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UNIVERSITY OF ALBERTA

EFFECT OF ANTI-LECTIN ANTIBODIES AND LECTIN HAPTEN INHIBITORS ON THE EARLY CHICK EMBRYO.

by

Jasmine Jeeva 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

DEPARTMENT OF ZOOLOGY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: Effect of Anti-lectin Antibodies and Lectin Hapten Inhibitors on the Early Chick Embryo, submitted by Jasmine Jeeva in partial fulfillment of the requirements for the degree of Master of Science in Zoology.

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Date: August 22/94

Dedication

This thesis is dedicated to my parents, Adam and Renee, in appreciation for their unquestioning support in helping me reach my goals and allowing me to find myself.

Abstract

Lectins are proteins, other than enzymes and antibodies, that bind specifically to carbohydrates. Two galactoside binding lectins, of 16 kD and 14 kD have been identified in the developing chick embryo In the gastrulating chick embryo, as well as during early organogenesis these lectins are expressed in the area pellucida, the area opaca the neural crest, the myotome and the primordial germ cells. These lectins are also present in the extracellular matrix of several organ primordia and at the surfaces of cells from the gastrulating embryo. Since the surfaces of the gastrulating chick embryo express cell surface glycoconjugates bearing ß-D galactoside groups, it is possible that these lectin could play a role in cell surface mediated functions such as cellular adhesion. To investigate the functional roles of these lectins in development, I determined the effects of sugar hapten inhibitors, neoglycoproteins (synthetic glycoproteins) bearing terminal galactose groups, and anti-lectin antibodies on the development of the gastrulating chick embryos cultured *in vitro*.

Embryos at early and late stages of gastrulation were cultured in defined medium containing the sugar hapten thiodigalactoside (TDG), the neoglycoprotein lactosylated bovine serum albumin (l-BSA), and anti-16 kD lectin antibodies. When compared to the controls, early gastrulating embryos cultured in the presence of the above-mentioned compounds underwent perturbed development. These embryos

showed lack of spreading with decrease in surface area of the blastoderm, poor demarcation between area pellucida and area opaca and failure of primitive streak development. Embryos at late stages of gastrulation, when cultured in the presence of these compounds, were able to continue further development and early organogenesis.

Since one of the events occurring during early gastrulation is the completion of hypoblast formation, it is possible that the inhibition of early gastrulation by TDG, lactosylated BSA and anti-lectin antibodies is due to the involvement of the endogenous lectins in hypoblast formation. Since these lectins are also present in the embryo at late stages of gastrulation, the lack of effect of the sugar hapten, the neoglycoprotein, and the anti-lectin antibodies in late gastrulation could reflect the presence of functional mechanisms that would compensate for the absence of lectin function.

Acknowledgements

I would like to express my gratitude to Dr. Sara Zalik for her guidance and encouragement, I will never forget her kindness. I would also like to thank Eva Dimitrov and Irene Ledsham, not only for their expert technical advice but also for their support and understanding, their friendship will always be a valuable part of my life. And I thank my family, friends, and fellow graduate students for their continued support throughout the years. And a special thanks to Don for his continued encouragement, even when the completion of my thesis seemed so far away. This project was supported in part by the Medical Reasearch Council of Canada.

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Introduction

The development of the embryo into a highly complex adult organism is dependent among other things, on the interactions between its cells and tissues. This intercellular communication provides the embryo with signals that regulate intracellular activities, which ultimately lead to synchronized morphogenesis, growth and differentiation. The subcellular events and molecular mechanisms underlying cell and tissue interactions during embryonic development and the identification of the multitude of factors mediating these interactions are of fundamental importance (Harrisson, 1989).

Two important embryonic developmental processes; gastrulation and neurulation, are of particular interest because they are responsible for the first visible signs of a distinct embryonic axis and the organization of the basic body plan of the embryo. Gastrulation results in the formation of the three germ layers. The cells that will form the endodermal and mesodermal organs are brought inside the embryo while cells capable of forming the skin and nervous system are spread over its external surface (Bellairs, 1982). Neurulation is the process whereby the chordamesoderm directs the overlying ectoderm to form the hollow neural tube (Martins-Green, 1988). The determination of the ectoderm, mesoderm, and endoderm by the end of gastrulation, as well as the subsequent development of the neural tube and neural crest, constitute an early building plan of the embryo that,

for its construction, requires dramatic changes in the physical and chemical properties of the embryonic cells.

The avian embryo provides a useful system to study the developmental processes involved during morphogenesis for a number of reasons, such as: i) availability of eggs at any time of the year ii) the possibility of delaying development by keeping the eggs cool iii) the availability of a wide range of culture and microsurgery techniques (Harrisson, 1989).

I. Description of Morphogenesis in the Early Chick Embryo.

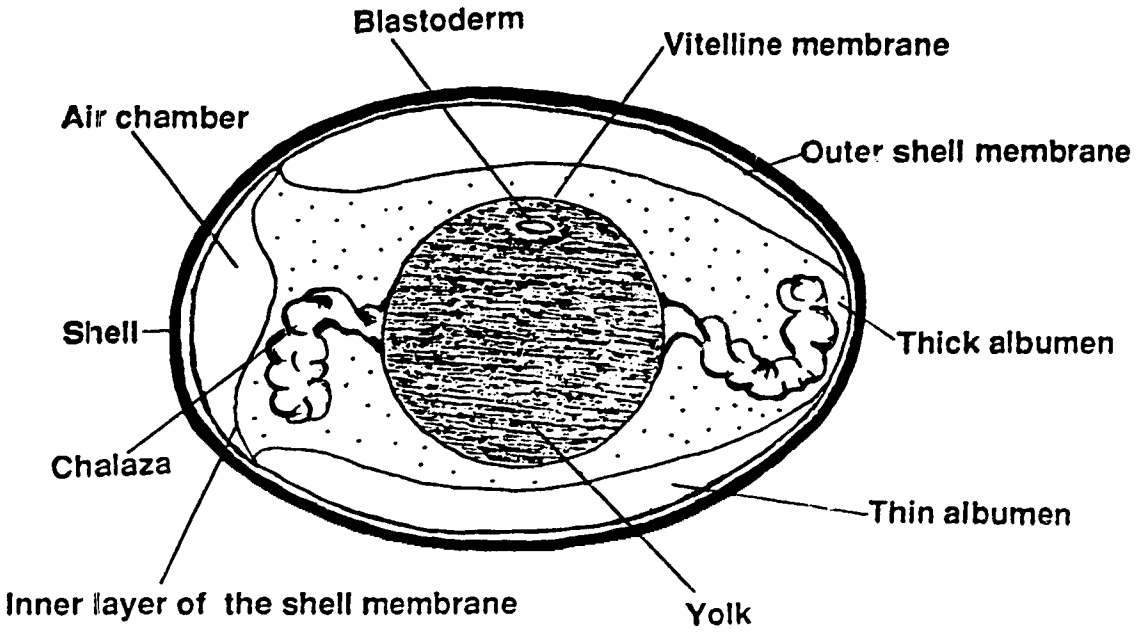
A. Cleavage and Blastula Formation.

The chicken ovum is mainly composed of reserve material, the yolk, on the upper surface of which rests a disc shaped drop of active cytoplasm, 2-3 mm. in diameter. The egg is fertilized as it enters the oviduct, and starts its 5 hour descent down this tract. During this time the yolk is gradually surrounded by the albumen and shell membranes secreted by different glandular regions of the genital tract (figure 1). On entering the uterus the egg starts a 20-hour rotation period around its long axis driven by the peristaltic contractions of the uterine muscles. During this period the calcareous shell forms around the soft shell membranes and the zygote starts to cleave (Eyal-Giladi, 1984).

The disc shaped active cytoplasm, called the germinal disc, starts to cleave around the time the egg enters the

Figure 1.

Sagittal section of a fertilized hen's egg at the time of laying. (Redrawn from Bellairs 1971).


Blastoderm
Vitelline membrane
Air chamber
Outer shell membrane
Shell
Thick albumen
Chalaza
Thin albumen
Inner layer of the shell membrane
Yolk

uterus of the mother hen (figure 2). The first cleavage furrows appear in the central area of the germinal disc. During the first 2 hours of cleavage, 5-6 cell divisions take place with all the furrows at a vertical position, therefore all the cells are continuous with the yolk. After 2 hours in the uterus some horizontal furrows have also occured, and the central cells start to separate from the yolk, due to the horizontal expansion of the deep ends of the cleavage furrows. This separation of the cells from the yolk gradually gives rise to a fluid-filled subblastodermic cavity, spreading from the center toward the periphery. After about 11 hours of cleavage the former germinal disc turns into an opaque 5-6 cell layer thick blastodisc (st. V, Eyal-Giladi, 1976) separated from the yolk by a fluid filled sub-blastodermic cavity (Eyal-Giladi, 1991) (figure 3).

The last 8-9 hours of intrauterine development, comprises stages VII-X (Eyal-Giladi and Kochav, 1976). At stage VII a transparent area starts to appear slightly central to the future posterior end of the blastodisc. The initially horseshoe-like area spreads during st. VIII and IX until the entire central area becomes transparent, and the blastodisc is turned into a blastoderm with two circular regions (Eyal-Giladi, 1984). A central region, the area pellucida that is separated from the yolk by a subgerminal cavity and appears to be clear; and a peripheral ring, the area opaca that is in contact with the yolk and appears to be opaque (Bellairs, 1982). The embryo proper forms in the area

Figure 2.

Cleavage in a chicken egg, viewed from the upper surface of the germinal disc. The cleavage furrows do not penetrate the yolk, and a blastodisc is produced. (Redrawn from Patten 1951).

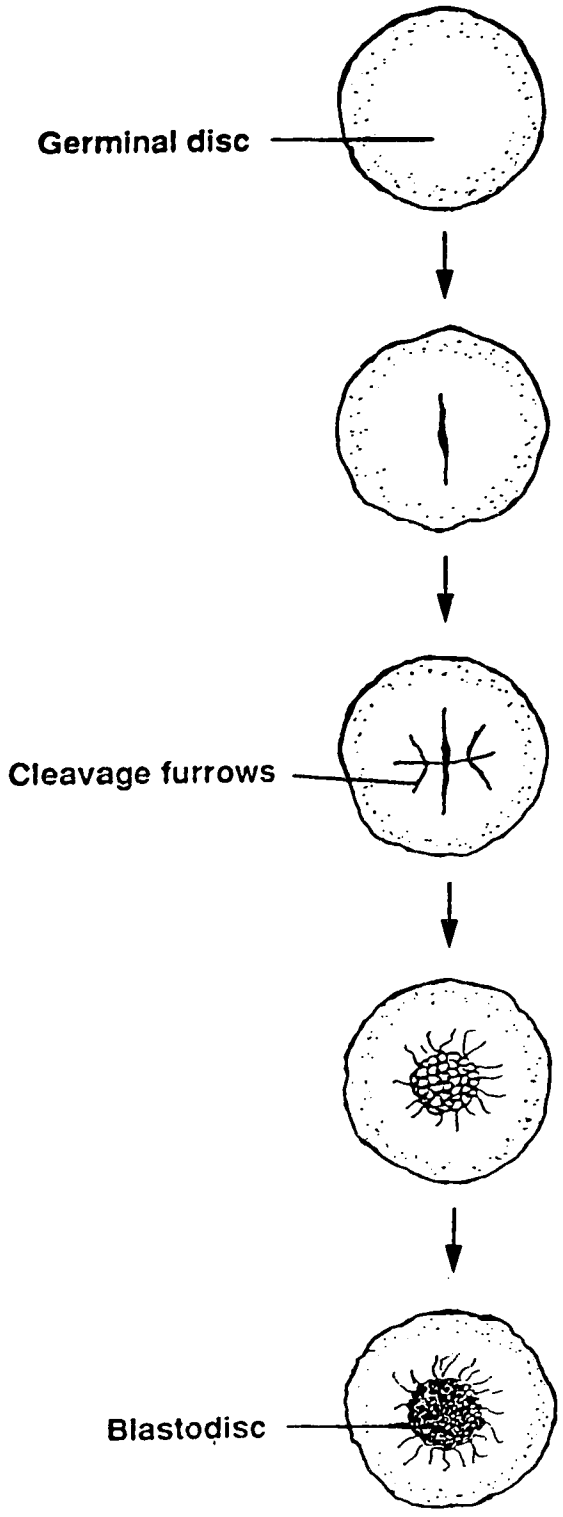
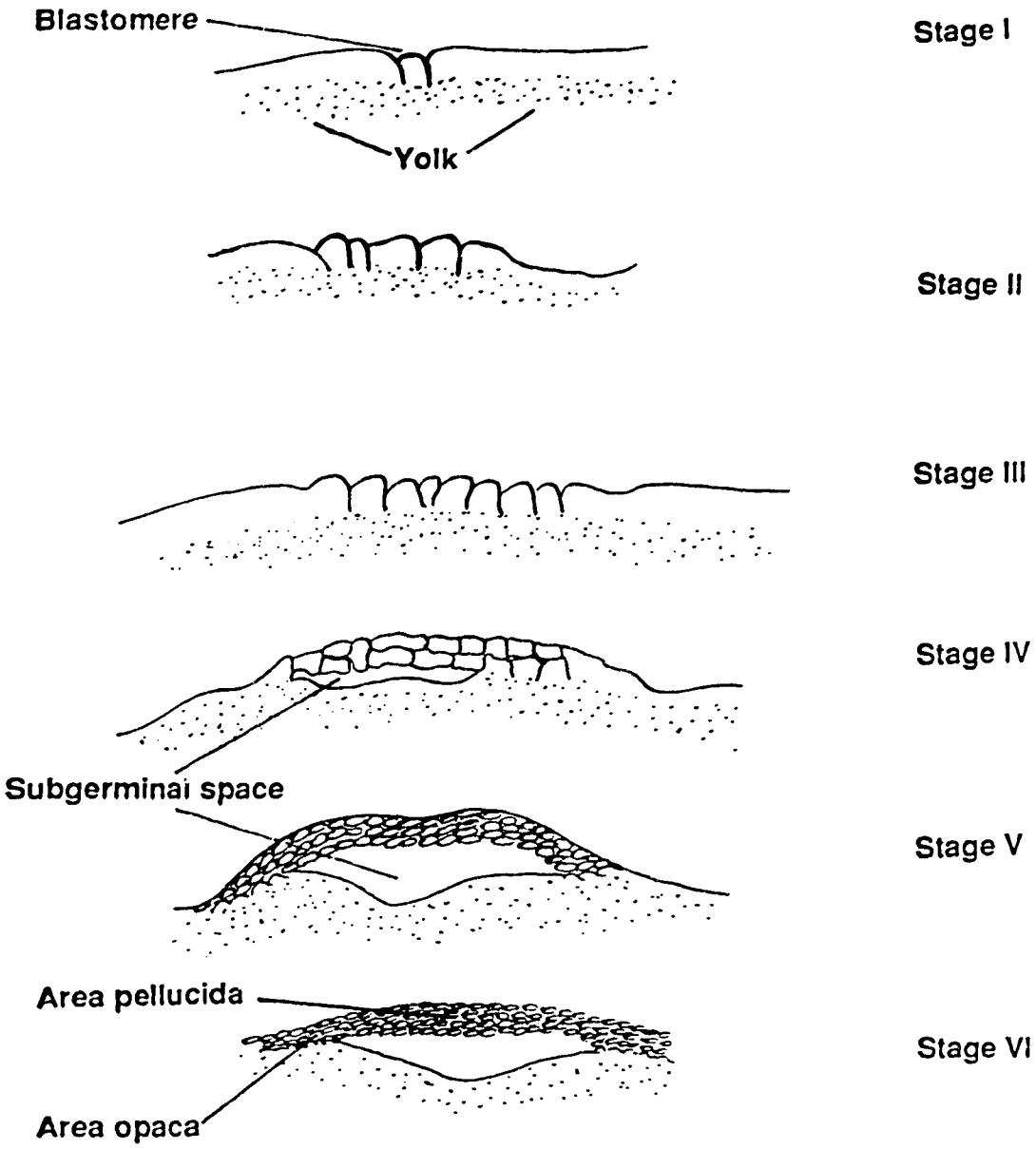

Germinal disc
Cleavage furrows
Blastodisc

Figure 3.

Median sagittal sections of cleaving chick blastodiscs. Stages indicated are based upon the staging system of Eyal-Giladi and Kochav (1976).

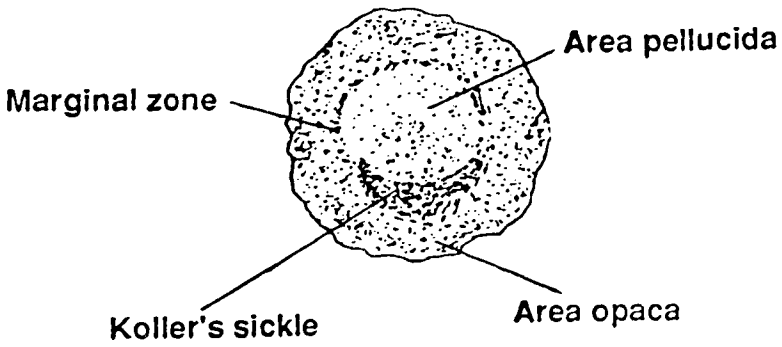

Blastomere
Yolk
Stage I
Stage II
Stage III
Stage IV
Subgerminal space
Stage V
Area pellucida
Area opaca
Stage VI

pellucida while the yolk sac and other extraembryonic membranes arise from the area opaca and the periphery of the area pellucida (Bellairs, 1982). The area pellucida forms as a result of a thinning out phenomenon involving the shedding of all the deep cell layers into the subblastodermic cavity, leaving only the transparent, one cell thick, upper epithelial layer. It is not known whether the shed cells have a morphogenetic role (Eyal-Giladi, 1984). The marginal zone, a single layered belt of cells, separates the area pellucida from the area opaca, and appears at stage X (Eyal-Giladi and Kochav, 1976) when the egg is laid. The marginal zone is visible at the posterior end of the stage X (Eyal-Giladi and Kochav, 1976) blastoderm in the form of a sickle-shaped ridge, Koller's sickle (Khaner and Eyal-Giladi, 1989) (figure 4). Azar and Eyal-Giladi (1979) have demonstrated that the marginal zone is instrumental in the formation of an inductive hypoblast.

After laying, subsequent development of the blastoderm depends on further incubation at 37-38°C. After one to two hours of incubation, the chicken embryo consists of two cell layers, an upper layer, the epiblast, and a lower layer, the hypoblast, (stage XIII Eyal-Giladi and Kochav, 1979), both developed from the area pellucida (Bellairs, 1982). The primary hypoblast is formed of cells derived from the epiblast by a process of vertical polyinvagination and also of cells which migrate horizontally in an anterior direction from Koller's sickle. The cells of the two different origins

Figure 4.

