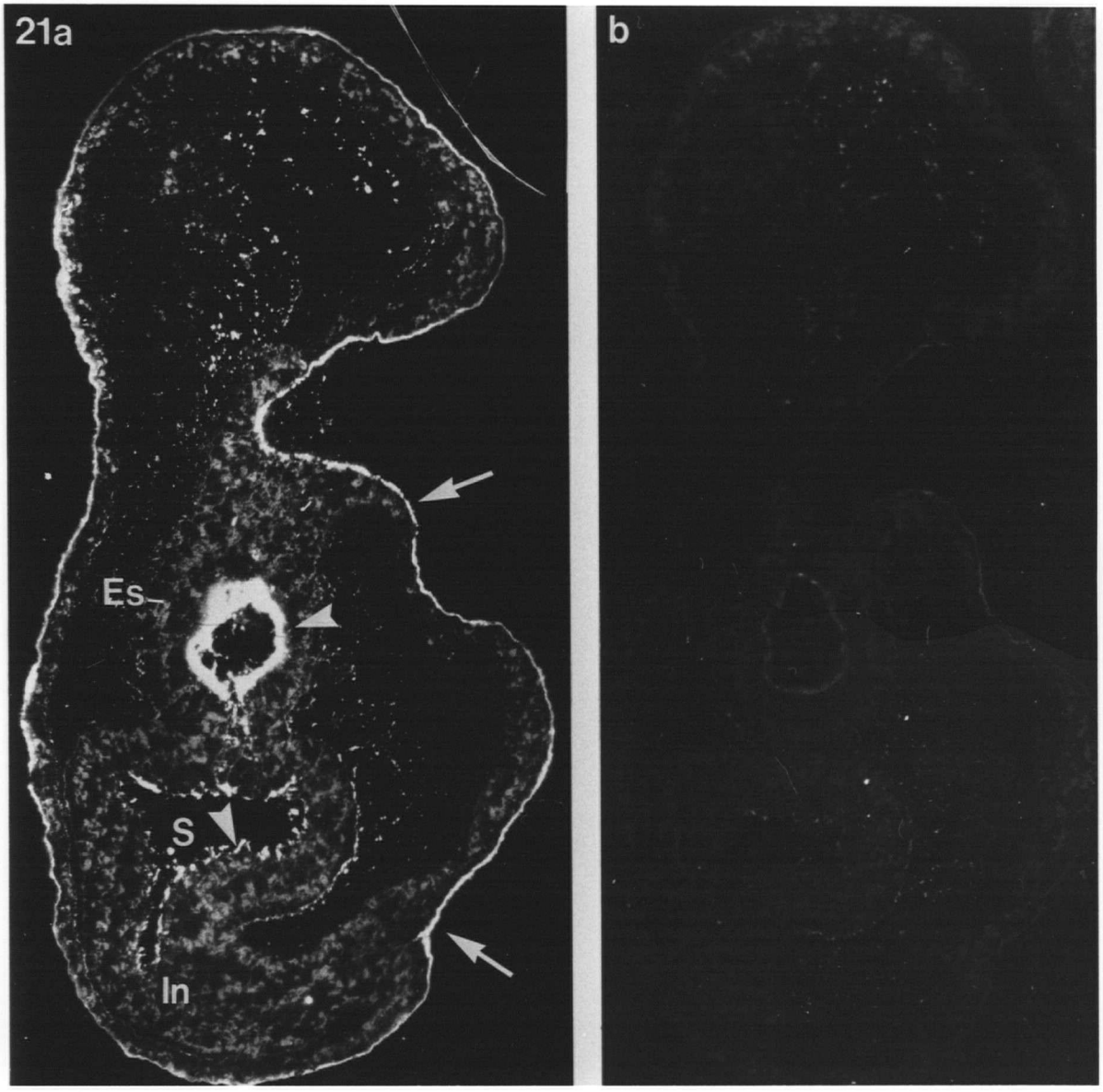
