The matrix of the optic vesicle-presumptive lens interface during induction of the lens in the chicken embryo

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SUMMARY

The cell coats of the presumptive lens cells and the extracellular interface between the lens rudiment and optic vesicle were investigated in the chicken embryo throughout the period during which lens induction is presumed to take place.

Histochemical methods showed that the cell coats contained both glycoproteins and glycosaminoglycans. Autoradiography after [³H]glucosamine injection indicated incorporation of the precursor with subsequent localization primarily at the cell surface. No obvious changes in the properties of the coat were noted with the progression of early lens morphogenesis.

The extracellular matrix at the interface between ectoderm and optic vesicle also contained glycoprotein and glycosaminoglycan. There was a heavy concentration of [³H]glucosamine-containing macromolecules in the area. Electron microscopy revealed that the interface consisted of the basement membrane systems of lens and optic vesicle, fused with their external fibrillar layers. In contrast to the findings on cell coats, the density of the interfacial matrix increases appreciably during the lens induction period. Evidence suggests that the cells of the two ocular epithelia are themselves the source of the matrix materials.

It is proposed that the macromolecules excreted by the epithelial cells into the interface interact at different concentrations to form aggregates of various structure by a process of self-assembly. This may be reflected in the different ultrastructure of the layers of the interfacial matrix.

Quantitative changes in the density of the matrix, leading to increased adhesion between lens rudiment and optic vesicle, may restrict the lateral spreading of the lens cells and so fix the basal area of the lens rudiment. This, together with continued cell replication, may produce the cell crowding, placode formation and invagination characteristic of lens morphogenesis.

INTRODUCTION

The idea that lens induction by the optic vesicle requires close contact between the cells of the inducing and responding tissue was based on demonstrations that materials interposed between the two blocked the process. Spemann (1905) excised the complete eye rudiment from Triton embryos in the optic vesicle stage and observed that a new presumptive retina was reconstituted.

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However, the latter failed to induce a lens if it was separated from the surface ectoderm by the invasion of a thin layer of mesenchyme. McKeehan (1951) described how in the 10- to 21-somite chick embryo the surface ectoderm and future retina adhered so strongly to each other that they could not be physically separated. Lens formation was restricted to the area of adhesion and could be prevented by placement of a cellophane strip between the interacting systems. In organ cultures, where ectoderm and optic vesicle had been partially separated, morphological signs of lens development were only seen in the regions in which direct contact between the two had been preserved (Langman, 1956).

The 'contact theory' of lens induction was first challenged by the demonstration that agar strips inserted between optic vesicle and surface ectoderm did not prevent lens differentiation (McKeehan, 1958). Millipore filters interposed between the two tissues similarly allowed lens formation to proceed (Muthukkaruppan, 1965).

O'Rahilly & Meyer (1959) provided morphological evidence for the absence of direct cellular contact when they found well-defined PAS-positive basement membranes on the basal surfaces of the optic vesicle and surface ectoderm, which appeared to fuse in the region of interaction. Electron microscopy, however, showed that both ocular primordia were enclosed by basement membranes, which were not in direct contact (Cohen, 1961; Hunt, 1961; Weiss & Fitton-Jackson, 1961).

In the meantime general concepts of the inductive process changed. Emphasis shifted from the importance of direct contact between the interacting tissues and from the role of postulated inducing factors to the possible influence of the micro-environment of the reacting cells. In particular, a number of observations (reviewed by Konigsberg & Hauschka, 1965, and Grobstein, 1967) suggested that extracellular matrix surrounding cells is of considerable importance in the regulation of their differentiation. Matrix components of potential interest in this regard include collagen (Kallman & Grobstein, 1965; Konigsberg & Hauschka, 1965; Bernfield, 1970; Cohen & Hay, 1971), acid mucopolysaccharides or glycosaminoglycans (Kallman & Grobstein, 1966; Bernfield & Banerjee, 1972; Bernfield, Banerjee & Cohn, 1972) and neutral glycoproteins, and, as morphological entities, epithelial basement membranes and the cell-surface-associated glycocalyx. Of these components the glycoproteins have so far received little attention.