The chick embryo at the time of laying. Stage XI (Eyal-Giladi and Kochav, 1976).


Area pellucida
Marginal zone
Koller's sickle
Area opaca

merge to form a uniform layer and are morphologically indistinguishable (Azar and Eyal-Gilad., 1979) (figure 5). The cells of the two layers of the blastoderm join at the margin of the area opaca and the space between the cell layers is the blastocoele. The early development of preincubation stages of the chick embryo have been studied in depth by Eyal-Giladi and Kochav (1976) and in subsequent descriptions, the early stages will be identified as EG & K using roman numerals.

B. Gastrulation

The main structure of the gastrulating avian embryo is the primitive streak. Development of the primitive streak occurs after approximately twelve hours of incubation, and enables cells from the outer layer of the embryo to pass into the blastocoele and become mesoderm and endoderm (Bellairs, 1986). The streak is first visible as a thickening of the cell sheet at the central posterior end of the area pellucida. This thickening is caused by the migration of cells from the lateral region of the posterior epiblast toward the center. As the thickening narrows it moves anteriorly and constricts to form the definitive primitive streak (figure 6). At the anterior end of the primitive streak is a regional thickening of cells called Hensen's node (Bellairs, 1986).

Although the hypoblast does not contribute any cells to the adult chicken it is essential for proper development of

Figure 5.

Formation of the hypoblast in the avian egg. (Redrawn from Gilbert 1991).

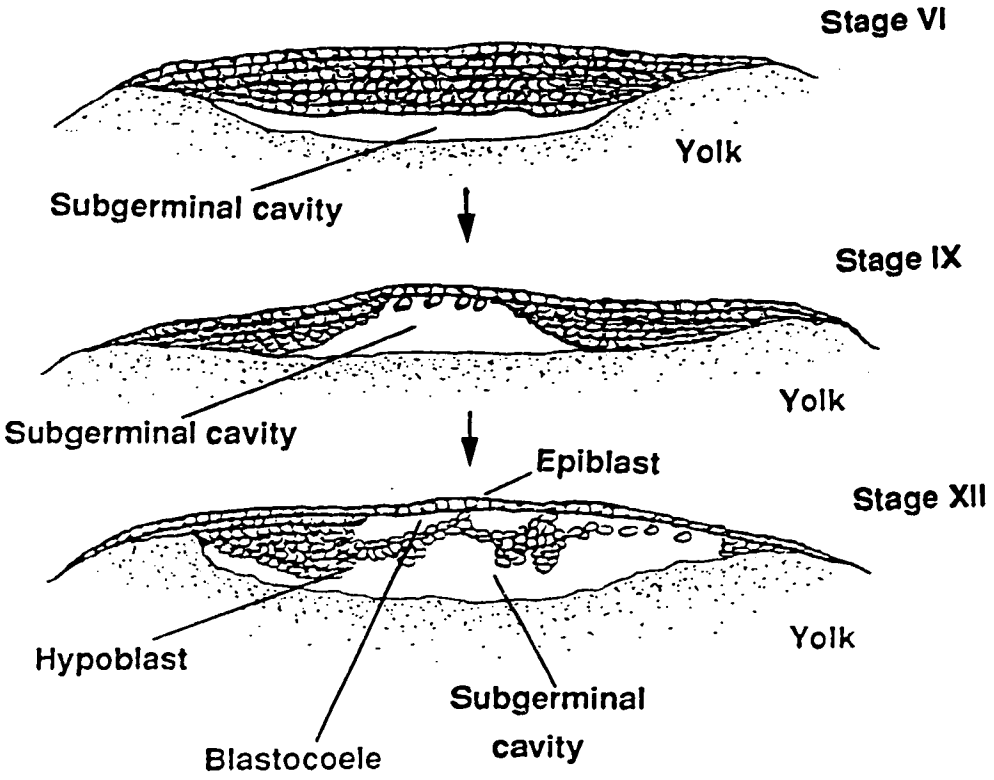
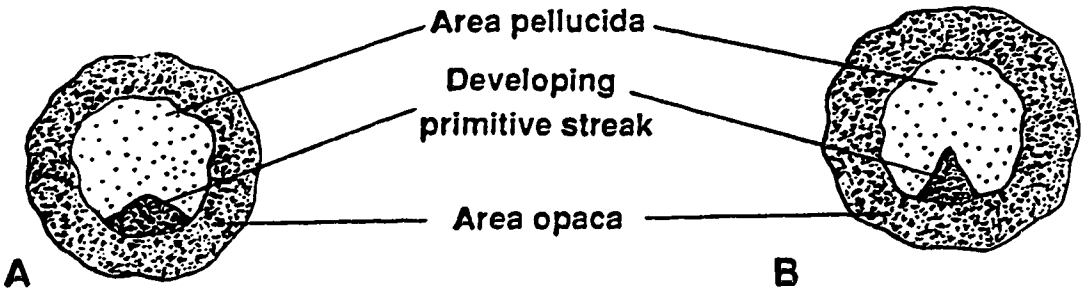
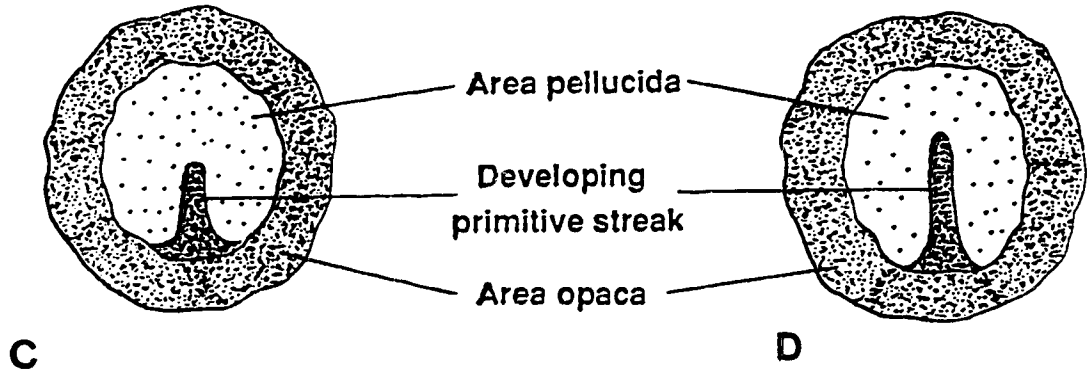
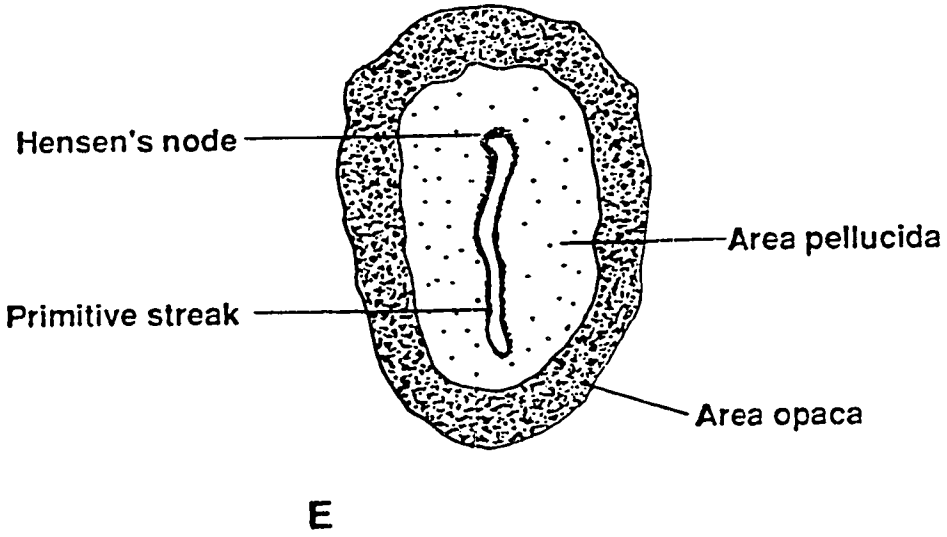

Stage VI
Yolk
Subgerminal cavity
Stage IX
Yolk
Subgerminal cavity
Epiblast
Stage XII
Yolk
Hypoblast
Blastocoele
Subgerminal
cavity

Figure 6.

Cell movements forming the primitive streak of the chick embryo. Dorsal view of the chick blastoderm after A) 3-4 hours of incubation B) 5-6 hours C) 7-8 hours D) 10-12 hours E) 15-16 hours. (Redrawn from Gilbert 1991).


Area pellucida
Developing
primitive streak
Area opaca
A
B


Area pellucida
Developing
primitive streak
Area opaca
C
D

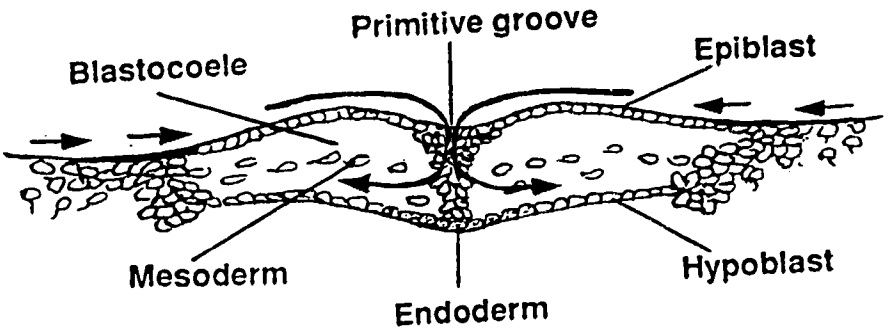

Hensen's node
Primitive streak
Area pellucida
Area opaca
E

the primitive streak. The posterior marginal zone (Koller's sickle) contributes cells to the hypoblast and this marginal zone derived hypoblast induces the formation of the primitive streak (Khaner and Eyal-Giladi, 1986). Experiments by Azar and Eyal-Giladi (1979) demonstrated the inductive ability of the hypoblast. When a stage XIII (EG & K) blastoderm had both the hypoblast and marginal zone removed it was incapable of forming a primitive streak. However, if only one of the two is removed that is, either the hypoblast or the marginal zone, a normal primitive streak is formed. The conclusion was that, in the first instance, the primitive streak arose as a result of an inductive effect of the existing hypoblast. In the second case, with the hypoblast removed and the marginal zone preserved, the inductor was the regenerated hypoblast, due to the contribution of marginal zone cells (Khaner and Eyal-Giladi, 1989). Khaner and Eyal-Giladi (1989) suggest that the marginal zone cells form a gradient of activity whose peak is at the posterior end of the marginal zone. These posterior cells will form the hypoblast and at the same time prevent any cells with lower activity from forming one of their own.

As cells converge to form the primitive streak, a depression forms within the streak. This primitive groove, serves as a blastopore through which the migrating cells can pass into the blastocoele (Bellairs, 1986) (figure 7). As cells of the embryonic endoderm pass through the primitive streak they insert themselves into the hypoblast. These

Figure 7.

Transverse section through a 17-hour embryo, illustrating the invagination of endodermal and mesodermal cells passing into the blastocoele. (Redrawn from Bellairs 1971).


Primitive groove
Blastocoele
Epiblast
Mesoderm
Endoderm
Hypoblast

cells give rise to all the endodermal organs and some of the extraembryonic membranes. Those cells migrating through Hensen's node pass down into the blastocoele and migrate anteriorly, forming head mesoderm and notochord (Bellairs, 1986). The remaining mesodermal cells migrate laterally after leaving the primitive streak and use the basement membrane of the epiblast as a substratum (Sanders, 1986).

C. Neurulation

The gradual disappearance of the primitive streak is accompanied by the appearance of the embryonic axis. The node regresses, the primitive streak disappears, and the area pellucida lengthens. Hensen's node regresses to the caudal end of the primitive streak and left in its place is the notochord, an axial concentration of mesoderm (Bellairs, 1971). While cells of the posterior portion of the 24-hour embryo are undergoing gastrulation, cells at the anterior end are already starting to undergo neurulation (Bellairs, 1986).

During neural induction, the chordamesoderm acts on the overlying adjacent ectoderm to stimulate it to form the neural tube. Neurulation can be described in terms of three major stages. First, the ectoderm overlying the notochord thickens and forms the neural plate. Second, a medial groove develops in the plate and the lateral portions elevate to form the neural folds. Lastly, these two folds come together and ultimately fuse, leading to the separation of the neuroepithelium from the epidermal ectoderm, and giving rise

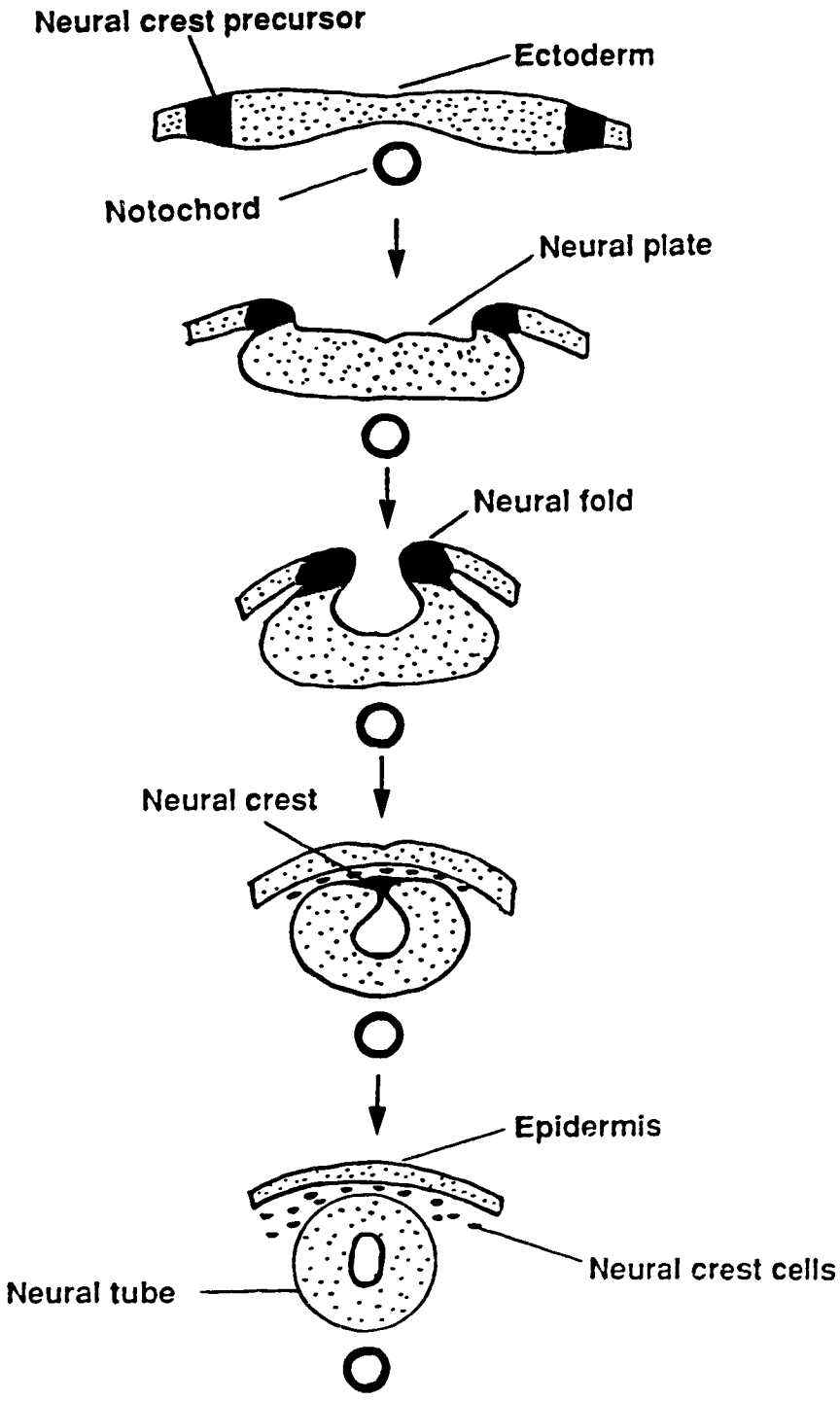
to the neural tube (Martins-Green, 1988) (figure 8). As a result, the original ectoderm has been divided into three groups of cells. The internally positioned neural tube, the epidermis of the skin and the neural crest cells (Bancroft and Bellairs, 1975). The cells at the dorsal-most portion of the neural tube become the neural crest cells. These neural crest cells will migrate through the embryo and will give rise to several cell populations, including pigment cells, membranous cartilage and bone, and cells of the peripheral nervous system (LeDouarin, 1982). Regionalization of the neural tube also occurs as a result of changes in the shape of the tube. In the cephalic end, where the brain will form, the wall of the tube is broad and thick. Here a series of swellings and constrictions define the various brain compartments. Caudal to the head region however, the neural tube remains a simple tube that tapers off toward the tail (Schoenwolf and Smith, 1990). At the same time the brain vesicles develop, the somites form, and the circulation begins. In general, the differentiation of each organ starts at the head end and gradually extends caudally so that the formation of the anterior regions of the embryo tends to be in advance of that of the posterior ones (Bancroft and Bellairs, 1975).

II. Cell Biology of Early Chick Embryo Development

Embryonic induction is the process where the fate of a particular tissue is determined through its interaction with

Figure 8.

Diagrammatic representation of neural tube formation. The ectoderm folds in at the most dorsal point, forming an outer epidermis, an inner neural tube, and the neural crest cells. (Redrawn from Gilbert 1991).


Neural crest precursor
Ectoderm
Notochord
Neural plate
Neural fold
Neural crest
Epidermis
Neural crest cells
Neural tube

another, adjacent tissue. Both gastrulation and neurulation are examples of inductive processes. During gastrulation, interaction between the epiblast and hypoblast results in the formation of the primitive streak which in turn forms the axial mesodermal structures (Khaner and Eyal-Giladi, 1979). Similarly, during neurulation the notochord acts upon the overlying ectoderm to form the neural tube. Embryonic induction brings about coordinated changes in cellular activity allowing cells to differentiate and form tissues and organs. The morphogenetic movements involved during gastrulation and neurulation require the coordinated and directed movements of several early cell populations (Sanders, 1982). The questions arise as to what forces direct cell movement and differentiation during morphogenesis of the chick embryo and what are the mechanisms involved in intercellular communication? The following discussion will focus on some of the factors controlling early chick morphogenesis, including growth factors, cell adhesion molecules, the extracellular matrix and lectins.