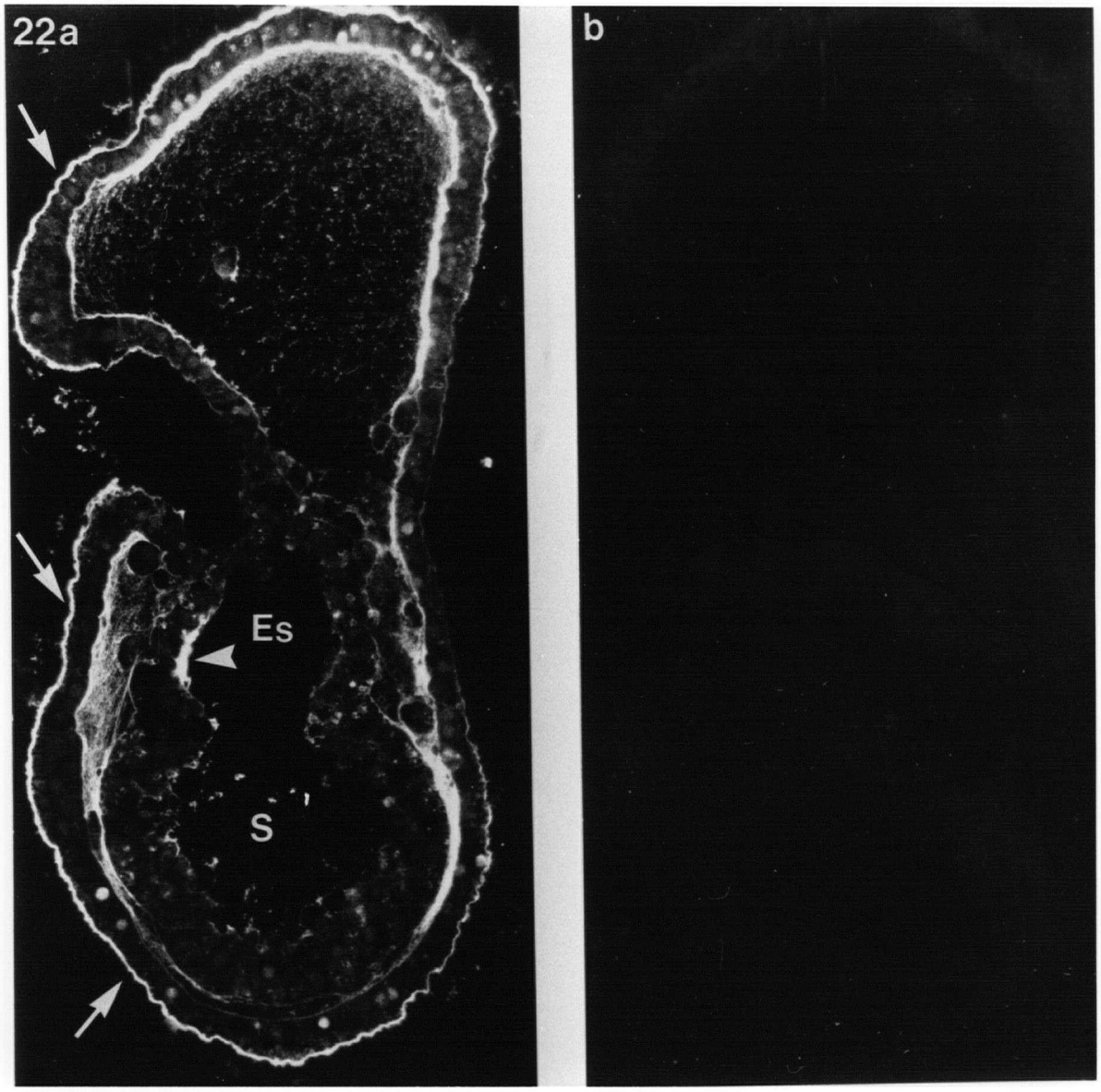