With this in mind we investigated the character and origin of the extracellular matrices of the eye rudiment of the chick embryo with emphasis on the interface between presumptive lens and optic vesicle during the period of approximation of the two tissues.
MATERIALS AND METHODS

Autoradiography

Eggs of White Leghorn chickens were incubated at 38 ± 1 °C for 44–55 h. Through a window in the shell radioactive precursors, dissolved in 0.05 ml buffered saline, were deposited on the embryo. Eggs were then resealed and returned to the incubator for an additional 0.5–6 h, at which time the embryos were removed from the eggs. They were fixed in Carnoy’s solution for 2–4 h, routinely dehydrated and embedded in paraffin. Sections of 4–5 μm were stained with Harris-Lillie hematoxylin. The slides were dipped in NTB-2 liquid emulsion (Eastman Kodak, Rochester, New York) diluted 1:1 with distilled water, air dried and stored at 4 °C in light-tight boxes containing silica gel. Optimum exposure times were found by trial experiments and were used consistently. Slides were developed in D-170, fixed in hypo, run through a standard alcohol/xylene series and coverslipped. Some slides were pretreated with periodic acid, exposed and developed, and then post-treated with Schiff reagent followed by Harris-Lillie hematoxylin.

A trial series was run using dosages of 5 μCi 3-[3H]-D,L-glutamic acid (specific activity 5 Ci/mM), 3,4-[3H]-L-proline (specific activity 5.86 Ci/mM) or 6-[3H]-D-glucosamine (specific activity 0.31 Ci/mM), all obtained from New England Nuclear Company (Boston, Massachusetts).

Based on the results of this series embryos were labelled with 10 μCi [3H]glucosamine to emphasize the labelling patterns for photomicrography. To facilitate grain counting a low dose of the label, 1 μCi [3H]glucosamine, was employed in conjunction with sectioning of the tissue at 3 μm. It was anticipated that these measures, giving thinner sections and lower grain densities, would increase the resolution of individual grains.

Histochemistry

A series of embryos was fixed in Carnoy’s solution, routinely dehydrated, embedded and cut at 4–5 μm. Sections were stained with PAS, Celestine blue and picric acid (Petri, 1968). PAS-reactive compounds are, in addition to glycogen, primarily neutral glycoproteins with free 1,2-glycol groups (Leblond, Glegg & Eidinger, 1957). Alcian blue staining was done according to Mowry (1956). This procedure demonstrates both sulfated and carboxylated acid mucopolysaccharides (Spicer, 1960; Lev & Spicer, 1964). To test the specificity of the labelling and staining patterns, sections were treated with salivary amylase or with crystalline alpha-amylase (10 mg per ml distilled water; Worthington Biochemical Corporation, Freehold, New Jersey) for up to 1 h at 37 °C prior to autoradiography or staining.

Electron microscopy

Embryos were removed from the eggs, the heads were dissected and split sagittally. The tissues were then processed according to the technique of Behnke
and Zelander (1970) with slight modifications. The sectioned heads were immersed in 1% Alcian blue 8GX and 2.3% glutaraldehyde in 0.1 M phosphate buffer, pH 6.2, overnight at 4°C, followed by a rinse in 0.1 M phosphate buffer plus 10% sucrose, pH 6.2. They were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h at 20°C and rinsed in 0.1 M phosphate buffer plus 10% sucrose, pH 6.2. After dehydration in a graded series of acetone the heads were embedded in Epon. Each head was cut frontally one-half the way through the eye rudiment at 0.5 μm on glass knives and the sections were stained with toluidine blue for light microscopy. Ultrathin sections were cut from the remaining half of the rudiment, mounted on Formvar and carbon-coated 100-mesh grids, stained routinely with 1% uranyl acetate and 0.2% lead citrate, and studied in a Philips EM-300 electron microscope. Behnke & Zelander (1970) have demonstrated that their method is based on enhanced fixation of mucosubstances, which are insolubilized by their binding with Alcian blue. The resultant dye-polysaccharide-protein complex is osmiophilic. In general there is good agreement between the results obtained with this method and those of other techniques for the ultrastructural demonstration of mucosubstances.

Staging and grain counting

In all cases the lens rudiments were staged after completion of the histological procedures according to the eye-staging series of O'Rahilly & Meyer (1959), which is based on the normal table of Hamburger & Hamilton (1951) (see also Zwaan, Bryan & Pearce, 1969, and Zwaan, 1974). For grain counting purposes the tissue sections were subdivided as indicated in Fig. 1. The areas counted were cellular regions of lens or optic vesicle, the separated basal regions (mostly basement membrane complex) of the two, the basal regions of lens and optic rudiment in the zone of approximation, and extracellular spaces away from the tissues studied.