A. Growth Factors

There is evidence indicating that the process of embryonic induction is controlled by specific soluble factors. The first system studied was the ectoderm of early amphibian embryos in the gastrula stage. During gastrulation, the ectoderm on the dorsal region of the embryo comes into contact with the layer of prospective mesoderm and is induced

to form the nervous system. When ectoderm isolated from the gastrula is cultured in a physiological salt solution, it fails to differentiate into nervous tissue and instead forms strands of epidermal cells. However addition of defined extracts to this salt solution results in the differentiation of the ectoderm explant into epidermis and neural tissue, or into muscle, notochord, renal tubules and gut epithelium. Therefore, the gastrula ectoderm has the ability to differentiate even into tissues which it does not form in normal development, and as a result can be used to test "factors" which might induce the formation of different tissue types (Tiedemann, 1966, Nieuwkoop, 1985, Gurdon, 1987). Further evidence for the importance of these growth factors was their identification in the early embryo (Whitman and Melton, 1989). Investigators continued to study the effects of these soluble factors on primitive streak and axis formation in the chick embryo.

Peptide growth factors are small soluble polypeptides capable of regulating cell proliferation and differentiation. The purification and sequencing of these factors and the genes encoding them has permitted their grouping into a relatively small number of families. Within each growth factor family, members are closely related structurally and in some instances may interact with similar or identical receptors (Whitman and Melton, 1989).

A number of growth factors have been identified in the developing chicken embryo (Engstom *et al.*, 1987, Seed *et al.*,

1988, Mitrani *et al.*, 1990). Recent work has shown that peptide growth factors can specify the fate of embryonic cells and may function as morphogens within the early embryo. An important step in elucidating the functions of growth factors in embryos is the identification of the factors present in the embryo and the investigation of their distribution and relationship with developmental processes (Ralphs *et al.*, 1990). Some of the growth factors found in the developing chick embryo include, insulin-like growth factor (Ralphs *et al.*, 1990), fibroblast growth factor (Seeds *et al.*, 1988), and activin (Ziv *et al.*, 1990).

Conditioned medium derived from the *Xenopus* XTC cell line (XTC-CM), secretes a mesoderm inducing factor (MIF), that is known to be an effective inducer of mesodermal structures in the ectoderm of early *Xenopus* embryos (Smith, 1987). Evidence indicates that XTC-MIF belongs to the transforming growth factor ß (TGF-ß) family since it is partially neutralized by certain antibodies to TGF-ß2 and that pure TGF-ß2 itself induces mesoderm in *Xenopus* (Woodland, 1989, Cooke and Wong, 1991).

The discovery that XTC-CM is a mesodermal inducer in *Xenopus* led to the further investigation as to whether medium conditioned by XTC could have similar effects in the avian embryo. XTC-CM induced the formation of full length notochord and rows of bilaterally symmetric somites in isolated epiblasts (Mitrani and Shimoni, 1990). However, TGF-ß1 and TGF-ß2 as such, were unable to induce axial mesodermal

structures. Therefore, the mesodermal inducing activity is thought to be due to a factor related to but distinct from TGF-ß2 (Mitrani and Shimoni, 1990).

A growth factor with properties very similar to fibroblast growth factor (FGF) has been detected in the yolk and white of unfertilized chick eggs and in the limb buds and bodies of 2.5 to 13-day chick embryos (Seeds *et al.*, 1988). FGF is a potent mitogen for a variety of mesodermal and neural crest-derived cells (Baird *et al.*, 1986). FGF may be involved in early muscle development (Seed and Hauschka, 1988), eye development (Mascarelli *et al.*, 1989), and angiogenesis (Folkman and Klagsbrun, 1987) of chick embryos. There is also evidence that the chick embryo contains a FGF-like growth factor throughout the initial phases of development. (Seeds *et al.*, 1988).

Mitrani *et al.* (1990) have isolated from the chick gastrula a genomic clone that shows a high degree of homology to the mammalian and *Xenopus* basic FGF gene. Immunolabeling analysis indicated that bFGF protein is present in the pregastrula stage of the chick embryo and is distributed evenly in the epiblast, hypoblast and marginal zone (Mitrani *et al.*, 1990). Since heparin and suramin inhibit the binding of FGF to its receptor (Slack *et al.*, 1987, Moscatelli and Quarto, 1989), Mitrani *et al.* (1990) applied these compounds to chick blastoderms grown in vitro under defined culture conditions (Mitrani *et al.*, 1990). Both substances were capable of blocking the formation of mesodermal structures in

a dose dependent manner, suggesting that FGF-like substances may be required throughout the process of axial mesoderm formation (Mitrani *et al.*, 1990). Unlike the conditioned medium derived from the *Xenopus* XTC cell line, bFGF cannot induce axial mesoderm in isolated epiblasts. These experiments suggest that bFGF is not sufficient to induce axial structures in the chick, but may still be necessary for this process to take place (Mitrani *et al.*, 1990).

The TGFß related factor activin, can also induce the formation of axial structures in isolated st. XIII (EG & K) epiblasts. Activin is also being transcribed in the chick hypoblast at the same stages in which axial mesoderm is being induced (Ziv *et al.*, 1990). It is therefore possible that activin acts as the inducer of axial mesoderm.

Scatter factor, a recently characterized protein secreted by some cultured fibroblastic cells, alters the locomotory behavior of certain cultured epithelial cells by increasing cell motility, the breakdown of cell junctions and cell scattering (Stern *et al.*, 1990). Among the developmental processes in which scatter factor could play a role is during epithelial-mesenchyme conversion such as in the de-epithelialization of the early epiblast to give rise to the mesoderm and the primitive streak. Several abnormalities were seen in gastrulating embryos that received either a graft of scatter factor-secreting cells or the purified factor. These abnormalities included, condensations of mesodermal cells, a second primitive streak-like structure, disruption of somitic

epithelium and, in some embryos, a neural plate-like thickening of the ectoderm (Stern *et al.*, 1990). It is possible that localized disruption in the continuity of the epiblast, due to the addition of scatter factor, results in an outlet through which the middle layer cells can ingress. Therefore scatter factor could mimic some process occurring during primitive streak formation. However, local disruption of epithelial continuity cannot account for the appearance of neural plate-like structures. Therefore, it is possible that scatter factor has inducing activity unconnected with de-epithelialization (Stern *et al.*, 1990).

The evidence cited above indicates that growth factors may be necessary for axial structures to develop. Growth factors are likely to be instrumental in the determination of cell tissue organization during early development (Whitman and Melton, 1989).The identification of growth factors as possible inducers indicates that they may function as morphogens in early embryonic induction.

B. Cell Adhesion Molecules

Cell-cell adhesion may regulate morphogenesis by constraining cell motion and controlling intercellular communications that are necessary for embryonic induction (Gallin *et al.*, 1986). The modulation of cell-cell adhesion is a major mechanism necessary for pattern development (Edelman, 1986).

Cell adhesion molecules (CAMs) function in a dynamic and highly regulated fashion to form and maintain tissue structure. CAMs are divided into three main groups; a)the cadherins, whose cell adhesive properties are dependent on calcium ions, b)the immunoglobulin superfamily of CAMs that are calcium independent and whose cell binding domains resemble those of antibody molecules and c)the saccharide CAMs in which the CAM protein recognizes a carbohydrate residue on an adjacent cell (Edelman and Crossin, 1991). All known CAMs are large intrinsic cell surface glycoproteins that are mobile in the plane of the membrane (Edelman and Crossin, 1991).

Cadherins are surface glycoproteins ranging in molecular weight between 120-140 kD. Most members of this family contain a large extracellular domain that mediates adhesion, a single hydrophobic transmembrane region, and a cytoplasmic tail (Geiger and Ayalon, 1992). Cadherins are divided into subclasses, including E-, N-, and P-cadherins. While all subclasses are similar in molecular weight, calcium and protease sensitivity, each subclass is characterized by a unique tissue distribution pattern. In development, the expression of each cadherin subclass is spatio-temporally regulated and associated with a variety of morphogenetic events (Takeichi, 1988).

In the chicken, two types of cadherins have been identified, L-CAM and N-cadherin (Hatta *et al.*, 1987). L-CAM was originally identified as a chicken liver CAM (Gallin *et*

al., 1983) and later found to be equivalent to a calcium dependent cell adhesion molecule termed E-cadherin identified in mouse cells (Yoshida-Noro *et al.*, 1984).

Expression of N-cadherin begins at the gastrulation stage in mesodermal and endodermal cells (Hatta and Takeichi, 1986). However, at the same stage, L-CAM is expressed in the epiblast but not in the mesoderm and endoderm (Thiery *et al.*, 1984). It has been suggested that expression of the calcium dependent CAMs is switched from L-CAM to N-cadherin in mesodermal and endodermal cells when they begin segregation from the epiblast (Hatta *et al.*, 1987). A similar transition in expression from L-CAM to N-cadherin was also observed in the process of separation of the neural tube and lens vesicle from the ectoderm (Hatta and Takeichi, 1986). Therefore N-cadherin and L-CAM seem to be expressed under a precise spatial and temporal control during morphogenetic events resulting in the separation of cell layers (Hatta *et al.*, 1987).

Neural cell adhesion molecule (N-CAM), a calcium independent CAM, was originally isolated from chicken retina cells. N-CAM is expressed as a number of major polypeptide chains each of which has three domains: an N-terminal cell-cell binding domain, a polysialic acid rich middle domain, and a cell associated or cytoplasmic domain (Rutishauser and Jessel, 1988). N-CAM possibly participates in cell-cell adhesion via homophilic interactions between N-CAM molecules

present on the surface of both interacting cells (Rutishauser and Jessel, 1988).

During development, N-CAM is broadly distributed. In neural tissues, N-CAM first appears in substantial amounts in the neural plate and is transiently expressed in morphogenetically active structures, such as the notochord, neural crest, somites, some epidermis, and mesenchyme (Rutishauser and Jessel, 1988). Since a substantial number of embryonic tissues are composed of cells that contain N-CAM, N-CAM mediated adhesion is probably not involved in the adhesive specification of individual cells. Instead N-CAM may act like a "glue" that is used where and when cells are required to establish adhesive contacts with N-CAM positive neighbors (Rutishauser and Jessel, 1988). Studies using antibodies to N-CAM have indicated a number of possible roles for N-CAM. For example, the initial innervation of skeletal muscle by motor axons appears to be regulated in part by N-CAM (Tosney and Landmesser, 1985). Also the establishment of N-CAM mediated adhesion may be involved in junctional communication between cells during early neural development (Keane *et al.*, 1987). N-CAM is not likely to participate directly in formation of junctional channels, its function may be required to maintain cell contact during junctional assembly (Rutishauser and Jessel, 1988).

Local cell surface modulation of CAMs may regulate primary processes of development, such as neurulation, therefore creating a specific pattern in early development.

During neural induction, the L-CAM originally present on the ectoderm disappears from the region that contacts the mesenchymal cells of the presumptive notochord. At approximately the same time N-CAM expression on this presumptive neural plate increases to a high level (Thiery *et al.*, 1982).Therefore, changes in the expression of L-CAM and N-CAM appear to be correlated with induction at the neural plate (Thiery *et al.*, 1984).

It is apparent that the appearance or disappearance of CAMs often occurs at sites of active cell rearrangement, segregation and association. There is not yet a clear understanding of the role of cell-cell adhesion in development, but it does appear that CAMs have a role in inductive processes.

C. The Extracellular Matrix

The extracellular matrix (ECM) provides a substratum for the movement and migration of cells. All matrices contain glycoproteins, collagens and glycosaminoglycans as major structural elements (Harrisson, 1989, Adams and Watt, 1993). The ECM appears to play a role in regulating the differentiated phenotype of cells (Adams and Watt, 1993).

The basement membrane is a special type of matrix that is deposited around epithelial and endothelial cells. The basement membrane may modulate morphogenesis (Ekblom *et al.*, 1986). During gastrulation, the epiblast layer modifies its own basement membrane at the primitive streak so that its

cells may migrate into the blastocoele (Sanders and Prasad, 1989). Cells which are initially part of the epiblast leave the epithelial sheet, become mesenchymal and undergo extensive migrations. Once they reach their destinations however, most of the cells become rearranged once more as epithelia (Bellairs, 1986). Cellular movement of these cells is correlated with the presence of a fibronectin meshwork in the extracellular basal lamina of the epiblast cells (Duband and Thiery, 1982). The basal lamina adjacent to the primitive streak lacks fibronectin. It is in this region that the basal lamina breaks up to allow the inward migration of presumptive mesoderm (Sanders, 1986). Fibronectin is present as strands under the epiblast just prior to the appearance of the streak (Duband and Thiery, 1982, Sanders, 1986). Observations by Toyoizumi and Takeuchi (1992) led to the conclusion that the mesoderm cells in the chick gastrula were guided to migrate towards the periphery of the area pellucida by a fibronectin rich ECM laid on the basal surface of the epiblast and that this movement was due to an in vivo locomotive mechanism using filopodia. Therefore it appears that the ECM appears to guide the direction of the migrating mesodermal cells.

Laminin is a major glycoprotein of basement membranes and has been shown to promote cell adhesion and movement of various non-epithelial cells and tumor cells (Zagris and Chung, 1990). The molecular weight of this protein is about 850 kD and it is apparently formed by several chains that interact to form a large, cross shaped molecule (Yamada *et*

al., 1985). At stage XIII (EG & K), laminin was detected at the ventral surface of the epiblast and in the entire hypoblast. In stage XIII (EG & K) blastoderms treated with laminin antibodies, the area pellucida collapsed and degenerated, while the rest of the blastoderm remained intact (Zagris and Chung, 1990). This suggests that laminin mediates adhesion of the cells of the area pellucida, which are destined to give rise to the embryonic axis. It appears that laminin plays a functional role in the directionality of cell migration and in cell adhesion during the first morphogenetic events in the early chick embryo (Zagris and Chung, 1990).

Some cell surface receptors for ECM molecules have been identified. One of the first molecules to be identified and characterized as a receptor for ECM molecules is the 140 kD avian integrin (Horowitz *et al.*, 1985), which possesses binding properties for both fibronectin and laminin (Harrisson, 1989). This receptor has now been localized on several cell types. It is present in the basal surface of epithelial cells, mesenchymal cells and neural crest cells (Duband *et al.*, 1986).

Hyaluronate is a complex polysaccharide that is a major component of the ECM surrounding proliferating and migrating cells in embryonic tissues (Toole *et al.*, 1989). Hyaluronate can be extensively hydrated resulting in expansion of the ECM, permitting cell proliferation and migration, and by reducing contact-mediated cell-cell communication, and postponing differentiation (Brown and Papaioannou, 1993). The

removal of hyaluronate leads to the compaction of the mesodermal cells, retraction of cell processes and appearance of intercellular junctions (Harrisson, 1989) which is often associated with the onset of differentiation (Brown and Papaioannou, 1993).

Microinjection of hyaluronidase (a hyaluronate degrading enzyme) into the blastocoele of gastrulating chick embryos resulted in the compaction of the middle layer cells in the area lateral to the primitive streak. These cells lost their mesenchymal shape and retracted their processes with the formation of intercellular junctions. In normal and saline injected-blastoderms, middle layer cells were rounded and linked to each other by small intercellular junctions in the primitive streak region. In saline treated embryos, these cells had migrated laterally along the basal lamina and appeared as typical mesenchymal cells, being separated by large intercellular spaces and surrounded by cell processes. Therefore hyaluronate, due to its space creating properties, promotes the detachment of ingressed primitive streak cells and preserves the mesenchymal form of the middle layer during the lateral migration of single cells along the basal lamina (Van Hoof *et al.*, 1986). It is possible that hyaluronate prevents cell-cell and/or cell matrix interactions necessary to trigger differentiation and the progressive reduction in accumulation of extracellular hyaluronate may be essential for the initiation of cellular

condensation that is necessary for differentiation (Kulyk and Kosher, 1987, Solursh, 1976).

ECM components appear to play a role in determining the type of differentiation a cell will undergo (Griffith and Sanders, 1991). Studies have shown that fibronectin and laminin differentially affect melanogenesis in cultures of avian neural crest cells (Rogers *et al., 1990*). It has also been demonstrated that when tail bud mesenchyme of chick embryos is cultured upon different ECM components it is able to differentiate into various cell types. For example, laminin promoted the differentiation of neural crest derived cells and neuroepithelial cells while type I collagen promoted myogenesis and chondrogenesis (Griffith and Sanders, 1991). It is therefore evident that cell matrix interactions play an important role during morphogenesis.

D. Lectins

Lectins are proteins, other than enzymes and antibodies, that bind specifically to carbohydrates (Barondes, 1986). Lectins are usually detected by their ability to agglutinate mammalian erythrocytes. A large number of animal lectins have now been described in embryonic and adult tissues (Barondes, 1986). Many of the lectins isolated from vertebrate tissues fall into one of two distinct structural classes (Drickamer, 1988). The first group, the C-type lectins, is a large family of both integral membrane and secreted proteins that require calcium for carbohydrate binding. The second class, the S-

type lectins, consists of smaller, calcium independent lectins characterized by their solubility in aqueous solution and their preferential binding to lactosamine structures (Hughes, 1992). Although a number of soluble vertebrate lectins have now been purified and characterized, the function of these lectins is still not fully understood (Barondes, 1986). Some lectins have been shown to interact with extracellular matrix proteins. For example, laminin appears to be a major glycoprotein ligand for a lactose binding lectin in differentiating mouse muscle cells (Cooper *et al.*, 1990, 1991). Studies using cultured cells have demonstrated a possible growth regulatory role for lectins. A ß-D galactoside-specific lectin purified from 14-day old chick embryos enhances chondrogenesis of cultured chick limb bud cells (Matsutani and Yamagata, 1982). Pitts and Yang (1981) have demonstrated that a ß-galactoside binding lectin from chick embryo kidney shows mitogenic activity towards lymphocytes. Studies have also indicated that ß-galactoside specific lectins, such as lung galaptin, have mitogenic activity toward vascular cells (Sanford and Harris-Hooker, 1990).

The concentration of lectin found in some tissues changes dramatically during development (Barondes, 1986). In the dorsal skin of the chick embryo in which feathers form, lectin activity first increased during the period of dermal condensation and then it decreased during the development of feathers (Kitamura, 1980). In the developing muscle of the

chick embryo, a lactose specific lectin is present in the embryo and declines in the adult, but in the liver it is relatively sparse in the embryo and abundant in the adult (Barondes, 1986). It has been suggested that those lectins that are expressed during embryonic development may play a specific role in tissue organization during embryogenesis, but those lectins that predominate in adult tissues may mediate other functions of the fully mature tissue (Barondes, 1986).