21a
b
Es
S
In

Fig. 22a: A sagittal section through a PF/CPC-fixed embryo which has been stained with FITC-WGA. Labelling of the HL is continuous and very intense (arrows). Some labelling of the ECM material lining the esophagus is also noted (arrowheads). Es=esophagus, S=Stomach. x480

Fig. 22b: A serial section of the above stained with the control sugar/conjugate solution (glcNAc/FITC-WGA) showing negative labelling of the HL. x480


22a
b
Es
S

SBA and RCA both labelled the HL in ethanol-fixed tissue. Labelling with SBA was discontinuous, and appeared as periodic densities around the embryo (Fig.23a). Unlike the other lectins which appeared to label the entire HL, RCA labelled only the innermost part of the HL (Fig.24b). With both SBA and RCA, there was little or no labelling of the ECM lining the stomach or intestine, although the ECM lining the esophagus labelled heavily with SBA (Fig.23a), and lightly with RCA (Fig.24a). Intense labelling of the mucous plug located at the junction between the esophagus and stomodeum was also noted with SBA (Fig.23a).

Control stains consisting of sugar/lectin conjugate solutions were performed on serial sections, and in all cases, labelling was negative (Figures 21b-24b).

Fig. 23a: A sagital section through an ethanol-fixed embryo, stained with FITC-SBA. The labelling of the HL is very intense, and has the appearance of periodic densities along the length of the HL (arrows). The ECM material lining the inside of the esophagus (Es) labels very strongly, as well as does the mucus plug, which is located just outside the stomodeum (arrowheads). Some labelling of the ECM lining the inside of the stomach (S) and intestine (In) is also evident. x480

Fig. 23b: A serial section of the above stained with the control sugar/conjugate solution (galNAc/FITC-SBA) showing no labelling of the HL. x480

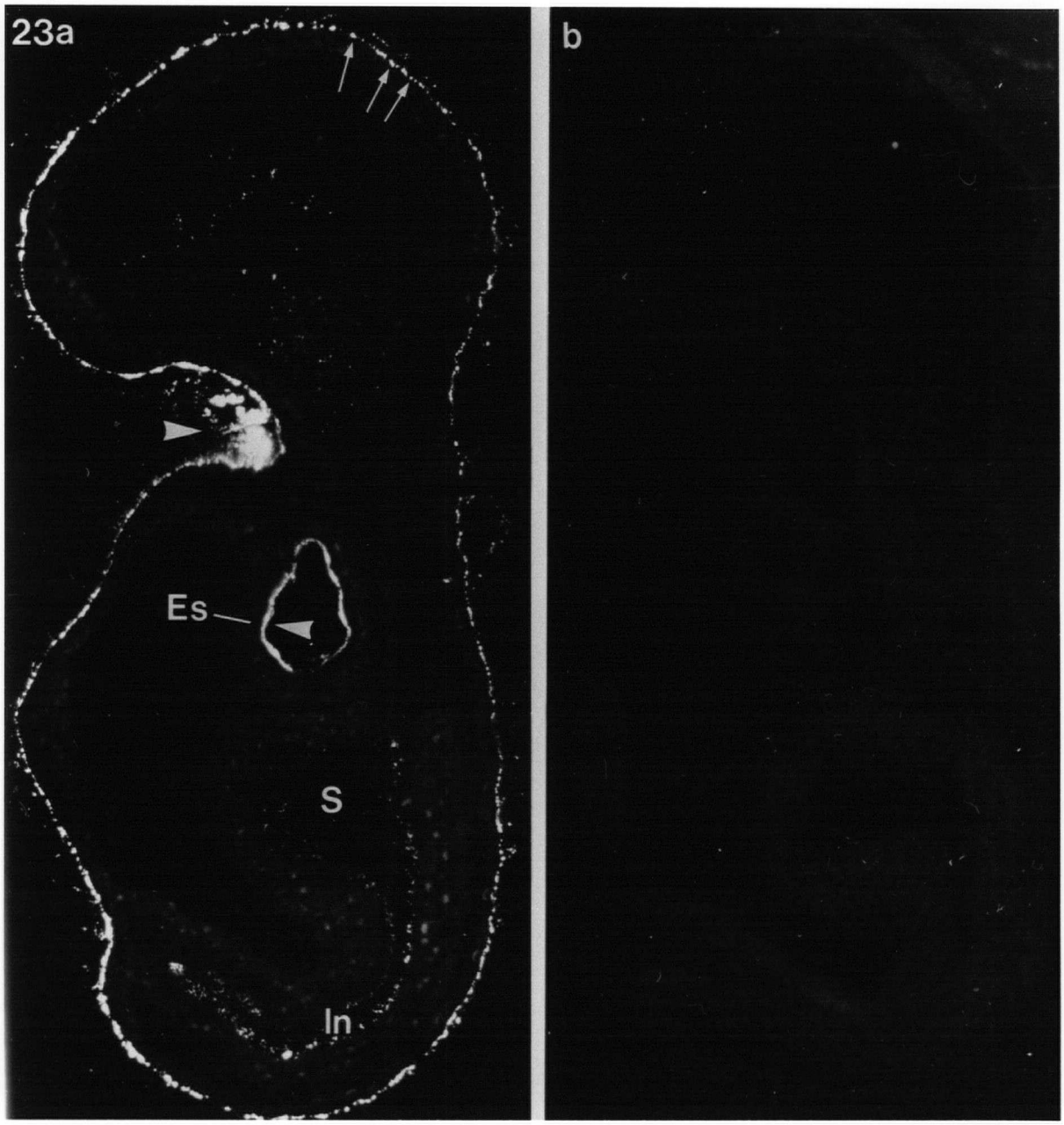
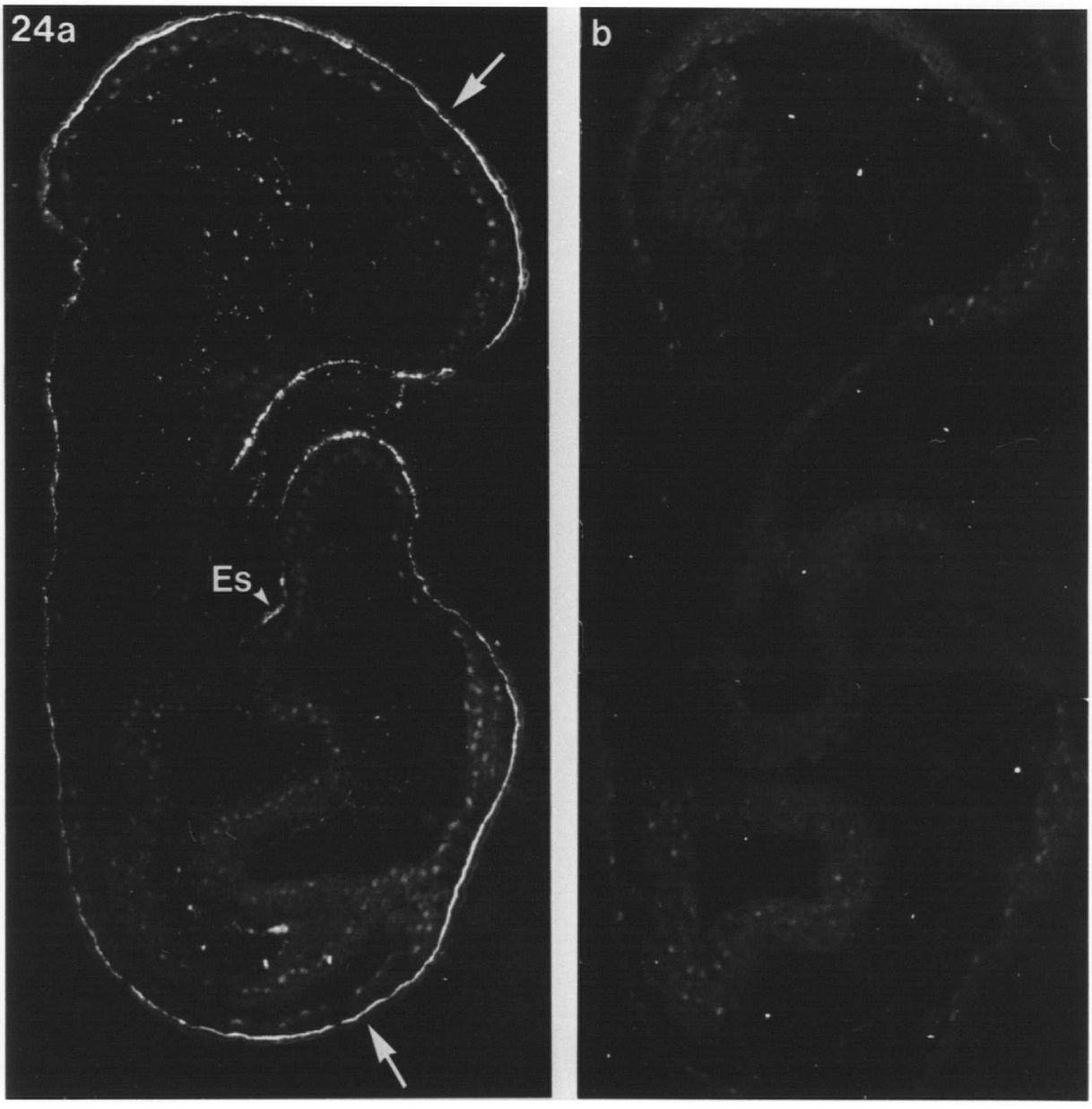