Photomicrography

To emphasize specific staining patterns appropriate filters were used for photomicrography of the specimens, i.e. a green filter for the PAS-stained slides and a red filter for the Alcian blue-stained ones.

RESULTS

The lens rudiment–optic vesicle interface

The term 'contact' will be used here in the sense of close approximation of the interacting tissues. We do not imply that direct cell-to-cell contact takes place across the interface. The area of contact between lens rudiment and optic vesicle is clearly delineated at the time when the presumptive lens cells are maximally elongated (stage 13) and invagination begins (stage 14). Thus, the character of the interface was most conveniently studied at these stages.
Extracellular matrix in lens development

FIGURE 1
Schematic drawing of the eye rudiment of a stage-14 chicken embryo, indicating the areas selected to count the densities of autoradiographic grains. Hatched boxes are examples of areas over the tissue of lens (L) or optic vesicle (ov). Open boxes represent extracellular space away from the tissues (1), areas over the extracellular matrix of the lens-optic vesicle interface with the adjacent basal regions of the tissues (2), and areas over the separate basal regions and underlying extracellular matrix of the lens (3) and the optic vesicle (4) away from the ‘contact’ zone. The stippled square indicates where background counts were obtained.

Autoradiographs of embryos exposed to [3H]glucosamine for 4 h revealed considerable incorporation of the precursor throughout the cells of the ocular epithelia. There was a striking accumulation of the label at the interface between lens rudiment and optic vesicle (Fig. 2B). In contrast with this, two other precursors used, [3H]proline (Fig. 2A) and [3H]glutamic acid (Fig. 2C) gave less intense labelling of the tissue and there was no indication at all of a concentration of grains over the basal regions and interface of the eye primordia. With PAS staining the reaction of the interface and basal surfaces was intense (Fig. 3), while weak staining was visible at the apical surface of the cells and over the tissue in general.

To ascertain if the accumulation of PAS-positive and glucosamine-containing materials at the interface of the rudiments was extra- or intracellular, sections tangential to the lateral tip of the lens cup, and particularly grazing sections, were very useful (Fig. 4). It was found that both PAS-positive material (Fig. 4A) and incorporated radioactivity (Fig. 4B) were mainly outside of the cells, in close association with their basal surfaces.
Extracellular matrix in lens development

There are no other tissues in the near vicinity, and we concluded therefore that the presumptive lens and optic vesicle just prior to and during invagination incorporate glucosamine into compounds which subsequently accumulate outside the basal surfaces of the cells. The same area is characterized by heavy PAS staining and is known from previous work to consist of the fused basement membrane systems of the ocular rudiments.

In stage 10, when contact between the surface ectoderm and the tip of the optic vesicle had just been established, there was a minimal PAS-positive reaction in the region where the tissues met. With the passage of time the staining grew stronger, becoming most intense at the onset of invagination.

\[^{3}H\]\text{glucosamine} was incorporated into materials excreted towards the interface throughout the period studied, i.e. from stage 10, the beginning of contact, through stage 17, the completion of lens invagination. We were unable to ascertain if there is a stage difference in the amount of label taken up, or alternatively if there is a steady rate of synthesis and excretion. This was due to the variability of the grain counts from one embryo to the next, undoubtedly a result of the vagaries of the \textit{in ovo} labelling procedure.

**Cellular surfaces**

Within the tissue the following different labelling and staining patterns were observed. Associated with the basal surfaces of cells rounded-up to divide (Zwaan \textit{et al.} 1969) we found darkly stained PAS-positive plaques (Fig. 5 A). In \[^{3}H\]\text{glucosamine}-treated tissue silver grains were seen over these plaques (Fig. 5 B).

There was a PAS reaction with and a grain deposition over the apical surface of the lenticular tissue. The PAS technique showed a 5–10 $\mu$m thick coat of amorphous, granular material (Fig. 6 A). \[^{3}H\]\text{glucosamine}-labelled tissue

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**Figures 2–4**

Fig. 2. A composite of portions of photomicrographs of three different stage-15 lens cups, each labelled with 5 $\mu$Ci of a different tritiated compound for 4 h. (A) \[^{3}H\]proline; (B) \[^{3}H\]glucosamine; (C) \[^{3}H\]glutamic acid. Note that there is a much greater concentration of grains over the lens (L), particularly over its basal region and the underlying matrix (arrow), after treatment with glucosamine than after injection of either of the amino acids. $\times$ 600.