Two galactoside binding lectins, the 16 kilodalton (kD) and 14 kD lectins have been identified in the developing chicken and extraembryonic membranes of the embryo. These lectins are present as early as the pre-incubated blastoderm stage (Zalik *et al.*, 1983 1989), and persist during development of the yolk sac (Mbamalu and Zalik, 1987, Guay, unpubl. results). The latter is an extraembryonic membrane that encloses the yolk and is involved in the transfer and processing of nutrients from the yolk to the embryo. Lectin activity increases at the stages when epibolic movements (movement of an entire sheet of cells) associated with the spreading of the yolk sac occur (Mbamalu and Zalik, 1987), and remains high throughout development (Guay, unpublished results).

In the gastrulating chicken embryo, the 16 kD and 14 kD lectins are present in the cells of the area pellucida and the area opaca, especially those cells undergoing migration and relocation (Zalik, 1991). The endodermal cells of the

area opaca, which are the precursors of the yolk sac, are rich in intracellular lectin (Sanders *et al.*, 1990). Lectin is also detected in matricular material at the cell surface (Sanders *et al.*, 1990). It has been suggested that the lectins are externalized by exocytosis at the leading edge of the migrating endoderm cells and could serve as a transitory substratum lining the subgerminal cavity (Zalik *et al.*, 1990, 1991).

After gastrulation, the lectins are also expressed in the external and internal limiting membranes of the neural tube, in the extracellular matrix surrounding the notochord and at the coelomic surface of the myocardium (Zalik, 1991, Zalik *et al.*, 1992). Cells of the neural crest that are dislodging from the ectoderm to migrate into the embryo and primordial germ cells of the germinal crescent are also rich in lectins (Didier *et al.*, 1990, Zalik, 1991). Although the blastoderm lectins have been isolated and purified (Zalik *et al.*, 1990), their role in embryogenesis has not been determined. Previous results, using plant lectins have shown that cell surface glycoconjugates bearing ß-D galactoside groups are present in the cells of the gastrulating chick embryo (Zalik and Cook, 1976). It is possible that the endogenous lectins interact with cell surface glycoconjugates that are detectable using plant lectins. The receptors for the endogenous lectins of the chick embryo remain to be isolated.

There are experiments suggesting that the ß-D galactoside binding lectins may play a role in cellular adhesion. Milos and Zalik (1982), measured adhesion in dissociated cell suspensions from the endoderm of the area opaca of the primitive streak embryo. They reported that suspension of cells in which low levels of lectin activity were detected in the medium, underwent rapid aggregation. In these cells a decrease in aggregation is observed when purified blastoderm lectin is added to the aggregation assay. Secondly, when cells are maintained at room temperature, their adhesive ability decreases. This decrease in adhesion is associated with an accumulation of lectin activity in the medium. Lastly, the inhibition of adhesion in the latter cells can be counteracted if either thiodigalactoside (TDG), a hapten inhibitor of the blastoderm lectin, or desialized fetuin, a glycoprotein with terminal galactose groups are added to the medium (Milos and Zalik, 1982). It has been suggested that when the concentration of active extracellular lectin is high, lectin molecules may bind to and mask the ß-D galactoside containing cell surface receptor important in adhesion. At low extracellular lectin concentrations the receptor may bind to a lectin molecule on an apposing cell surface and contribute to an adhesive bond (Milos and Zalik, 1982). Although experiments using cell suspensions indicate that the presence of lectin in the medium affects adhesion of presumptive extraembryonic cells, further information on the

role that these molecules may play in early embryonic development is needed.

The present series of experiments represents an approach to determine the physiological roles of these lectins in early chick development, particularly in the process of gastrulation. The effects of lectin hapten inhibitors and anti-lectin antibodies on early and late gastrulating embryos cultured in defined medium were determined.

Materials and Methods

I. Culture of Chick Blastoderms on Defined Medium

A. Preparation of Solutions

Modified Pannett and Compton's (1924) Saline (PCS) consisted of 4.8 g. NaCl; 0.62 g. KCl; 0.31 g. $CaCl_2$; 0.51 g. $MgCl_2$ and 4.0 g. Hepes (N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)) (Sigma: cell culture tested); dissolved in 1 liter of double distilled water. The pH was adjusted to 7.5 with HCl and sterilized. In this saline, hepes has been added instead of phosphate.

Preparation of the defined medium is as follows. Roswell Park Memorial Institute (RPMI) 1640 Medium containing 25 mM hepes and sodium bicarbonate (Sigma) was modified as follows. To 100 ml of medium, the following compounds were added: L-glutamine (Sigma) to a concentration of 2 mM; 200 µg/ml of gentamicin solution (Sigma); 50 µg/ml of conalbumin (Sigma). This defined medium can support embryo development for up to 48 hours, incubation at 37°C, 1.2% CO_2 and 95% humidity (figure 12).

B. Preparation of Treatments for Cultured Blastoderms

The haptens utilized in this study were maltose and thiodigalactoside (TDG). Both were purchased from Sigma. Maltose and TDG were dissolved immediately before use in defined medium to the appropriate concentration.

The neoglycoproteins, bovine serum albumin (BSA), maltosylated-BSA, and lactosylated-BSA, all purchased from

Sigma, were dissolved immediately before use in defined medium to the appropriate concentration.

Nonimmune rabbit IgG (Sigma) and purified anti-16 kd polyclonal IgG (See procedure C and D) were dissolved in defined medium (that contained an additional 150 µg/ml conalbumin) immediately before use to the appropriate concentration.

All media were subjected to sterile filtration prior to use.

C. Preparation of the 16 kD Lectin Antiserum

The 16 kD lectin preparations used for preparation of the 16 kD antiserum was obtained from livers of adult laying hens, homogenized and purified by affinity chromatography on aminophenyl-ß-D-lactoside (APL) sepharose (Sigma) (Zalik *et al.*, 1983, 1990). The antiserum was raised in rabbit. The antiserum to the 16 kD lectin was raised against the 16 kD band cut from sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels in which the affinity purified liver lectin was separated. Approximately 140 µg of protein was injected subcutaneously 4 times at monthly intervals. For the first injection the gel band was freeze dried, ground, and suspended in Freund's complete adjuvant, subsequent injections were given in incomplete adjuvant and antiserum was collected two to three weeks after the last injection. For immunoblot analysis the anti-16 kD lectin

antiserum was used at dilutions of 1:1000 (Zalik *et al.*, 1990).

D. Purification of Anti-16 kD IgG Using Protein A

The purification of IgG was performed according to the manufacturer's instructions using the Biorad Econo-Pac Protein A Kit #732-2020, with the following modifications.

The 16 kD antiserum sample was dialyzed in 50 times its volume of binding buffer for one and half hours at 4°C. The concentrated sample was then added to the protein A column and incubated for two hours at room temperature. The pH of the acid eluants of IgG was neutralized with NaOH. Subsequently, the eluted fractions were dialyzed against PCS and concentrated using Amicon centriprep concentrators.

E. Culture of Blastoderms on the Vitelline Membrane

Fertilized eggs from White Leghorn chickens were obtained from the University of Alberta Poultry Farm. Embryos were harvested from both pre-incubated eggs and eggs incubated for 18 hours at 38°C and cultured according to the procedure described by New (1955) with several modifications as follows.

Sterile technique was used throughout the following procedure. The egg was rinsed with 70% alcohol and opened onto a modified egg separator (separating the yolk from the albumen). A hole 3 cm. in diameter was cut in No.1 Whatman filter paper 4.25 cm. in diameter. The paper was placed on

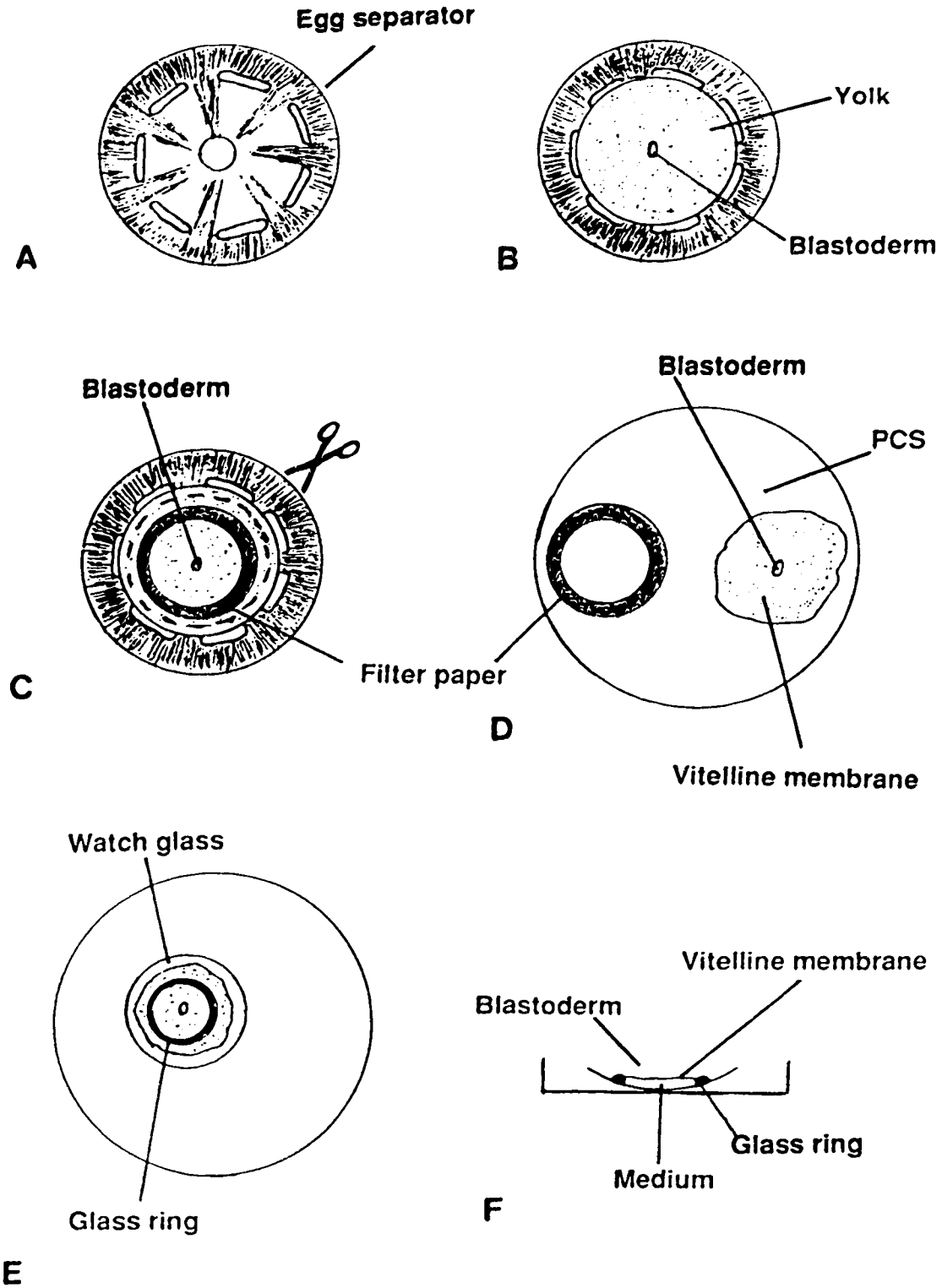
the yolk with the hole centered around the blastoderm. The vitelline membrane was then cut around the external periphery of the filter paper. Using forceps, the filter paper with the attached vitelline membrane and blastoderm were slowly removed from the yolk and submerged in a petri dish containing cool (10-15°C) PCS. The filter paper and associated vitelline membrane and embryo were left to soak in PCS for approximately two minutes to facilitate removal of the vitelline membrane from the filter paper and to remove any excess yolk. Once the vitelline membrane was removed from the filter paper, it was placed with the blastoderm uppermost on a 5 cm. watch glass. A glass ring (2.5 cm diameter) was then centered around the embryo. The free edges of the vitelline membrane were folded around the ring so that the membrane was reasonably tight and flat (Figure 9). To prevent the embryo from drowning, as much saline from above the membrane was removed. The saline under the vitelline membrane was then replaced with 1 ml. of defined medium containing the appropriate compound. Once the preparation was completed, the watch glass was placed in a 100 x 15 mm. sterile petri dish and incubated for up to 48 hours at 37°C, 1.2% CO_2 and 95% humidity.

F. Harvesting of Embryos

After 48 hours, the embryo cultures were rinsed briefly with 37°C PCS. The embryos were observed and photographed

Figure 9.

Diagrammatic representation of the technique used for culturing whole blastoderms. A) egg separator B) egg opened on to egg separator C) filter paper ring centered on the yolk around the embryo; the vitelline membrane is cut around the external periphery of the filter paper ring D) the vitelline membrane and attached embryo are immersed in PCS to remove the filter paper and any excess yolk E) the vitelline membrane and attached embryo are placed on the watch glass ventral side up and the glass ring is centered around the embryo F) the watch glass is placed on a sterile 15 X 100 petri dish and the edges of the vitelline membrane are wrapped tightly around the glass ring. Any excess PCS is removed, and the medium is pipetted under the membrane.


Egg separator
A
Yolk
Blastoderm
B
Blastoderm
C
Filter paper
Blastoderm
PCS
D
Vitelline membrane
Watch glass
Glass ring
E
Vitelline membrane
Blastoderm
Glass ring
Medium
F

with a Zeiss stereomicroscope. Photograph were taken with Kodak technical pan 2415 film.

II. Fluorescent Staining

After the embryos had been observed, they were fixed in freshly prepared 3.7% paraformaldehyde in PCS for 1 hour at 4°C. Following fixation, the embryos were washed briefly with phosphate buffered saline (PBS: 0.15M NaCl in 0.005M $NaKPO_4$ buffer, pH 7.1) at room temperature, and dehydrated sequentially in 50%, 75% and 100% aqueous polyethelene glycol (PEG) 400 (Sigma): blastoderms remained in each PEG solution for 30 minutes. Blastoderms were then transferred to a 1:1 (v/v) mixture of PEG 400/PEG 1000 at 45°C for 30 minutes, followed by PEG 1000 and by PEG 1500 at 45°C each for 1 hour. Embryos were then embedded in PEG 1500 and sectioned (5 µm) on a Reichert-Jung microtome. Sections were mounted on slides coated with rubber cement (Lepage's) thinned with ethyl acetate and kept at room temperature until stained (Zalik *et al.*, 1987).

To compare the overall cell arrangement of control and experimental embryos, sections were stained with DAPI (4-6-diamino-2-phenylendole), a drug that binds to DNA (Coleman *et al.*, 1981, Russel, 1975). After treatment for 2 minutes in acetone, sections were washed with three changes, 5 minutes each, of PBS. The sections were then incubated for 1 minute in 0.5 µg/ml. DAPI in PBS in the dark. The sections were then briefly washed with PBS and mounted in

Mowiol mounting medium (Osborn and Weber, 1982) (Mowiol 4-88 Calbiochem) modified by the addition of 0.5 g. DABCO (2-4-diazabicyclo (2,2,2)octane, Sigma) per 24 ml. of Mowiol solution. Sections were observed with a Zeiss photo-microscope. Photographs were taken with Kodak Tmax film P3200 film.

Results

I. Embryos cultured in defined medium

Although embryos can be cultured on thin albumen, albumen contains many proteins and glycoproteins which may overshadow the effect of the addition of any possible inhibitor of the lectin. A defined medium was therefore developed to provide the minimum amount of nutrients necessary for relatively normal embryonic growth within a time period. It was reasoned that these culture conditions would allow us to detect the effects of agents added to the medium on embryonic development.

A. Stage XI (EG & K) embryos cultured with defined medium vs. albumen

Stage XI (EG & K) embryos (figure 10) were cultured on thin albumen and compared to stage XI (EG & K) embryos cultured on defined medium to determine if the defined medium provided sufficient nutrients necessary for proper development.

Stage XI (EG & K) embryos cultured on thin albumen for 24 hours at 37°C developed to stage 4+ (H & H) (figure 11). The primitive streak developed to its maximum length, the area opaca and area pellucida were well defined and the blastoderm increased from a width of 4 mm. and a length of 4 mm. to an average width of 8 mm. and length of 8.2 mm.

Stage XI (EG & K) embryos cultured on defined medium also developed to stage 4 (H & H) (figure 12). In this medium

Figure 10.

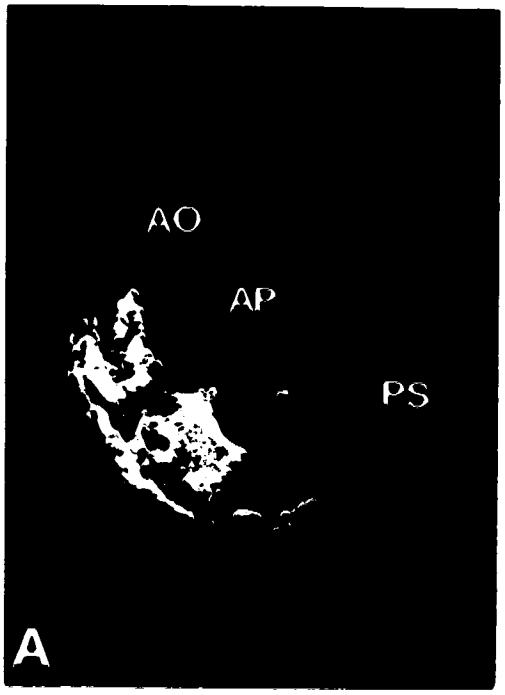
A & B) Unincubated embryos (stage XI, EG & K) prior to culturing with various treatments. (AP, area pellucida: AO, area opaca). The posterior section of the area pellucida in figure (A) is covered with yolk. A) Magnification 35x B) Magnification 46x


AP
A


B

Figure 11.