23a
b
Es
S
In

Fig. 24a: A sagittal section through an ethanol-fixed embryo, stained with FITC-RCA. Heavy labelling of the HL is observed (arrows), and appears to label the innner region of the HL, and not the outer region. Some labelling of the ECM lining the esophagus (Es) is also faintly present (arrowheads). x480

Fig. 24b: A serial section of the above stained with the control sugar/conjugate solution (galactose/FITC-RCA) showing no labelling of the HL. x480


24a
Es
b

$\underline{Au_{25}}$-Lectin Labelling of the Hyaline Layer

Gold particles conjugated to SBA, a galNAc-binding lectin, were located over the intervillus, supporting and outer meshwork regions (Fig.25a). In addition, all of the ECM material lining the alimentary canal also labelled with SBA-Au_{25}.

Con A, representing the mannose/glucose-binding lectins, did not label the HL heavily, as it did at the light microscopic level when conjugated to FITC. The somewhat scattered labelling was, however, above the level of background labelling, and most frequently was located over the supporting layer (Fig.26a,26b). Very few gold particles were located over the ECM material lining the alimentary canal, after staining with this gold lectin conjugate.

The 2 lectins WGA and PNA, representing the glcNAc-binding and galactose-binding group of lectins respectively, had very similar labelling patterns in the HL. Unlike SBA, which labelled all regions of the HL, labelling with WGA and PNA was almost exclusively limited to the supporting layer. (Fig.27a,28a). Whereas PNA labelling was also found over the ECM material lining the alimentary canal, WGA labelling was negligible over the same region.

The labelling patterns of the Au_{25}-lectins in the HL are summarized in table 12.

TABLE 12: Au_{25}-LECTIN LABELLING* OF THE HYALINE LAYER: A REGIONAL BREAKDOWN

	REGIONS OF THE HYALINE LAYER		
LECTIN	INTERVILLUS	SUPPORTING	OUTER MESHWORK
WGA	+	++	+
PNA	+	++	/
SBA	++	++	++
Con A	/	+	/

*LEGEND

++ = heavy labelling
\+ = moderate labelling
/ = little or no labelling

Control stains (0.1M blocking sugar/Au_{25}-lectins conjugates) were performed on serial sections, and with all the lectins, labelling was negative (Fig.25b,26b,27b,28b).

Fig. 25a: A TEM through the ectodermal region of the embryo, showing the hyaline layer on the outer side of the epithelium. This section was stained with Au_{25}-SBA, and shows heavy labelling of the intervillus (In), supporting (Su) and outer meshwork (OM) regions of the HL. x29,400

Fig. 25b: A TEM as above, stained with the control sugar/conjugate solution (galNAc/Au_{25}-SBA), showing very low background labelling over the HL. x31,500

Fig. 26a: A TEM through the ectodermal region of the embryo, showing the hyaline layer which surrounds the ectodermal epithelium. This section was stained with Au_{25}-Con A, and shows moderate labelling of the HL, predominantly over the supporting layer (arrows), but also over the other regions. x34,000

Fig. 26b: A TEM as above, stained with the control sugar/conjugate solution (mannose/Au_{25}-Con A), showing very little background labelling. x26,000