Fig. 3. A stage-14 lens cup stained with PAS, Celestine blue and picric acid. The solid black line between lens (L) and optic cup (ov) is the PAS-positive double basement membrane complex (bm). The apical or luminal surface (Lu) reveals a lightly PAS-positive granular coat (arrow). $\times$ 500.

Fig. 4. Paratangential sections of stage-15 lenses cut as indicated by the broken line in the inset. (A) Stained with PAS, Celestine blue and picric acid. The central region (arrow) is the PAS-positive basement membrane of the lens. $\times$ 500. (B) A similar section from material labelled with 10 $\mu$Ci \[^{3}H\]glucosamine for 6 h shows a halo of many grains (arrow) surrounding only lightly labelled lens cells. $\times$ 650.
revealed incorporation into the apical coat (Fig. 6B). It was noted that the autoradiographic procedure consistently disrupted and removed part of the apical material (compare Fig. 6A and 6B). Finally, linear arrays of stain or grains appeared to be associated with lateral cell surfaces in approximation to each other and/or the intercellular space (Figs. 5A and 5B, 7A and 7B). No essential differences were observed in the intensity of staining and labelling of the surfaces of presumptive lens cells obtained from the various embryonic stages.

Incorporation over time

A series of embryos was treated with a single dose of \[^{3}\text{H}]\text{glucosamine}\) at stage 12. Grains appeared over the optic vesicle and lens cells at \(\frac{1}{2}-1\) h (Fig. 8). By 1 h the first grains were present in association with the PAS-positive basal regions (Fig. 9). Inspection at 2 h revealed an increased accumulation of grains over the basal poles (Fig. 10). Furthermore, grains were first seen over regions removed from the ocular tissues and PAS-positive zones. By 6 h the basal area of the tissue was darkened with grains (Fig. 11), and there was more grain accumulation over extracellular spaces away from the tissues. Progressively greater grain deposition over intra-tissue and apical surfaces was noted; however, the resolution at the light microscope level was not sufficient for a detailed study.

Grain counts were carried out as indicated in Fig. 1 and confirmed the visual

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**Figures 5-7**

Fig. 5. Stage-12 lens primordia and optic vesicles above and below the darkly stained basement membrane, respectively. 
(A) Stained with PAS, Celestine blue and picric acid. Note the PAS-positive nature of the basement membrane complex and of the plaques at the basal poles of two lens cells, one of which is rounded-up for mitosis (m). \(\times\) 1000. 
(B) Autoradiograph of a lens treated with 10 \(\mu\text{Ci}\) \[^{3}\text{H}\text{glucosamine}\) for 6 h. There is a heavy concentration of grains over the basement membranes and over the PAS-positive plaque associated with a cell in prophase (arrows), but individual grains are difficult to resolve in the photograph, because of their density and because of the staining of the underlying structures. \(\times\) 1000.

Fig. 6. Sections of the apical regions of stage-15 lenses. (A) Stained with PAS, Celestine blue and picric acid. The region above the cells is the PAS-positive apical coat (arrow). \(\times\) 1300. (B) An autoradiograph of a similar section from tissue labelled with 10 \(\mu\text{Ci}\) \[^{3}\text{H}\text{glucosamine}\) for 4 h. Note the grains over the cells and extracellularly over the apical coat (arrows). More external regions of the apical coat have been fragmented and partially stripped off, presumably an artifact of the tissue processing. \(\times\) 1300.

Fig. 7. Sections of lenses illustrating lateral cell boundaries. (A) A stage-15 lens stained with PAS, Celestine blue and picric acid and showing PAS-positive lateral cell coats (arrows) near the luminal surface. \(\times\) 1400. (B) A stage-13 chick lens treated with 10 \(\mu\text{Ci}\) \[^{3}\text{H}\text{glucosamine}\) for 6 h. Arrows indicate rows of grains, which appear to be over the lateral cell surfaces. \(\times\) 1350.
Extracellular matrix in lens development

Table 1. Counts of [\( ^3H \)]glucosamine grains (per 100 \( \mu m^2 \)) in the early chick eye rudiment