A & B) Stage 4+ (Hamburger and Hamilton, 1951) embryos that have been cultured for 24 hours with thin egg albumen from initial stage XI (EG & K) embryos. Further elongation of the area pellucida (AP), presence of the primitive streak (PS), and spreading of the area opaca (AO) are evident. A) Magnification 22x B) Magnification 25x


AO
AP
PS
A

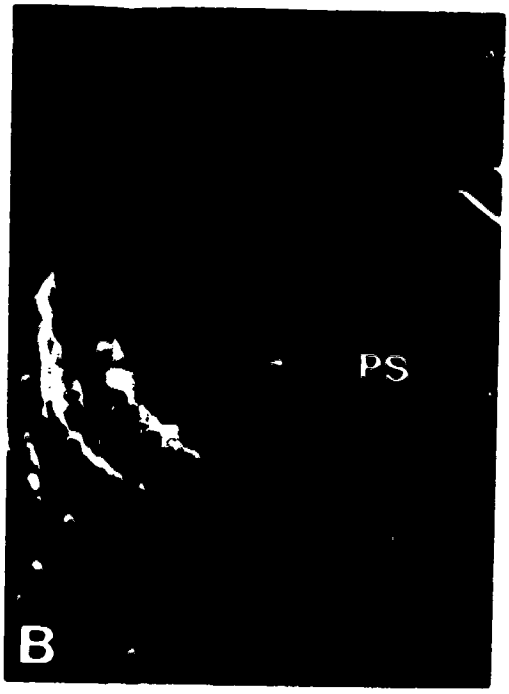
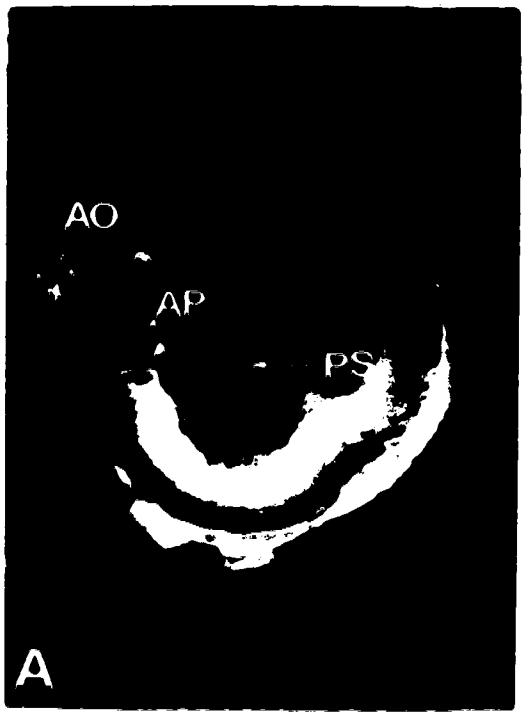
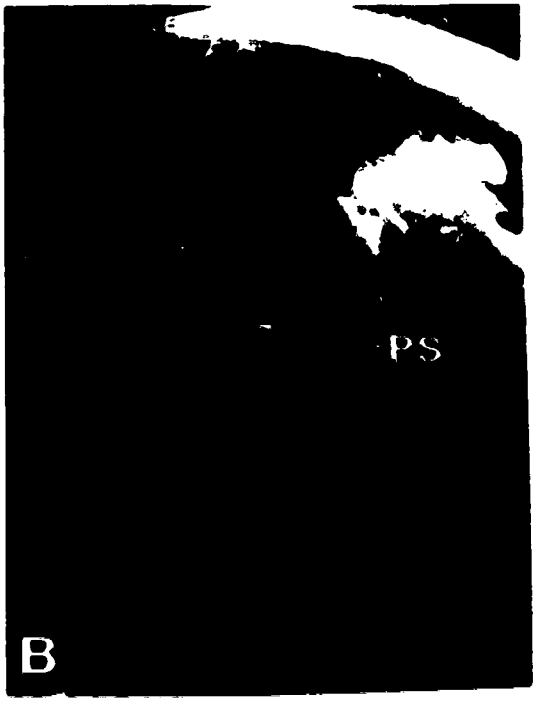
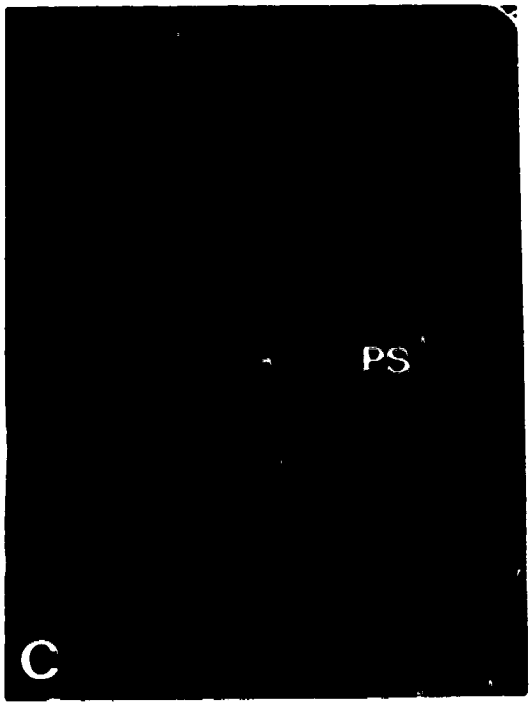

PS
B

Figure 12.

A,B & C) Stage 4 (Hamburger and Hamilton, 1951) embryos that have been cultured for 48 hours with defined medium from initial stage XI (EG & K) embryos. Further elongation of the area pellucida (AP), presence of the primitive streak (PS), and spreading of the area opaca (AO) are evident.
A) Magnification 28x B) Magnification 26x (The creases in this photograph are due to the fact that this embryo was photographed below the vitelline membrane.) C) Magnification 25x


AO
AP
PS
A


PS
B


PS
C

it was necessary to culture the embryos at 37°C with 1.2% CO_2 for 48 hours. Although growth was slower, after 48 hours, the embryos had well formed primitive streaks, well differentiated area opacas and area pellucidas, and the blastoderm increased from a width of 4 mm. and a length of 4 mm. to an average width of 7.5 mm. and length of 7.8 mm.

B. Stage 4 (H & H) embryos cultured with defined medium vs. albumen

Stage 4 (H & H) embryos (figure 13A) cultured on thin albumen for 24 hours at 37°C developed to stage 11 or 12 (H & H) (figure 13B). The optic vesicles were well formed and the brain was becoming regionalized. The heart was well developed and beating and usually 12 somites were visible (see figure 14 for reference).

Stage 4 (H & H) embryos cultured on defined medium for 48 hours at 37°C with 1.2% CO_2; developed to stage 12-12+ (H & H) (figure 13C). The optic vesicles were well developed, the brain had begun to regionalize although, in 80% of the embryos. However, the neural tube was characteristically narrow in embryos cultured in defined medium when compared with those cultured in albumen. Usually 8-10 somites were visible, although these did not appear as discrete.

II. Effect of Thiodigalactoside on Embryonic Development

These experiments were conducted in order to determine whether TDG, the most effective hapten inhibitor of the

Figure 13.

A) Stage 4+ (Hamburger and Hamilton, 1951) embryo that has been incubated for 20 hours *in ovo*, prior to culturing with various treatments. (area pellucida, AP: area opaca, AO: primitive streak, PS) Magnification 24x

B) Stage 4 (Hamburger and Hamilton, 1951) embryo that has been cultured for 24 hours with thin egg albumen. The embryo has reached stage 12 (Hamburger and Hamilton, 1951). The neural tube (NT), heart (H) and somites (S) are well defined. (see figure 14) Magnification 50x

C & D) Stage 4 (Hamburger and Hamilton, 1951) embryos that have been cultured for 48 hours with defined medium. The embryo has reached stage 12 (Hamburger and Hamilton, 1951). The neural tube (NT) and the heart (H) are well formed although the somites (S) are not as well defined.
C) Magnification 53x D) Magnification 63x

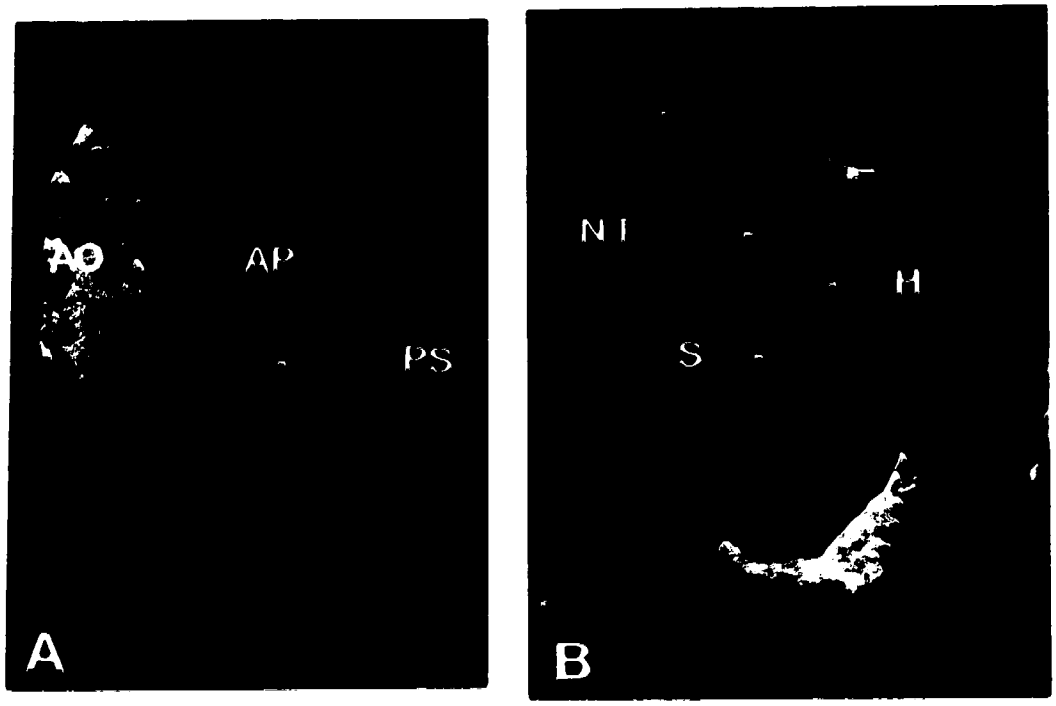
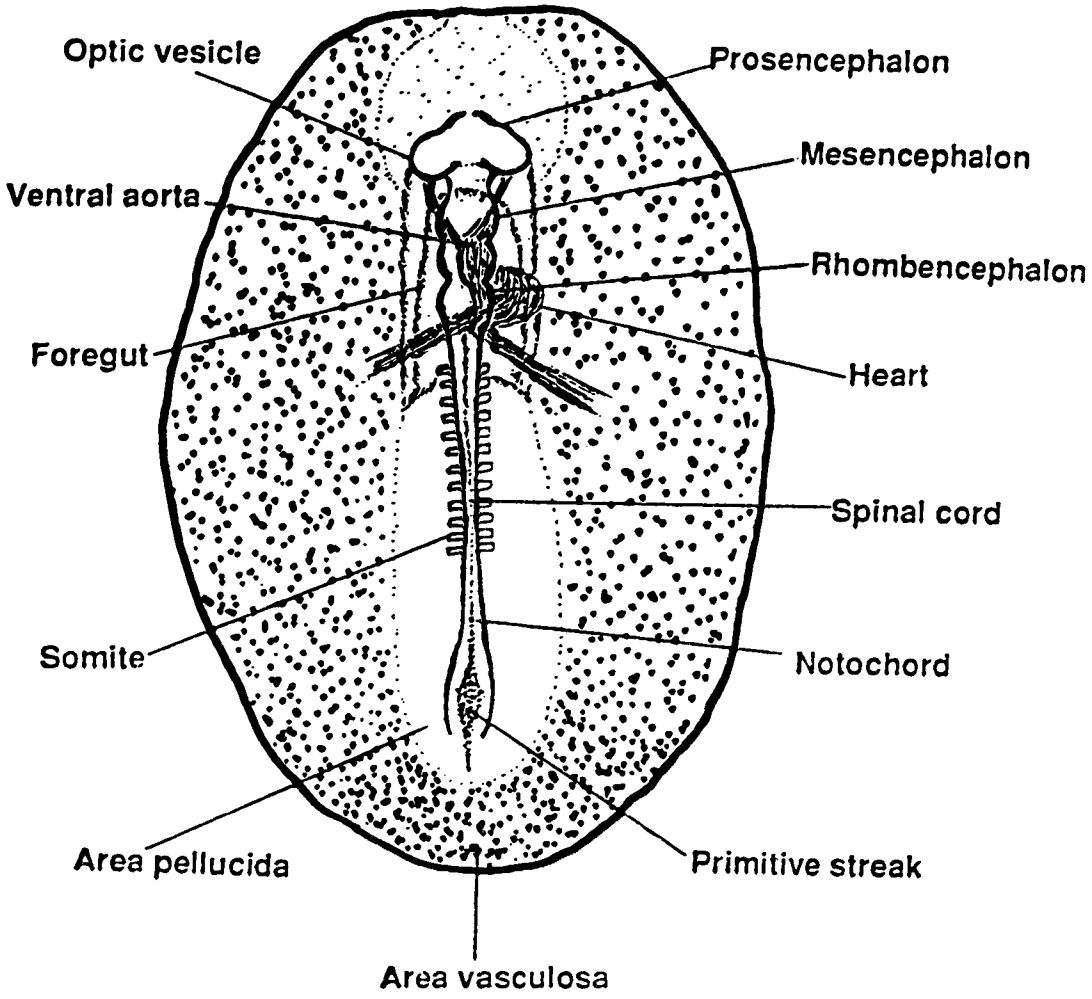

AO
AP
PS
A
NT
H
S
B
NT
H
S
C
D

Figure 14.

Diagrammatic representation of a stage 12 (Hamburger and Hamilton, 1951) embryo showing major morphological features. (Redrawn from Gilbert, 1991).


Optic vesicle
Prosencephalon
Mesencephalon
Ventral aorta
Rhombencephalon
Foregut
Heart
Spinal cord
Somite
Notochord
Area pellucida
Primitive streak
Area vasculosa

endogenous blastoderm lectins, had any effects on development. Control embryos were cultured in defined medium alone or in defined medium in the presence of maltose at the same concentration as TDG. Preliminary experiments were conducted in order to establish the effects of maltose on development and determine concentrations of this sugar that were not deleterious to further development. A final concentration of 0.05M was used in the control embryos.

A. Stage XI Embryos

Stage XI (EG & K) embryos cultured for 48 hours with 0.05M maltose developed into stage 4 (H & H) embryos (figure 15). The appearance of these embryos was similar to those embryos cultured on defined medium alone. The area opaca and area pellucida were well differentiated, a primitive streak characteristic of stage 4 embryos was also present. The embryos increased in size from a width of 4 mm. and length of 4 mm. to an average width of 7.2 mm. and length of 7.6 mm.

Stage XI (EG & K) embryos cultured for 48 hours with 0.05M TDG were altered in their development (figure 16). At this concentration, stage XI (EG & K) embryos failed to progress to stage 4 (H & H)). These embryos failed to develop a primitive streak, although sometimes an ill defined cell mass in the center of the blastoderm could be distinguished. There was poor demarcation between the area pellucida and the area opaca, and the area opaca appeared to be retracting (table 1). From a starting width of 4 mm. and length of

Figure 15.

A,B & C) Representative stage XI (EG & K) embryos that have been cultured for 48 hours with defined medium and 0.05M maltose. Embryos have developed to stage 4 (Hamburger and Hamilton, 1951). The well defined area pellucida (AP), area opaca (AO) and primitive streak (PS) are evident. (In figure (A) the posterior portion of the primitive streak is covered by a yolk aggregate.) A) Magnification 24x B) Magnification 23x C) Magnification 23x

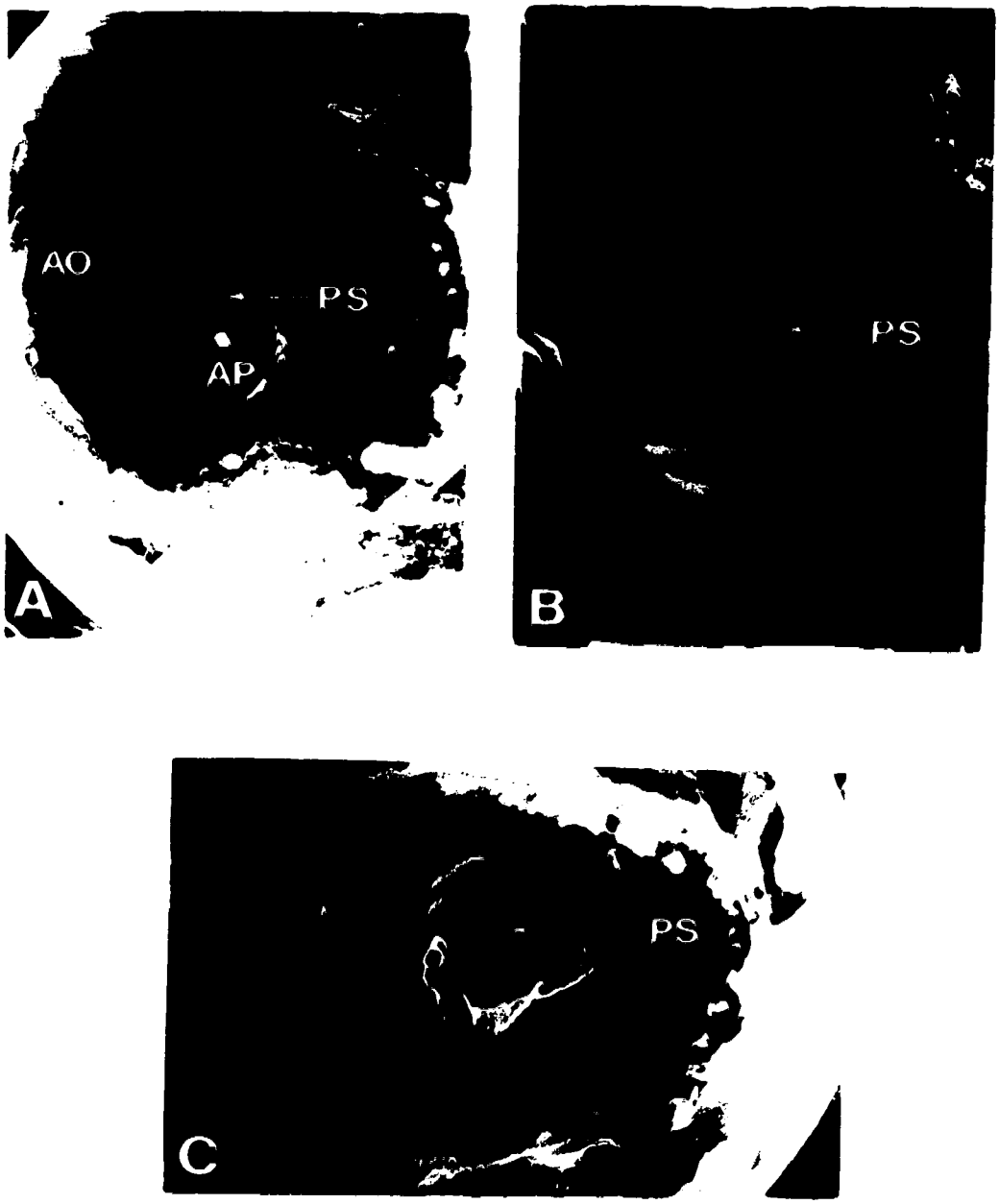

AO
PS
AP
A
PS
B
PS
C

Figure 16.