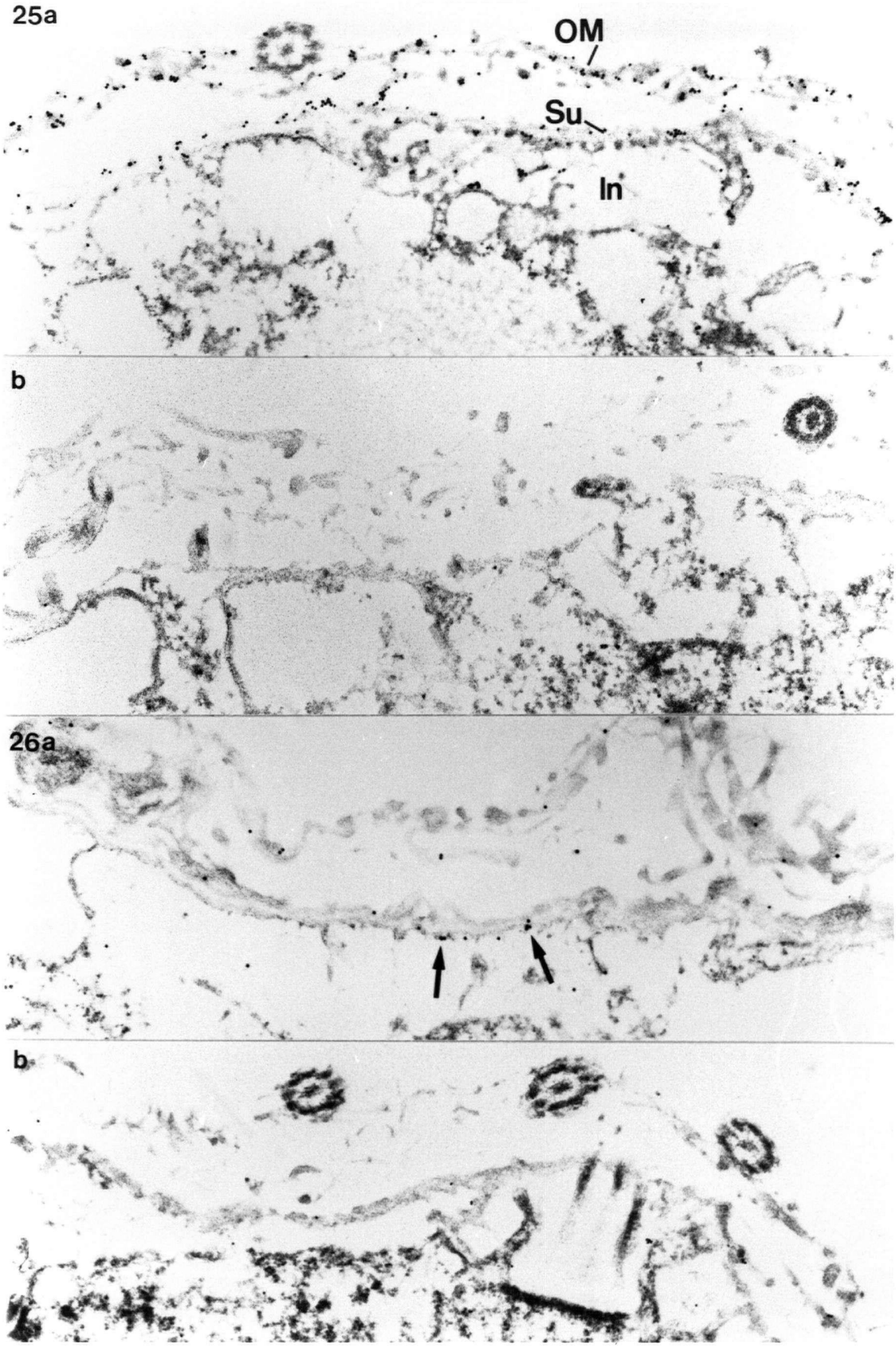
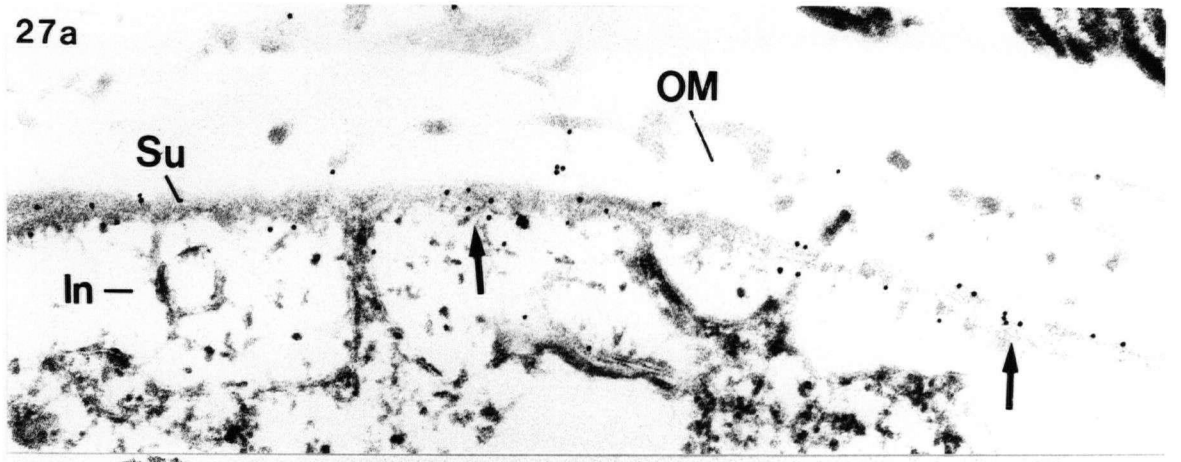

25a
OM
Su
In
b
26a
b

Fig. 27a: A TEM through the ectodermal region of the embyro, stained with Au_{25}-WGA, showing the labelling pattern over the HL. The gold particles are restricted primarily to the supporting layer (Su, arrows), although an occasional particle is seen over other regions of the HL.
In = intervillus, OM = Outer Meshwork x38,500

Fig. 27b: A TEM as above, stained with the control sugar/conjugate solution (glcNAc/Au_{25}-WGA), showing a very low background level of labelling. x35,700

Fig. 28a: A TEM through the ectodermal region of the embyro, stained with Au_{25}-PNA, showing the labelling pattern over the HL. Labelling of the supporting layer is very heavy (arrows), while labelling of the intervillus and outer meshwork regions is very light and scattered. x55,000

Fig. 28b: A TEM as above, stained with the control sugar/conjugate solution (galactose/Au_{25}-PNA), showing no background labelling. x46,200