<table>
<thead>
<tr>
<th>Time after labelling (h)</th>
<th>Lens (tissue)</th>
<th>Optic vesicle (tissue)</th>
<th>Lens (basal region)</th>
<th>Optic vesicle (basal region)</th>
<th>Interfacial matrix</th>
<th>Extracellular zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6 (1-11)</td>
<td>7 (1-12)</td>
<td>8 (0-17)</td>
<td>6 (0-11)</td>
<td>17 (5-32)</td>
<td>1 (0-4)</td>
</tr>
<tr>
<td>3</td>
<td>7 (1-17)</td>
<td>11 (1-23)</td>
<td>22 (4-34)</td>
<td>24 (8-30)</td>
<td>43 (32-51)</td>
<td>12 (0-34)</td>
</tr>
<tr>
<td>6</td>
<td>46 (27-61)</td>
<td>40 (20-55)</td>
<td>71 (57-82)</td>
<td>72 (55-82)</td>
<td>119 (93-144)</td>
<td>42 (9-89)</td>
</tr>
</tbody>
</table>

The areas over which counting was performed are defined in Fig. 1. Average values are given with their range indicated in parentheses. The background (non-tissue) grain count was 0-2-0-4 grain per 100 \( \mu m^2 \). The basal regions are mainly the extracellular regions, adjacent to the basal cell membrane, but they may include minor contributions from the basal cytoplasmic areas. Label was applied in a single dose at 45 h of incubation (stage 12). Six h later most embryos had reached stage 14.

observations (Table 1). From these we deduce that both optic vesicle and lens are synthesizing glucosamine-containing macromolecules and that over time there is a progressive appearance of these materials first in the tissues, next in the adjacent PAS-positive basement membranes and finally in extracellular areas away from basement membranes. In those areas where the two PAS-positive basement membranes of optic vesicle and lens are in apposition there appears to be a continuous piling up of radioactive macromolecules.

The considerable grain density in the area of contact between the rudiments makes accurate counting difficult and the values listed in Table 1 for this area are probably underestimated. For this reason, not too much significance should be attached to the fact that the counts over the interface are about twice as high.

Figures 8-11

Fig. 8. A stage-12 lens treated \( \frac{1}{2} \) h before with 10 \( \mu Ci \) [\( ^3H \)]glucosamine shows only a few grains over the lens (L) tissue (arrows), and no grains over the optic vesicle (ov). \( \times 750 \).

Fig. 9. A stage-13 eye rudiment treated 1 h previously with 10 \( \mu Ci \) [\( ^3H \)]glucosamine. There are grains over the lens (L) and optic vesicle (ov) cells and over the joined basement membrane system. \( \times 700 \).

Fig. 10. A stage-14 lens cup 2 h after exposure to 10 \( \mu Ci \) [\( ^3H \)]glucosamine. Note the increase in the concentration of grains over the ocular tissues and basement membranes. \( \times 600 \).

Fig. 11. Stage-15 eye treated 6 h before with 10 \( \mu Ci \) [\( ^3H \)]glucosamine. The concentration of grains over the cells of the lens cup (L) and the optic vesicle (ov) has again increased, and the basal areas are black with grains. \( \times 600 \).
Fig. 12. Stage-15 eye primordium of the chicken embryo, stained with Alcian blue to demonstrate the presence of acid mucopolysaccharides.

(A) The basement membrane system (bm) between the lens cup (L) and optic vesicle (ov) is strongly positive. Weaker reactions are seen over the tissues; they are most notable at the free surface of the lens (Lu). × 700.

(B) Higher magnification of the lens cells shows distinct Alcian blue-positive deposits at the apical surface (Lu) and at the basal pole (cp) of a cell rounded-up to the free tissue surface for mitosis (m). × 1800.

(C) Lateral surfaces of the columnar lens cells (arrows) react with the Alcian blue stain. A nucleus (n) at the basal extremity of a cell is presumably in the S-phase of the cell cycle. × 1800.

as those over the separate basement membranes. It also appears that a single dose of [3H]glucosamine remains available for incorporation for a long time, because even at 6 h after the injection label over the tissues is still increasing.

Glycosaminoglycans

Alcian blue, a polyvalent cationic dye, was used to study the distribution of acid mucopolysaccharides or glycosaminoglycans. These substances are polyanions with sulfate ester and carboxylic acid residues. They interact with the dye to form insoluble complexes.