A,B,C & D) Stage XI (EG & K) embryos that have been cultured for 48 hours with defined medium and 0.05M TDG showing abnormal development. Observe the lack of expansion of the area opaca (AO) as evidenced by the embryo diameter. At times the area pellucida (AP) has completely retracted as in figure D. The primitive streak is absent but sometimes an ill defined cell mass (arrow) is present on the area pellucida. A) Magnification 17x B) Magnification 17x C) Magnification 18x D) Magnification 17x

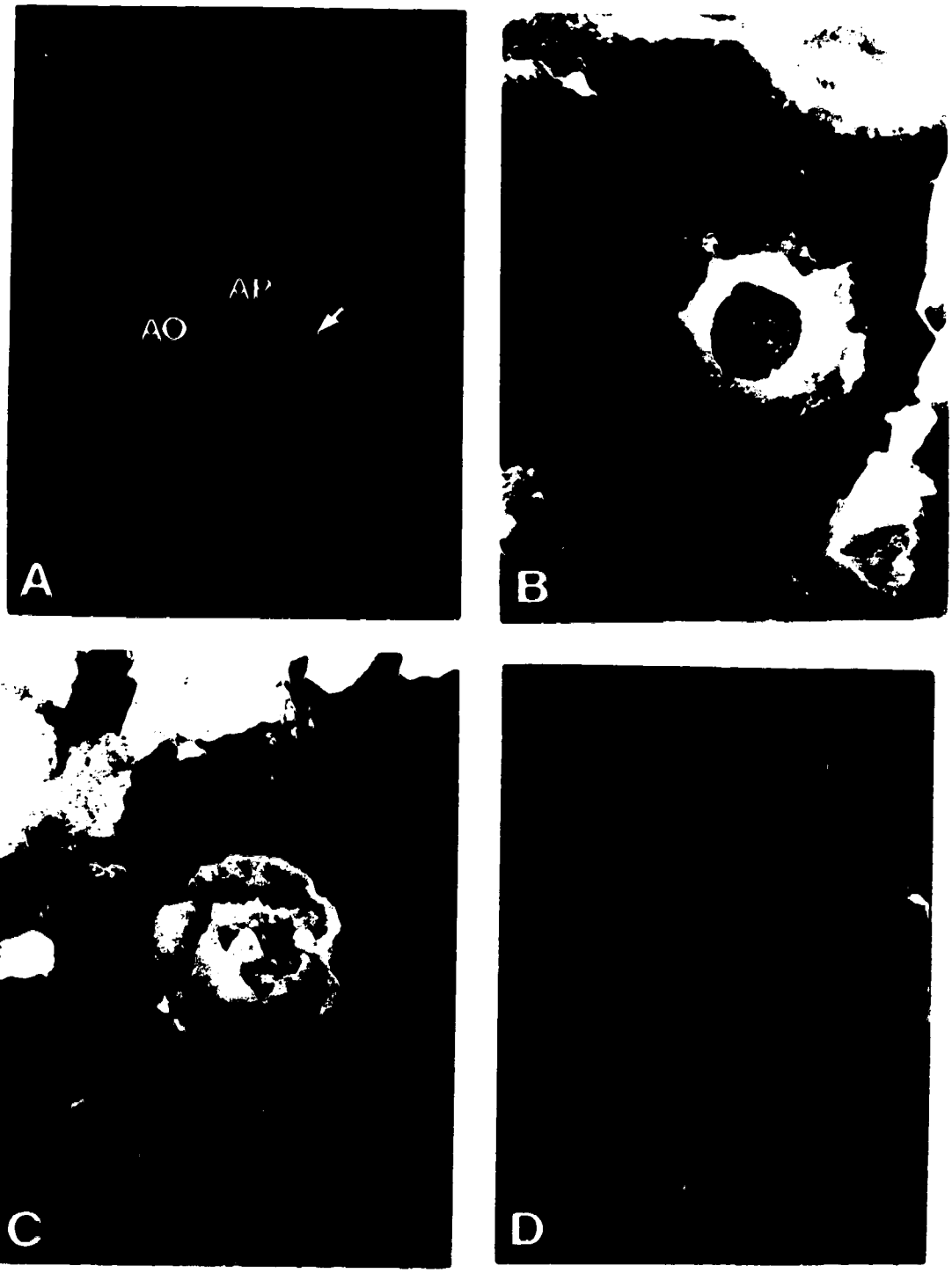

AP
AO
A
B
C
D

Table 1.

Comparison of characteristics of stage XI (EG & K) embryos treated with haptens and cultured for 48 hours. (PS: primitive streak, AP: area pellucida, AO: area opaca). The average size of the embryos after 48 hours is a measurement of the width and length.

Treatment	Number of Embryos Treated	Average Size after 48 hours	% of embryos with PS	% of embryos with AP & AO
DM	16	7.1 x 7.4 mm	100%*	100%
0.05M Maltose	18	7.2 x 7.6 mm	100%*	100%
0.05M TDG	23	5.1 x 5.0 mm	0%**	30%

* Stage 4-4+ PS present
** Mass present on AP 22% of the time

4 mm., the embryos on average only increased to a width of 5.1 mm. and length of 5.0 mm (figure 17). These embryos were significantly smaller ($P<0.0001$; t-test with 99% confidence limits) than the control embryos.

Transverse sections of stage XI (EG & K) embryos cultured for 48 hours with 0.05M maltose and 0.05M TDG were stained with DAPI, which stains the nuclei of the cells. The cell arrangements of these embryos were then compared. The transverse section through the center of the embryo treated with maltose (figure 18) shows the differentiation of the epiblast. The cells in this area have spread out in an uniform layer. In the transverse section through the center of the embryo treated with TDG (figure 19), the cells of the blastoderm have failed to spread and as a result the embryo is thicker and more compact.

B. Stage 4 (H & H) Embryos

Stage 4 (H & H) embryos cultured for 48 hours with 0.035M TDG developed into well formed stage 12 embryos. In these embryos, maltose was used at a concentration of 0.035M, higher concentrations of this sugar inhibited regionalization of the neural tube. There was no distinguishable differences between the TDG treated embryos and the control embryos (figure 20). Therefore, under the present experimental conditions, TDG appeared to have no effect on the development of stage 4 embryos, when compared to the maltose treated embryos.

Figure 17.

Histogram represents the mean increase in the width (w) and length (l) of stage XI (EG & K) blastoderms after 48 hours of culture with hapten treatments (0.05M maltose, 0.05M TDG and defined medium: DM). Average initial size of embryos was width=4 mm. and length=4 mm. Embryo growth is a measurement of the initial width and length of the embryo subtracted from the final width and length. Error bars represent standard error of the mean.

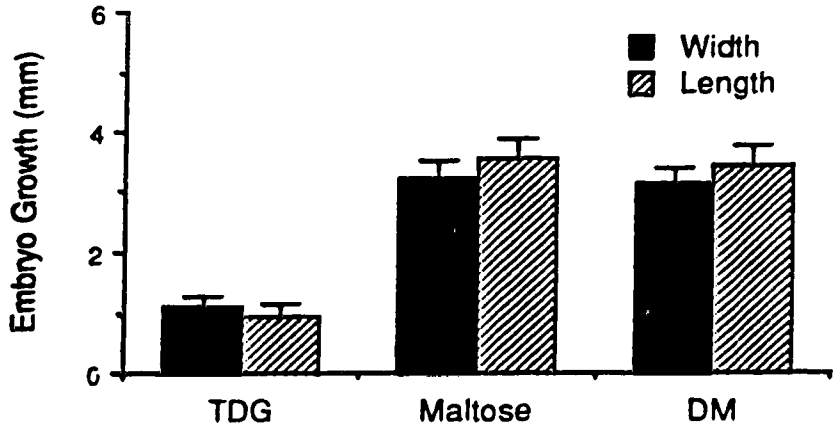

Embryo Growth (mm)
6
4
2
0
Width
Length
TDG
Maltose
DM

Figure 18.

Transverse section through the area pellucida of a stage XI (EG & K) blastoderm cultured for 48 hours with 0.05M maltose and stained with DAPI. The cells of the blastoderm have spread out in an uniform layer. Bar is equal to 20 µm.

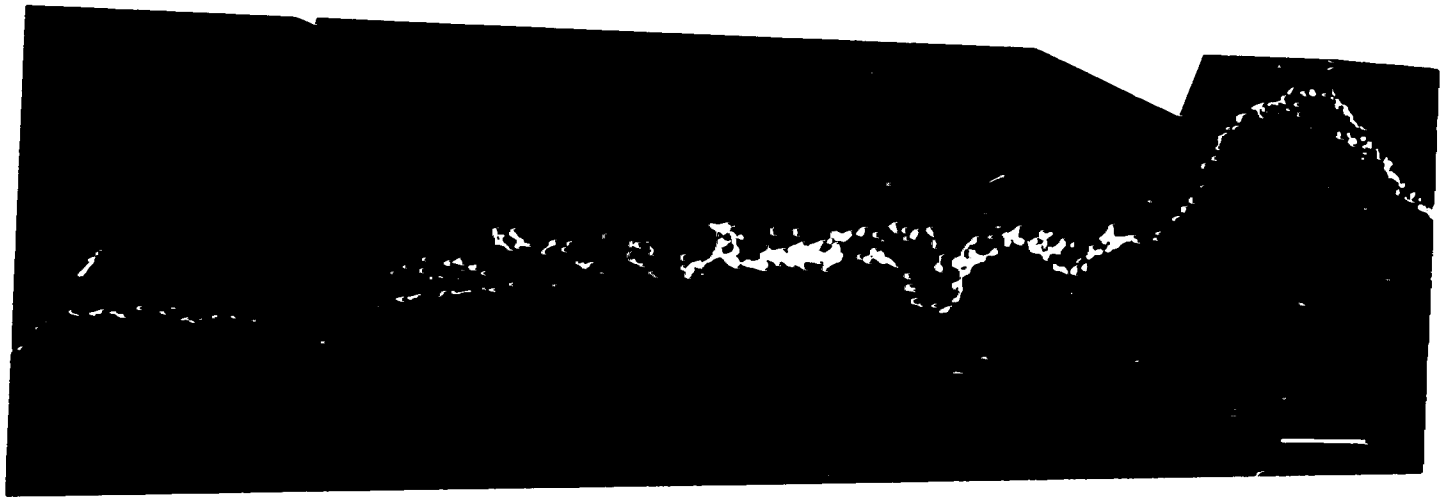


Figure 19.

Transverse section through the center of a stage XI (EG & K) blastoderm cultured for 48 hours with 0.05M TDG and stained with DAPI. It is evident that the cells of the blastoderm have failed to spread and as a result the embryo is thicker and more compact. Bar is equal to 20 μm.

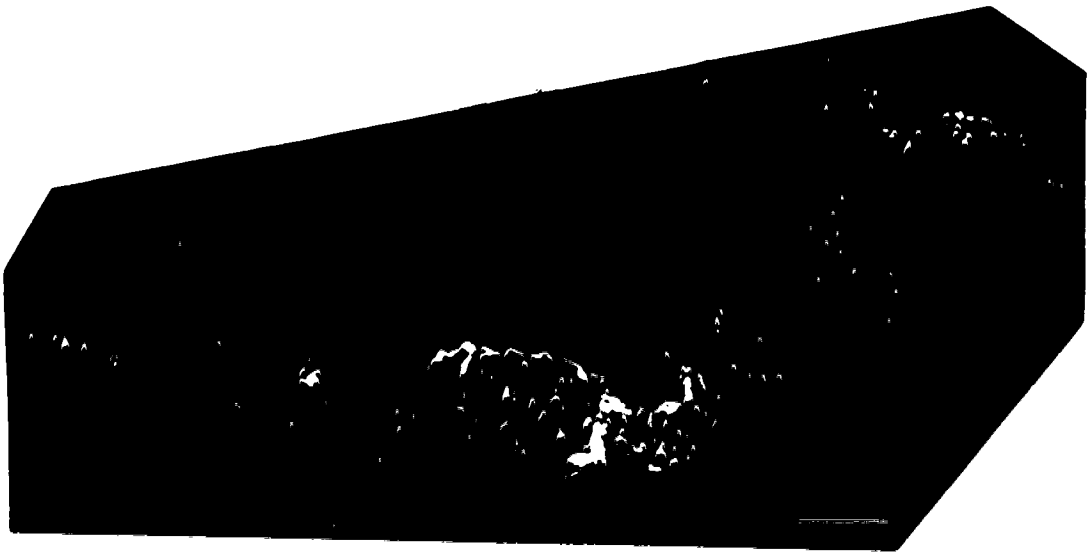
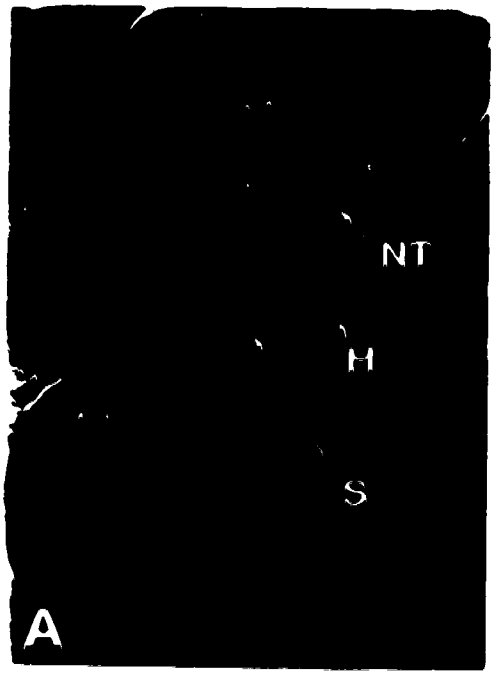


Figure 20.

A) Stage 4 (Hamburger and Hamilton, 1951) embryo that has been cultured for 48 hours with defined medium and 0.035M maltose. Embryo has progressed to stage 12+ (Hamburger and Hamilton, 1951). The well defined neural tube (NT), heart (H) and somites (S) are evident. Magnification 40x

B) Stage 4 (Hamburger and Hamilton, 1951) embryo that has been cultured for 48 hours with defined medium and 0.035M TDG. The embryo shows similar characteristics as that shown in A. Magnification 48x


NT
H
S
A

III. Effect of Neoglycoproteins on Embryonic Development

A synthetic glycoprotein containing terminal galactose (lactosylated-bovine serum albumin: l-BSA) was added to embryo cultures to determine its effects on development. Controls consisted of embryos cultured in defined medium alone, embryos cultured in the presence of BSA, or embryos cultured in the presence of maltosylated-BSA (m-BSA). Preliminary experiments were conducted to establish the concentrations of BSA and control neoglycoproteins in the medi that did not induce deleterious effects on development. A concentration of 500 µg/ml of m-BSA was used in the controls.

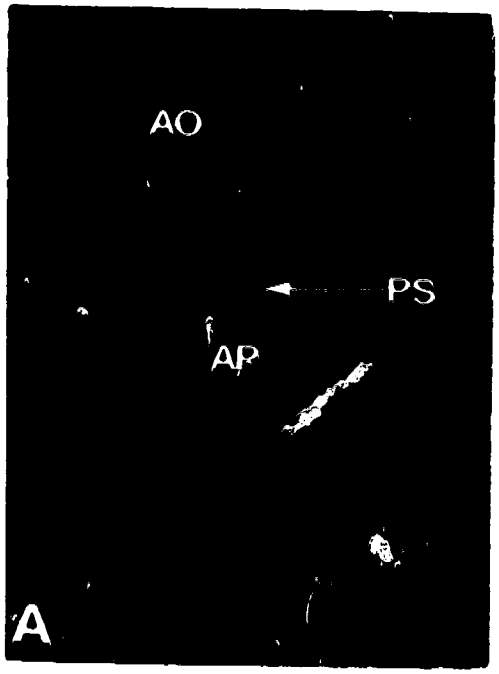
A. Stage XI (EG & K) Embryos

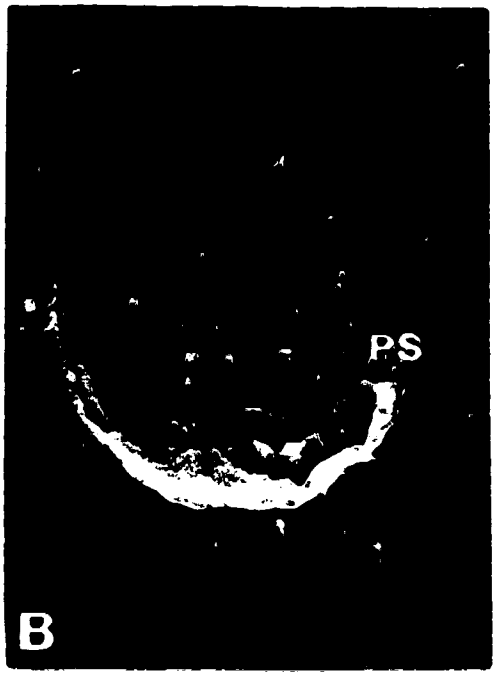
Embryos treated with BSA (500 µg/ml) alone or m-BSA (500 µg/ml) developed into well formed stage 4 (H & H) embryos (figure 21 and 22). Embryo development was comparable to embryos cultured on defined medium alone.

Embryos treated with l-BSA (500 µg/ml) were perturbed in their development. After 48 hours, these embryos resembled those treated with TDG (figure 23). These embryos onlyincreased in size from a width of 4 mm. and a length of 4 mm. to an average width of 5.1 mm. and length of 5.5 mm. (figure 24). These embryos were significantly smaller ($P<0.0001$; t-test with 99% confidence limits) than the control embryos. There was no primitive streak formation, but

Figure 21.