27a
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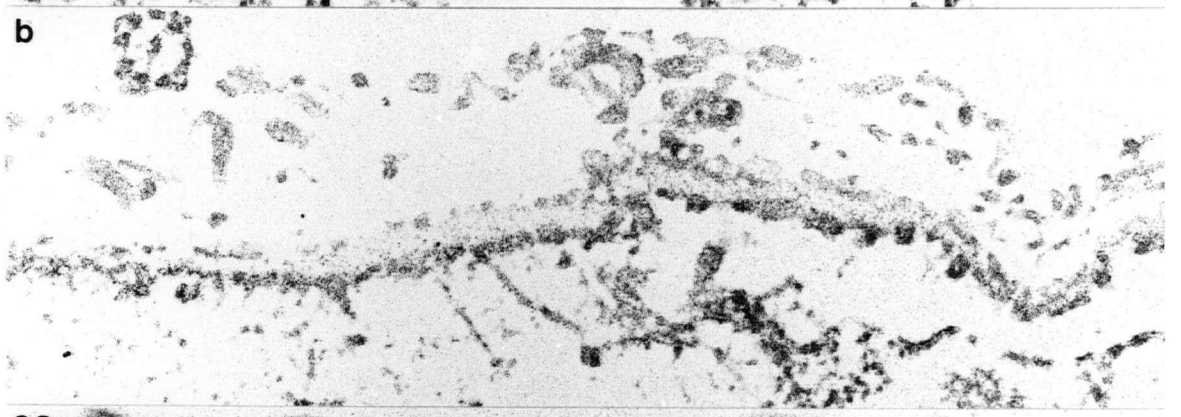
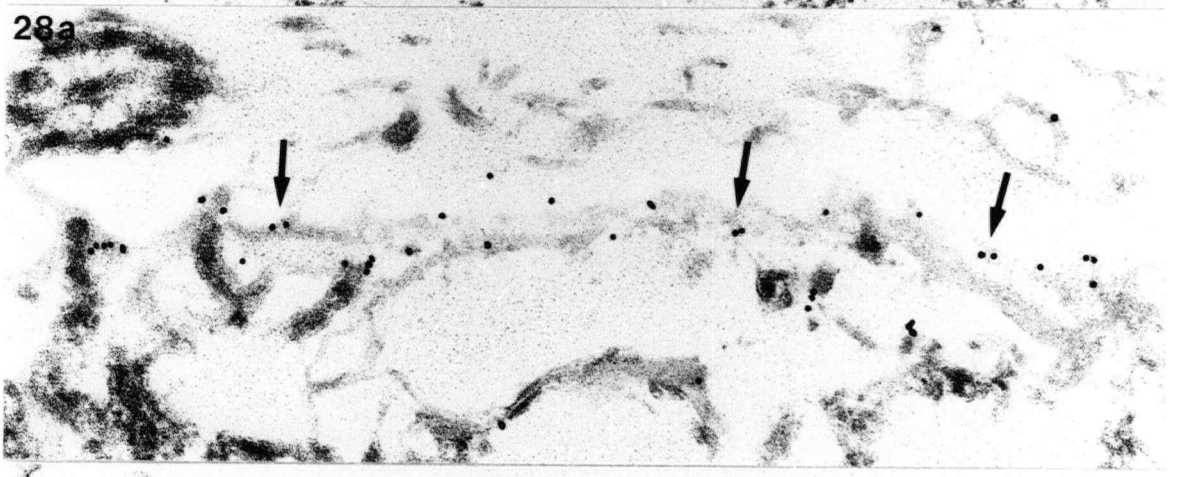
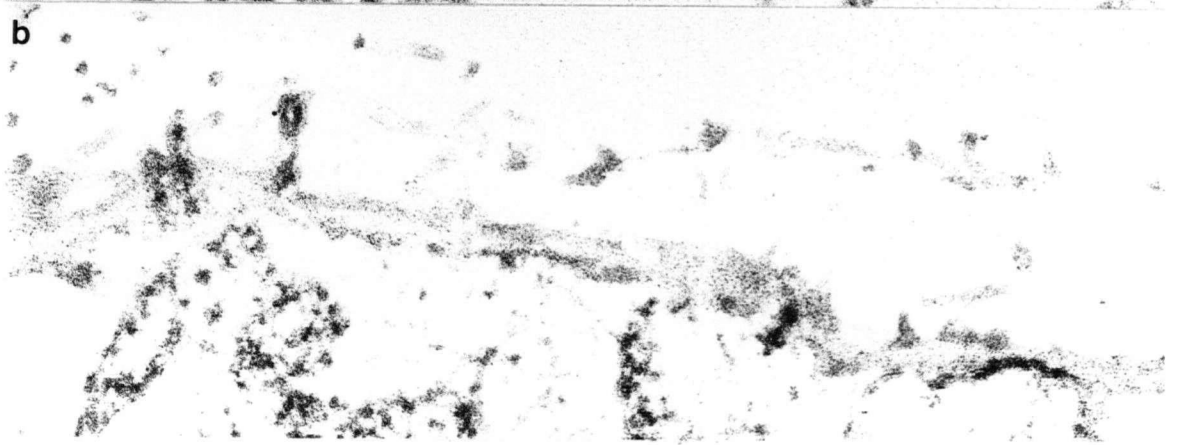