The observed staining patterns were similar to those seen with the PAS reagent. The basement membrane system between the lens rudiment and the optic vesicle went from weakly reactive in the earliest phases of contact to
strongly positive prior to and during invagination (Fig. 12A). No attempts were made at this time to differentiate between various classes of acid mucopolysaccharides by such methods as the 'critical electrolyte concentration' technique of Scott & Dorling (1965). It is possible, therefore, that the denser staining reflects not only an increase in the quantity of these acid glycosaminoglycans, but also a shift in their character.

The apical, lateral and basal surfaces of the presumptive lens cells reacted with the Alcian blue at all stages studied (Fig. 12B, C).

Treatment with amylase

To clarify whether the PAS-staining and labelling, apparently at and outside of the basal cell surfaces, was actually due to intracellular glycogen accumulating at the basal cell poles, extra-ocular and ocular tissue sections were treated with amylase or distilled water. To ascertain the activity of the enzyme the PAS-positive materials in embryonic heart cell cytoplasm and in association with extra-embryonic membrane, which are known to consist of glycogen, were studied. Neither was labelled after [3H]glucosamine treatment. Their PAS reaction became negative after amylase treatment but did not change appreciably after incubation with water. Examination of PAS-stained autoradiographs of ocular tissues revealed no apparent difference in grain densities or in intensity of staining between the distilled water-treated controls, the enzyme-treated ocular tissues or non-treated sections. We conclude that the radioactive glucosamine incorporation and the PAS reactivity at the lens–optic vesicle interface is not caused by the presence of glycogen.

Electron microscopy

From the results described above we concluded that the chick optic vesicle and lens rudiment produce PAS-positive glycoproteins and acid glycosaminoglycans that appear to become part of the cell surfaces of the tissues of origin and of the double basement membrane complex between the two tissues. Either one or both types of macromolecules contain glucosamine. Because this study was conducted at the light microscope level, the precise location of the PAS and Alcian blue reactions and the origin of the [3H]glucosamine grains remained somewhat uncertain. To ascertain if these loci were in fact extraplasmalemmal, and consequently parts of the matrix covering the cells, we investigated the ultrastructure of the ocular primordia. Particular attention was paid to the double basement membrane region between the two rudiments.

Earlier reports utilizing routine electron microscopic procedures indicated the presence of far less material between the basal plasma membranes of the optic vesicle and lens cells than we expected because of the PAS intensity in the same area. This can be explained on the basis of the solubility of glycoproteins in routine fixatives and consequently their loss during processing of the tissue. We anticipated that the method of Behnke & Zelander (1970), based on the
En bloc precipitation of mucosubstances by Alcian blue, would result in a more precise ultrastructural localization of these substances and in the retention of significantly more material in the double basement complex than had been previously demonstrated.

Electron microscopy of the interface

In regions where the lens and optic vesicle rudiments were closely approximated the distance between their cell membranes was about 7200 Å. Adjacent to the basal plasma membranes of lens cells (Fig. 13) an amorphous, lightly stained layer about 800 Å thick was observed. The basal cell membranes of the optic vesicle had a similar layer about 800 Å thick on their external side. Between the two was a third layer of material, approximately 5600 Å thick and composed of darkly stained amorphous granular aggregates and lightly stained filamentous structures. In the filaments no banding pattern was detected.

The basal regions of lens not in contact with the optic vesicle (Fig. 14) revealed from the cells outward two layers: a lightly stained, amorphous layer about 800 Å wide adjacent to the plasma membrane and a 5600 Å thick layer of darkly stained globular and lightly stained filamentous material. The latter bordered on a very large space, which contained very little detectable material and which probably constitutes the forming vitreous humor. The non-apposed basal optic vesicle had two similar layers, but they were 800 Å and a more variable 0–5600 Å in thickness, respectively (Fig. 15).

Ultrastructure of lens cell surfaces

The cells of the lens rudiment are either in the interphase of the cell cycle and stretched from one surface to the other or in mitosis and rounded-up at the apical surface (Zwaan et al. 1969). The lateral borders of cells in interphase

Figures 13-15

Fig. 13. Electronmicrograph of the contact region between the lens rudiment (L) and the optic vesicle (ov) in stage 14. The tissue was treated with Alcian blue after the method of Behnke & Zelander (1970). The interfacial matrix shows the following characteristics. The basal plasma membranes of the organ rudiments are lined by lightly stained, amorphous layers (zi), on average about 400 Å thick for the optic vesicle and up to twice as wide for the lens. The zone between them (ze), around 5000 Å wide, has numerous darkly stained globular aggregations (g) as well as thin filamentous structures (f). ×20500.