A,B & C) Stage 4 (H & H) embryos that have been cultured for 48 hours with defined medium and bovine serum albumin (BSA) (500µg/ml) from initial stage XI (EG & K). The well defined area pellucida (AP), spreading of the area opaca (AO), and the primitive streak (PS) are evident.
A) Magnification 23x B) Magnification 28x (White spot on the primitive streak is yolk.) C) Magnification 27x

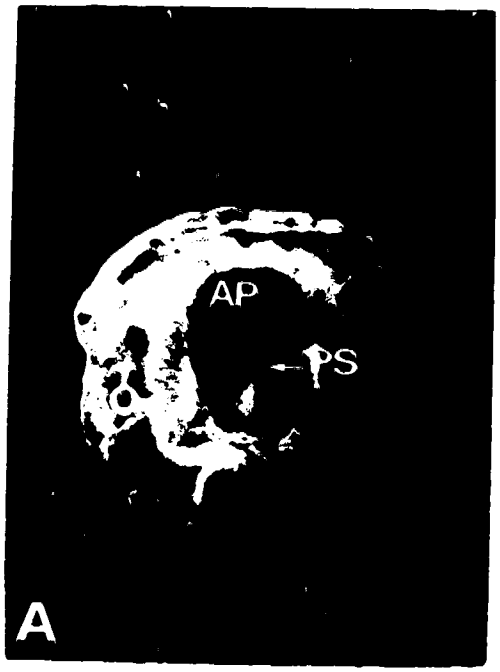

AO
PS
AP
A


PS
B


C

Figure 22.

A,B & C) Stage 4 (Hamburger and Hamilton, 1951) embryos that have been cultured for 48 hours with defined medium and maltosylated-BSA (500 µg/ml) from initial stage XI(EG & K) embryos. The well defined area pellucida (AP), the spreading of the area opaca (AO) resulting in the increase of the blastoderm, and the primitive streak are evident.
A) Magnification 28x B) Magnification 29x C) Magnification 30x


AP
PS
A


B


C

Figure 23.

A,B,C, & D) Embryos that have been cultured for 48 hours with defined medium and lactosylated-BSA (500 µg/ml) from initial stage XI (EG & K) embryos showing abnormal development. Observe the inhibition of expansion of the area opaca (AO) as evidenced by the embryo diameter. The primitive streak (PS) is absent and sometimes the area pellucida (AP) has completely retracted as in figure C. Arrow in figure D indicates an ill defined cell mass that is sometimes present on the area pellucida. A) Magnification 26x B) Magnification 28x C) Magnification 27x D) Magnification 23x

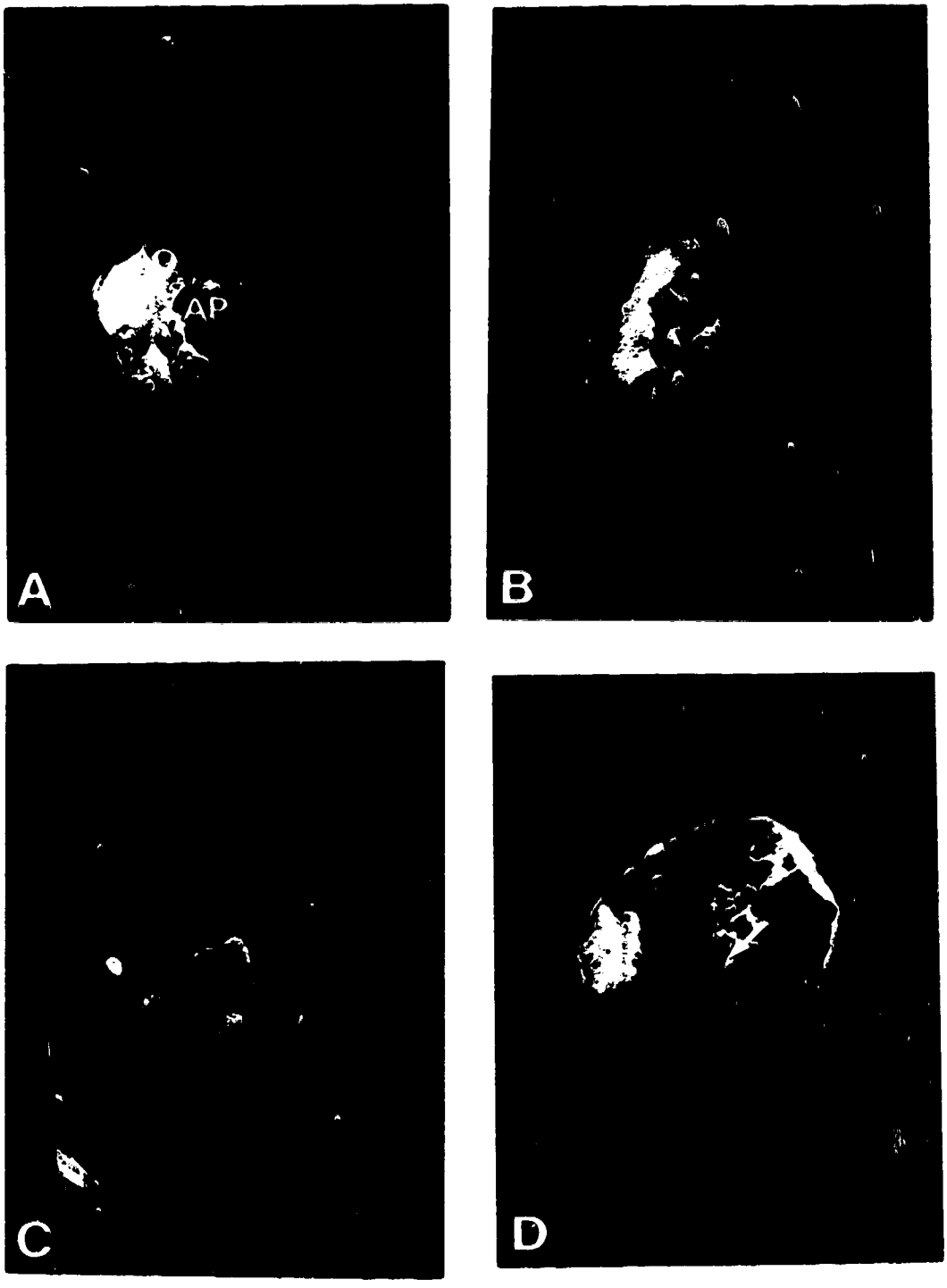
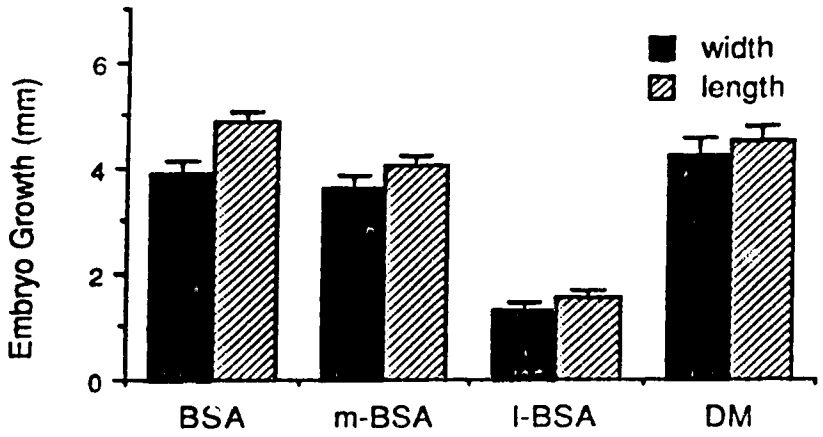

AP
A
B
C
D

Figure 24.

Histogram represents the mean increase in the width (w) and length (l) of stage XI (EG & K) blastoderms after 48 hours of culture in the presence of neoglycoproteins (i.e. maltosylated-BSA, lactosylated-BSA, and BSA all at a concentration of 500 µg/ml, and defined medium, DM). The average initial size of the stage XI (EG & K) blastoderms was width=4 mm. and length=4 mm. Embryo growth is a measurement of the initial width and length of the embryo subtracted from the final width and length. Error bars represent standard error of the mean.


Embryo Growth (mm)
6
4
2
0
width
length
BSA
m-BSA
I-BSA
DM

but an ill defined cell mass was sometimes present in the center of the blastoderm. Differentiation between the area opaca and area pellucida was not always evident (table 2).

B. Stage 4 (Hamburger and Hamilton, 1951) Embryos

Stage 4 (H & H) embryos cultured for 48 hours with 1-BSA, at concentrations of 500 to 600 µg/ml advanced to a well developed stage 12 (H & H) embryos. There was no distinguishable differences between the 1-BSA treated embryos and the control embryos (figure 25). Therefore, under these experimental conditions, 1-BSA appeared to have no effect on stage 4 (H & H) embryos.

IV. Effect of Anti-lectin IgG on Embryonic Development

Purified anti-16 kD lectin IgG was added to embryo cultures to determine if these antibodies had any effect on embryonic development. Control embryos were cultured in defined medium alone or in defined medium containing non-immune purified rabbit IgG (Sigma). For these experiments, the concentration of conalbumin in the medium was increased to 200 µg/ml. It was observed that the addition of normal IgG at a concentration of 600 µg/ml induced a delay in development of the embryos, when compared to the control embryos cultured in defined medium alone. This developmental delay could be overcome by increasing the concentration of conalbumin. Embryos cultured in defined medium with

Table 2.

Comparison of characteristics of stage XI (EG & K) embryos treated with neoglycoproteins and cultured for 48 hours. (PS: primitive streak, AP: area pellucida, AO: area opaca). The average size of the embryos after 48 hours is a measurement of the width and length.

Treatment	Number of Embryos Treated	Average size after 48 hours	% of embryos with PS	% of embryos with AP & AO
DM	21	8.2 x 8.5 mm	100%*	100%
BSA	24	7.9 x 8.8 mm	100%*	100%
m-BSA	26	7.6 x 8.0 mm	100%*	100%
l-BSA	28	5.1 x 5.5 mm	0%**	43%

* Stage 4-4+ PS present
** Mass present in center of AP 29% of the time

Figure 25.

A) Stage 4 (Hamburger and Hamilton, 1951) embryo that has been cultured for 48 hours with defined medium and BSA (600 µg/ml). Embryo has developed up to stage 12+ (Hamburger and Hamilton, 1951). The neural tube (NT) and heart (H) are well formed although the somites (S) are not as well defined. Magnification 61x

B) Stage 4 (Hamburger and Hamilton, 1951) embryo that has been cultured for 48 hours with defined medium and maltosylated-BSA (600 µg/ml). Embryo has developed to a stage 12+ (Hamburger and Hamilton, 1951) embryo exhibiting similar characteristics as in A. Magnification 63x

C) Stage 4 (Hamburger and Hamilton, 1951) embryo that has been cultured for 48 hours with defined medium and lactosylated-BSA (600 µg/ml). Embryo has developed to a stage 12+ (Hamburger and Hamilton, 1951) embryo exhibiting similar characteristics as in A. Magnification 66x


NT
H
A


B


C

concentrations of 200 µg/ml of conalbumin were similar to those cultured in defined medium containing 50 µg/ml of this glycoprotein. For these experiments, control embryos in defined medium contained conalbumin concentrations of 200 µg/ml.

A. Stage XI (EG & K) Embryos

IgG purified from the preimmune serum did not inhibit development (figure 26A) but due to the low amount of preimmune serum collected and for convenience, purified rabbit IgG purchased from Sigma was used as the control. Embryos treated with 600 µg/ml rabbit IgG developed to stage 4 (H & H), with a well formed primitive streak and increased in average size from a width of 4 mm. and a length of 4 mm. to a width of 8.1 mm. and a length of 8.8 mm (figure 26B).

At a concentration of 600 µg/ml, anti-16 kD IgG effected embryonic development (figure 27). After 48 hours, embryos failed to develop a primitive streak but the area opaca and area pellucida were still distinguishable (table 3). The blastoderms only increased from an initial width of 4 mm. and length of 4 mm. to a width of 5.0 mm. and length of 5.1 mm. (figure 28). These embryos were significantly smaller ($P<0.0001$; t-test with 99% confidence limits) than the control embryos.

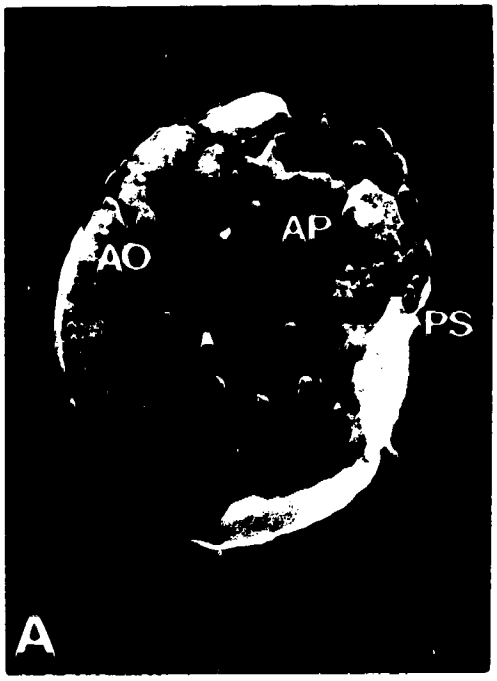
Transverse sections of stage XI (EG & K) embryos cultured for 48 hours with rabbit IgG (600 µg/ml) and anti-16 kD lectin IgG were stained with DAPI, which stains the nuclei

Figure 26.

A) Effect of preimmune rabbit IgG (600 µg/ml) on development of stage XI (EG & K) embryos after 48 hours of culture. The area opaca (AO), area pellucida (AP) and the primitive streak are evident. Magnification 33x

B) Effect of purified rabbit IgG (600 µg/ml) purchased from Sigma exhibits similar development as in A. Magnification 32x

Conalbumin concentration in the above cultures was increased to 200 µg/ml.


AO
AP
PS
A

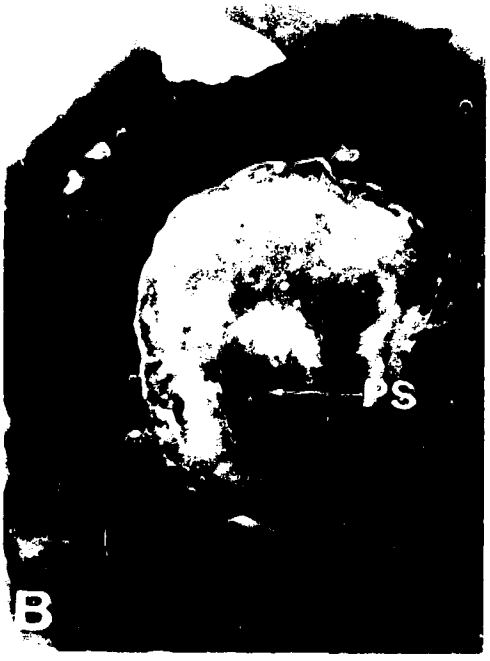
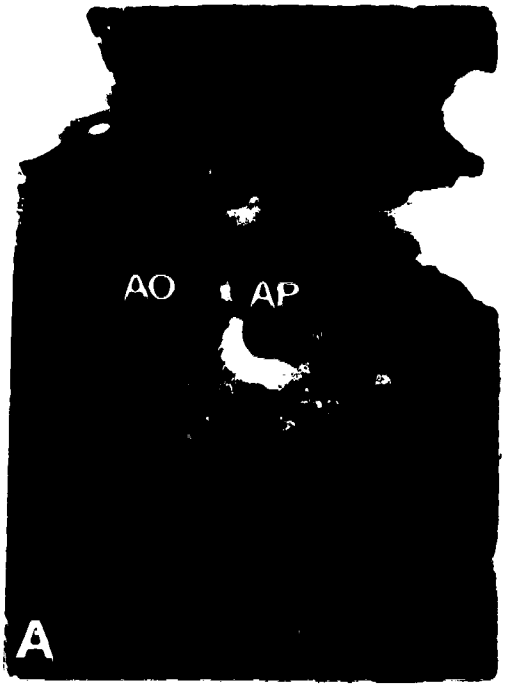

PS
B

Figure 27.

A,B & C) Effect of anti-16 kD IgG (600 µg/ml) on development of stage XI (EG & K) embryos, after 48 hours of culture. Conalbumin concentration was increased to 200 µg/ml. The area opaca (AO) has failed to spread and the primitive streak (PS) is absent but the area pellucida is still present. Arrow in figure C, indicates an ill defined cell mass. A) Magnification 18x B) Magnification 17x C) Magnification 16x


AO
AP
A


C

Table 3.

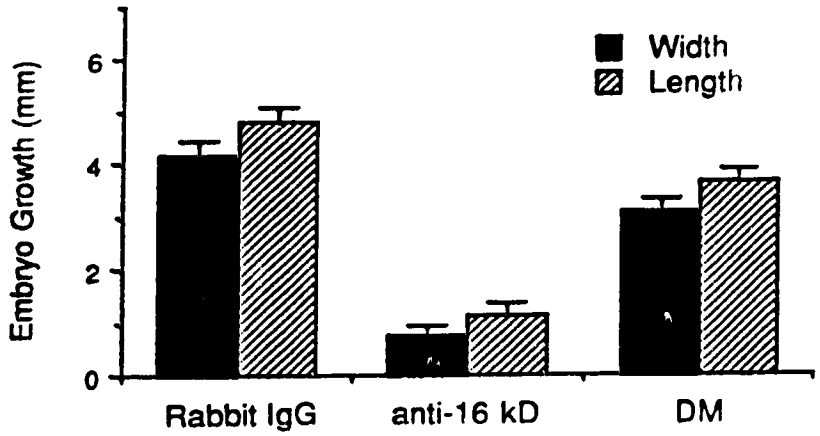
Comparison of characteristics of stage XI (EG & K) embryos treated with anti-16 kD lectin IgG and rabbit IgG and cultured for 48 hours. (PS: primitive streak, AP: area pellucida, AO: area opaca). The average size of the embryos after 48 hours is a measurement of the width and length.

Treatment	Number of Embryos Treated	Average Size after 48 hours	% of embryos with PS	% of embryos with AP & AO
DM	16	7.1 x 7.6 mm	100%*	100%
Rabbit IgG	14	8.1 x 8.8 mm	100%*	100%
Anti-16 kd IgG	15	5.0 x 5.1 mm	0%**	100%

* Stage 4-4+ PS present
** Mass on center of AP 30% of the time

Figure 28.

Histogram represents the mean increase in the width (w) and length (l) of stage XI (EG & K) blastoderms after 48 hours of culture with rabbit IgG (600 µg/ml), anti-16 kD lectin IgG (600 µg/ml), and defined medium (DM). The average initial size of stage XI (EG & K) embryos was width=4 mm. and length= 4 mm. Embryo growth is a measurement of the initial width and length of the embryo subtracted from the final width and length. Error bars represent standard error of the mean.