b


28a


b

5.3 DISCUSSION

The present study demonstrated that lectins from 4 different carbohydrate-binding groups are found to label the HL. These include galNAc-binding (SBA), galactose-binding (RCA,PNA), glcNAC-binding (WGA), and mannose/glucose-binding (Con A) lectins. The labelling patterns vary from lectin to lectin, suggesting that the distribution of their conjugate sugars in the HL is not uniform. At the LM level, FITC-conjugated SBA, RCA, WGA, and Con A all label the HL. Although with the LM it is not possible to resolve the 3 regions of the HL clearly, it appears that labelling with RCA is restricted to the inner region of the HL, suggesting that this region is rich in galatose residues, whereas SBA, WGA and Con A labelling occurs over the "entire" HL, suggesting that galNac, glcNAc and mannose/glucose reisidues are more evenly distributed.

Ultrastructural studies using colloidal gold conjugated lectins confirm the results described above. While all the layers label heavily with SBA, suggesting that galactosamine is present throughout this structure, WGA and PNA are present almost exclusively in the supporting layer suggesting that this region is much richer in glucosamine and galactose. Labelling with Con A is moderate in intensity, and indicates the presence of some mannose/glucose residues primarily in the supporting region of the HL.

There are some discrepencies in labelling patterns between LM (FITC-lectin) and TEM (Au_{25}-lectin) staining. For example, with the LM, WGA labelling appears to span the entire width of the HL, whereas with the TEM, significant labelling is seen only over the supporting layer. This can most likely be attributed to the different fixations used for these two methods. It is quite possible that the PF/CPC

fixation used for FITC-WGA labelling did not fix the entire HL, permitting only the supporting layer to be labelled. More likely, labelling with WGA, although only present over the supporting layer, might have obscured the other regions since the marker, FITC, amplifies the signal to a certain degree. It must be remembered that the 3 regions present in the HL are really ultrastructural entities, and thus trying to distinguish them with the LM is not really warranted. It is probable that the fixative used for TEM (glutaraldehyde/alcian blue), which fixes the entire HL, gives a more accurate representation of lectin binding sites throughout the HL than do ethanol or formlin fixatives.

As discussed earlier, chondroitin sulfate and dermatan sulfate contain large amounts of galactosamine. Since SBA, which labels all regions of the HL, binds strongly to galactosamine, it is possible that CS and DS are present in the HL. PNA and WGA bind strongly to glucosamine and galactose residues which are present in large quantities in hyaluronic acid and keratin sulfate suggesting that these may be present in the supporting layer. PNA and WGA can also be expected to bind to laminin and collagen, two molecules which have been found in the sea urchin HL (Spiegel et al., 1983). It is possible that the supporting layer of asteroid HL may also contain these substances. Alternatively, the carbohydrates may be part of new types of macromolecules. Isolation and biochemical characterization of some of the sugar containing molecules of the HL may help to sort out this problem.

It has been suggested that the HL is involved in maintaining cellular polarity, adhesion and organization during early embryonic development (Dan, 1960; Wolpert & Mercer, 1967; Schroeder, 1988) as well

as protection (Lundgren, 1973) and lubrication (Crawford & Abed, 1986). The identification of adhesive molecules in the sea urchin HL plus the cross-reactivity of many vertebrate basal laminae antibodies in the HL suggests that it serves a function similar to that of the BL. The fact that the different morphological regions of the asteroid HL contain different carbohydrate moieties suggests that there are different glycoconjugates in these regions, which may reflect differences in adhesive properties within these regions. Since it is possible to isolate relatively pure populations of living ectoderm, endoderm or mesenchyme cells from asteroid embryos (Crawford, 1988), it should now be possible to isolate and purify molecules from the different regions of the HL using solid phase lectin affinity chromatography. This may permit the elucidation of the role of the HL in the embryo by enabling the effect of these molecules on isolated embryonic cells to be monitored.

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