Embryo Growth (mm)
6
4
2
0
Width
Length
Rabbit IgG
anti-16 kD
DM

nuclei of the cells. The cell arrangements of these embryos were then compared. The transverse section through the center of the embryo treated with rabbit IgG (figure 29) shows the differentiation of the epiblast. The cells in this area have spread out in an uniform layer. In the transverse section through the center of the embryo treated with anti-16 kD lectin IgG (figure 30), the cells of the blastoderm have failed to spread and as a result the embryo is more compact.

B. Stage 4 Embryos

Stage 4 embryos cultured for 48 hours with 600 µg/ml anti-16 kD IgG developed to well formed stage 12 (H & H) embryos. There was no distinguishable differences between the anti-16 kD IgG treated embryos and the control embryos (figure 31). Above this concentration, non-immune rabbit IgG was deleterious to embryonic development.

Figure 29.

Transverse section through the area pellucida of a stage XI (EG & K) blastoderm cultured for 48 hours with rabbit IgG (600 µg/ml) and stained with DAPI. The cells of the blastoderm have spread out in an uniform layer. Bar is equal to 20 µm.

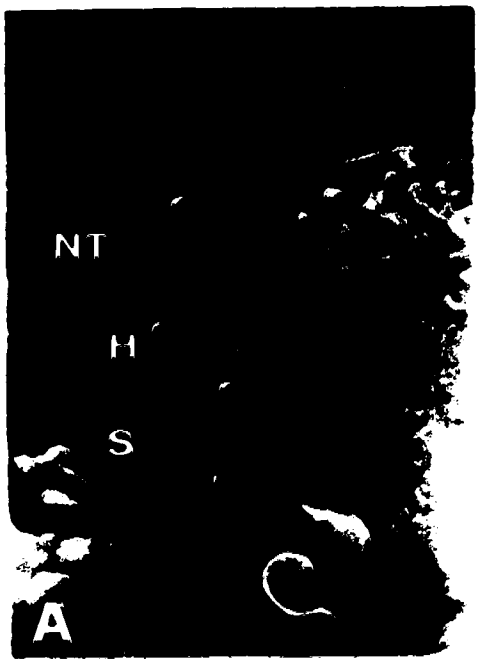
Figure 30.

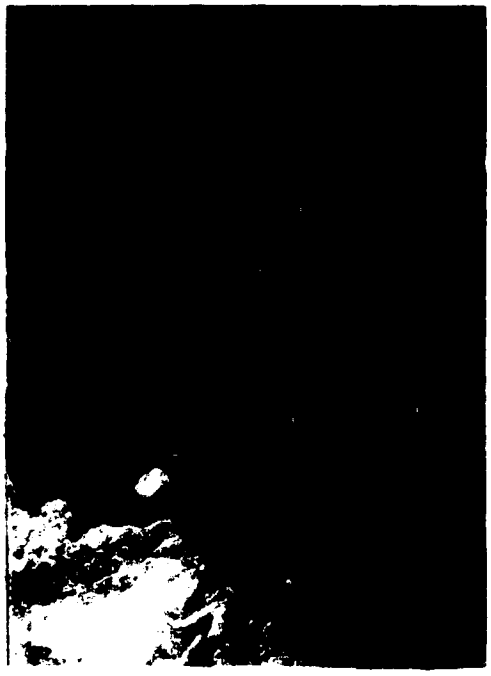
Transverse section through the center of a stage XI (EG & K) blastoderm cultured for 48 hours with anti-16 kD lectin IgG (600 µg/ml) and stained with DAPI. It is evident that the cells of the blastoderm have failed to spread and as a result the embryo is thicker and more compact. Bar is equal to 20 µm.

Figure 31.

A) Effect of rabbit IgG (600 µg/ml) on the development of stage 4 (Hamburger and Hamilton, 1951) embryos after 48 hours of culture. The embryo has developed to stage 12 (Hamburger and Hamilton, 1951). Conalbumin concentration has been increased to 200 µg/ml. The neural tube (NT) and the heart (H) are well defined although the somites are not as well formed. Magnification 56x

B) Effect of anti-16 kD IgG (600 µg/ml) on development of stage 4 (Hamburger and Hamilton, 1951) after 48 hours of culture. Conalbumin concentration has been increased to 200 µg/ml. The embryo has developed to stage 12 (Hamburger and Hamilton, 1951) exhibiting similar characteristics as in A.


NT
H
S
A



Discussion

In this study the possible role(s) of the endogenous galactose-binding lectins in the gastrulating chick embryo were examined. The goal of this investigation was to determine whether interference with these lectins, through the addition of competitive hapten inhibitors or lectin antibodies, had any effect on early embryogenesis in the chick embryo. The hapten inhibitors could compete to a certain extent with the endogenous lectin ligand. The anti-lectin antibodies could bind directly to the sugar binding region or to other regions of the lectin and interfere with lectin-ligand binding.

When early chick embryos are cultured *in vitro*, the culture medium of choice is the thin albumen of the egg. Albumen contains a series of glycoproteins, such as ovalbumin, ovotransferrin and ovomucoid (Burley and Vadehra, 1989), which may mask the effect of any added sugar hapten inhibitors. Therefore, a defined medium was developed to provide the minimum amount of nutrients necessary for relatively normal embryonic growth within a time span in culture. Development of embryos cultured under these conditions duplicated only to a certain extent embryonic development *in ovo* because there was some differences in the development and growth rate of the nervous system. Nevertheless, embryos cultured under these conditions allowed us to determine some of the developmental perturbations induced by the lectin inhibitors. In addition to a measure of

control over the components of the medium, these experiments were conducted within defined time intervals, this allowed for a valid comparison of the developmental effects of these lectin inhibitors. A number of pilot experiments were conducted to determine the concentrations of the compounds used as controls, i.e. maltose, maltosylated-bovine serum albumin (m-BSA), BSA, and rabbit IgG that did not affect embryonic development. In every experiment embryos cultured in the corresponding control medium were included. It is also known that there are slight variations in the range of embryonic stages among batches of eggs. Therefore, a second set of controls consisting of embryos cultured in defined medium alone were conducted in order to confirm that embryo development was consistent within each individual experiment.

In these experiments, lectin inhibitors were added to cultures from embryos at the preincubation stage (stage XI; EG & K) and after 18 hours of incubation (stage 4; H & H). At stage XI (EG & K), the blastoderm is divided into a thick peripheral ring, the area opaca, and a central, thin area pellucida. The area pellucida is beginning to separate into the upper epiblast and the lower hypoblast (Azar and Eyal-Giladi, 1979). Although the hypoblast does not contribute any cells to the adult chick, it is essential for proper formation of the primitive streak, which is the main structure of the gastrulating avian embryo (Bellairs, 1986). At stage 4 (H & H) gastrulation is fully underway. Cells of the epiblast have converged to form the primitive streak and

cells are migrating through the primitive groove into the blastocoele to form the endoderm and mesoderm. In our studies, development of embryos at stage XI (EG & K) was affected, however no effect was observed in stage 4 (H & H) embryos.

Stage XI (EG & K) embryos treated with TDG and lactosylated-BSA were perturbed in their development. Compared to the controls, embryos failed to develop a primitive streak, showed poor demarcation between the area pellucida and the area opaca, and displayed evidence of contraction and decrease in area of the blastoderm. In addition, these embryos sometimes had a group of cells aggregated in the center of the blastoderm. Compared to embryos treated with nonimmume IgG, anti-16 kD IgG also effected gastrulation. In antibody treated embryos, blastoderms were significantly smaller than controls and did not develop a primitive streak. Anti-16 kD IgG treated embryos differed from hapten treated embryos in that, the area opaca and the area pellucida remained distinguishable. In contrast, stage 4 (H & H) embryos underwent further morphogenesis. Based on these results, it is apparent that under the present experimental conditions, the lectin inhibitors used in these experiments affected the processes involved in early gastrulation but had no noticeable effects on the processes involved in late gastrulation.

I. Possible Mechanisms of Action of Lectin Inhibitors

The stage that was most susceptible to the lectin hapten inhibitors and anti-lectin antibodies was stage XI. During stages X through XIII (EG & K), the primary hypoblast is forming. Two different cellular components contribute to the assembly of the hypoblast. One component, already present at the time of laying, is the isolated islands of polyingressed cells that originated in the epiblast. The second component appears as an advancing front of cells from the posterior region of the marginal zone i.e. the boundary between the area opaca and the area pellucida (Eyal-Giladi, 1991). One of the main functions of the hypoblast is the induction of the primitive streak in the epiblast. Once the primitive streak is formed, cells leave the epiblast, advance towards the primitive streak, penetrate the streak and then migrate laterally from the streak to form the mesoderm and endoderm (Bellairs, 1986).

During gastrulation, the extraembryonic endodermal cells derived from the area opaca also become relocated. At the primitive streak stage these cells are located at the ventral surface of the area opaca in a multilayered array. During subsequent development, they become distributed over the yolk to enclose it during the process known as epiboly (Bellairs, 1971).

The 14 and 16 kD galactose-binding lectins are already present in the chick blastoderm at the time of gastrulation (Didier *et al.*, 1993). These proteins are abundant in the

extraembryonic endoderm, the endodermal and some of the mesodermal cells spreading from the primitive groove, the area opaca, the vitelline membrane and the perivitelline space (Zalik et al., 1990, 1994).

One of the possible processes that could be affected by the lectin inhibitors is hypoblast formation. The primary hypoblast derived from the marginal zone is necessary for the induction of the primitive streak (Khaner and Eyal-Giladi, 1986). In embryos at stage 3-4 (H & H) the hypoblast has already been formed and cells of the epiblast have started to migrate and congregate in the area of the nascent primitive streak. At these stages, embryos can continue to gastrulate without further support from the hypoblast (Eyal-Giladi *et al*, 1992). Studies, using scanning electron microscopy, clearly demonstrate that hypoblastic cells near the posterior marginal zone have bifurcated protrusions which are indicative of an active amoeboidal movement (Eyal-Giladi, 1984). Little is known as to how the cells from the posterior marginal zone migrate anteriorly to coalesce with the islands of polyingressed cells that are already present in the developing hypoblast. Didier *et al.* (1993) and from experiments in our own laboratory, have shown that the 16 kD lectin is present in the early hypoblast of the unincubated embryo. It is possible that the marginal zone cells release galactose binding lectins and these lectins may be necessary for the migration of these cells by allowing for transitory adhesion and deadhesion on a galactose rich substratum

(Zalik, 1991). If lectin activity is necessary for the formation of the hypoblast this would explain why lectin inhibition effected early gastrulation and not late gastrulation. Formation of the primitive streak during stages XI (EG & K) and stage 2 (H & H) depends upon the inductive influence of the hypoblast but after stage 2 (Hamburger and Hamilton, 1951) the primitive streak can continue to develop without further support from the hypoblast (Eyal-Giladi, 1991). It is possible that embryos cultured at stage XI (EG & K) were unable to develop to the primitive streak stage because formation of the hypoblast was disrupted, while embryos cultured at stage 4 (H & H) were not effected by the addition of lectin inhibitors.

Another possible reason why lectin inhibition effected early gastrulation but not late gastrulation is that the association of these lectins with the vitelline membrane may be necessary in the expansion of the chick blastoderm. It is known that the association of the blastoderm with the inner side of the vitelline membrane is crucial for blastoderm expansion (New, 1959).

Evidence indicates that externalized blastoderm lectin in which the 14 and 16 kD lectin predominate inhibits the adhesion of cells of early embryos (Milos and Zalik, 1982). It is possible that the galactose binding lectins in the vitelline membrane as well as those released from cells could be involved in blastoderm spreading by allowing for transitory adhesion and de-adhesion (Zalik *et al.*, 1994). In

the results presented here, it is possible that inhibition of lectin function during early gastrulation may have affected the transitory adhesion necessary for the blastoderm to spread. However, blastoderm expansion was not inhibited in embryos cultured at stage 4 (H & H). It is possible that stage 4 (H & H) embryos also depend on lectin activity for blastoderm expansion, but other mechanisms that would compensate for loss of lectin function could have developed by this stage.

Some of the first cells to migrate through the primitive streak are the endodermal cells. The substrate these cells use is largely unknown. It has been suggested that the lectin and associated glycoconjugates released at the apical surfaces of these cells acts as a transitory substratum for the endodermal cells to migrate on. The lectins may be used in the adhesion and deadhesion of cells to the lactosaminyl residues of extracellular molecules necessary for migration. The addition of lectin inhibitors to early gastrulating embryos resulted in compact blastoderms with little evidence of cell spreading. If the endodermal cells do rely on these galactose binding lectins as a substratum for migration, this would explain why the early stages of gastrulation are inhibited in these experiments.

Stage 4 (H & H) embryos cultured in the presence of lectin inhibitors underwent neurulation. Yet, during neurulation these lectins are expressed extracellularly in the apical and basal surfaces of the neural tube, the

notochord, the apical and basal surfaces of the endoderm and the coelomic surfaces of the heart primordium (Zalik *et al.*, 1994). It has been suggested that these lectins could bind to the poly-N-acetyllactosamine residues present in the inner limiting membrane of the neural tube or to similar saccharide groups in the proteins of the extracellular matrix, such as fibronectin or laminin (Zalik et al., 1994). Released lectin from the neural crest could serve as a bridge between the lactosamine residues of the extracellular matrix proteins and allow for transitory adhesion (Zalik, 1991).

It is possible that the interference with lectin function during late gastrulation might have stimulated a functional mechanism that would compensate for the absence of lectin function. The lack of any phenotypic abnormalities in embryos cultured at late gastrulation may be due to the coexpression of proteins with similar functions. If several proteins with similar functions are coexpressed in the same cell or tissue, knocking out any one of them may have no effect on that tissue (Erickson, 1992).

There is evidence in other systems that indicates that loss of supposedly important genes might up-regulate compensatory pathways (Erickson, 1992). The disruption of the transforming growth factor-ß1 (TGF-ß1) gene in homozygous transgenic mice did not result in gross developmental abnormalities (Shull *et al.*, 1992). Proteins of the TGF-ß family play important roles in growth control in the adult organism and display several isoforms, TGF-ß1, TGF-ß2, and

TGF-ß3 (reference). These isoforms exhibit overlapping patterns of expression during development. Shull *et al.* (1992), suggest that expression of these isoforms compensates for the lack of TGF-ß1. The myogenic transcription factor family of genes, composed of MyoD, myogenin, Myf-5, and Myf-6 are thought to regulate skeletal muscle differentiation (Rudnicki, 1992). Mice lacking MyoD were viable, fertile and show no morphological abnormalities. However, Myf-5 mRNA levels are elevated in postnatal mutant mice. Normally Myf-5 expression becomes markedly reduced at day 12 of gestation, when MyoD mRNA first appears. It has been suggested that Myf-5 expression is repressed by MyoD in normal development, and may compensate for the absence of MyoD. These results indicate that MyoD is dispensable for skeletal muscle development in mice, revealing some degree of functional redundancy in the control of skeletal myogenic development (Rudnicki, 1992).

It has been suggested that the L-14 S-type lectin present in the mouse embryo may play multiple roles during early embryogenesis. These include attachment of the blastocyst to the uterine wall, the differentiation of the muscle cell lineage and the development of the central nervous system (Poirier and Robertson, 1993). However, homozygous mutant mice that lack the L-14 lectin develop normally and are viable and fertile. The absence of any major phenotypic abnormalities in these mutant animals suggest that other proteins potentially compensate for the absence of the

L-14 lectin. A related protein L-30, a lectin that has carbohydrate binding specificity similar to that of L-14 is present in the same embryonic cell populations as L-14 at the time of implantation. It has been suggested that the two S-type lectins may be capable of functional substitution at this early stage of embryogenesis (Poirier and Robertson, 1993).

A protein that appears to have a similar distribution and could play a functional role similar to the galactose binding lectins during neurulation is tenascin. Tenascin (also called cytotactin) is a large oligomeric extracellular matrix protein (End *et al.*, 1992), and is synthesized at specific times and locations during embryonic development(Erickson, 1989). Tenascin appears at the time of gastrulation in the basement membrane separating the epiblast from the underlying mesoderm (Crossin *et al.*, 1986), and is found along the neural tube and notochord. It is also found in pathways for neural crest cell migration, in particular in the anterior portion of somites (Erickson, 1989).

Different cells react in different ways to tenascin. Some cells stick to it, while other cells round up and detach from this protein. While cells flatten and spread on fibronectin or laminin substrates, those cells attached to tenascin maintain a rounded or branched morphology depending on the cell line (Erickson, 1989). The distinctive morphology of cells on tenascin, the lack of cell spreading and the

weakness of cell attachment suggest a role quite different from that of other adhesion molecules (Erickson, 1989).

In quail embryos, tenascin is present in the migratory pathways of neural crest cells (Mackie *et al.*, 1988). According to Mackie *et al.* (1988), tenascin may be involved in directing a migratory pathway of neural crest cells within a meshwork of tenascin. The distribution of fibronectin is too widespread to mediate directional migration. Tenascin could make the fibronectin rich matrix less adhesive for the neural crest and allow cells to migrate by loosening the grip on fibronectin (Mackie *et al.*, 1988). Saga *et al.*(1992), generated mice in which tenascin gene expression was completely disrupted. The homozygous null mutant mice were born live, no anatomical or histological abnormalities were detected in any tissues, and no major changes were observed in distribution of fibronectin, laminin, collagen, and proteoglycan. The existence of these mutant mice, lacking tenascin yet phenotypically normal casts doubt on the theory that tenascin plays an essential role in normal development (Saga *et al.*, 1992).

From this information on tenascin, it is possible that the loss of lectin activity during neurulation might up-regulate a compensatory protein such as tenascin. It is possible that the galactose binding lectins and tenascin have similar roles during neurulation, by modifying the extracellular matrix proteins to make them less adhesive to allow the directed migration of cells. It is possible that

loss of lectin function during late gastrulation may have been compensated for by tenascin. The modification of the extracellular matrix glycoproteins to allow for a weaker adhesion could have been taken over by tenascin.

II. Future Studies

A number of further studies are necesssary to discern the exact role of the galactose binding lectins during chick embryogenesis. It would be very beneficial to do further histological examinations of the embryos described in this study to determine the location of the lectins before, during and after the addition of lectin inhibitors.

The anti-16 kD polyclonal antibodies used in this research has some cross reactivity, however our laboratory has recently developed monoclonal antibodies specific to the 16 kD galactose binding lectin. Early and late gastrulating embryos could then be cultured with these monoclonal antibodies and any developmental perturbations could be compared to those found in the experiments described in this study.

Another possible experiment would be to inhibit both tenascin and lectin activity with the appropriate antibodies, in the early and late gastrulating embryo. Such experiments would assist in determining whether or not tenascin acts as a compensatory mechanism for the lectins.

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