Fig. 14. Electronmicrograph, similar to the one of Fig. 13, of the free basal surface of the lens rudiment (L) at stage 15, away from the area of ‘contact’ with the optic vesicle. The underlying extracellular matrix can be divided into two zones, a thin amorphous one (zi) immediately adjacent to the plasma membranes and a much wider outer zone (ze), globular and filamentous in structure. ×32000.

Fig. 15. Electronmicrograph, comparable to the one of Fig. 14, but of the optic vesicle (ov). Note that the outer zone (ze) is much less prominent than is the case for the lens. ×32000.
may be in apposition to adjacent cells or to intercellular space. Free lateral borders were composed of an intermittent layer of darkly staining, globular material, about 300 Å thick, and external to the plasma membrane (Fig. 16). Where the lateral surfaces of cells were apposed, the two darkly stained layers appeared to merge and were indistinguishable as to their origins (Fig. 17). Investigation of lens cells rounded-up for mitosis, i.e. having no contact with the basement lamina, indicated large aggregations of globular, darkly stained material along their basal borders (Fig. 18). The material on the surfaces of both interphase and mitotic cells appeared to be no different in structure from the dark-staining component of the interfacial matrix, but there were no filaments.

The apical borders of lenticular cells (Fig. 19) were characterized by the presence of a layer of amorphous material about 300 Å thick and external to the plasma membrane. This surface by its amorphous nature was different from the lateral and basal regions. No specific staining reaction was observed within lens cell cytoplasm. The observed pattern was similar in intensity and locations to that seen after routine uranyl acetate and lead citrate staining (see Fig. 18).

Within the cytoplasm of both presumptive lens and retina cells we frequently observed small membrane-limited vacuoles (Fig. 15), adjacent to the basal membrane. Some appeared empty, while others contained material with no definite structure, but with the same staining characteristics as the cell coat. Sometimes the vacuoles appeared to be fused with the basal cell membrane (Fig. 14) or open to the extracellular space (Fig. 13), suggesting that they were discharging their contents. Others (Trelstad & Coulombre, 1971; Hay & Dodson,

Figures 16–19

Fig. 16. This and the following figures demonstrate the electronmicroscopic appearance of various parts of the cell coats of presumptive lens cells at stage 14 after treatment according to the method of Behnke & Zelander (1970). Here the lateral surfaces of several interphase cells show reactions of varying intensity, ranging from rows of very distinct globular deposits external to the plasma membranes adjacent to intercellular space (a) to areas of very light deposits (b) and to regions of plasmalemma without associated globules. ×14000.

Fig. 17. The cell coats of the lateral surfaces of three neighbouring cells (C1, C2, C3) show up as a darkly stained inverted Y. The nucleus (n) and a mitochondrion (mi) of one cell are indicated. ×40,500.

Fig. 18. At the basal pole of a lens cell in mitosis (m), rounded up towards the free surface of the tissue in preparation for division, an accumulation of intensely stained material (arrow) can be noted external to the plasma membrane. ×8000.

Fig. 19. The free apical surface of the lens tissue shows a fuzzy, amorphous, lightly stained cell coat (arrows). The coat is much thinner than in comparable light-microscopic preparations (see Fig. 6A and B) and the part not immediately associated with the apical plasma membranes was probably stripped off during the preparation of the tissue. ×24000.
Fig. 20
1973) have described similar structures in corneal epithelial cells and have proposed that these vacuoles are vehicles for the excretion of collagen from the corneal cells.

**DISCUSSION**

There is an increasing awareness that macromolecules, which were up to recently considered constituents of the extracellular matrix, are also found in close association with cell surfaces. These macromolecules typically consist of carbohydrate units covalently linked to peptides and can be divided into glycoproteins and proteoglycans. In the first group one or more often extensively branched heterosaccharides with a relatively low number of sugar residues, i.e. certain hexoses, hexosamines and neuraminic acids, are bound to a protein chain. The glycoproteins are histochemically characterized by their PAS-positive reaction (Leblond et al. 1957). The carbohydrate parts of the second group, termed acid mucopolysaccharides or more recently glycosaminoglycans, are always considerably larger than those of glycoproteins and consist of long linear sequences of sugar residues, usually with a repeating disaccharide pattern of alternating hexosamine and hexuronic acid molecules. They are often sulfated. Because their highly negative electrostatic field repulses the negative periodate ions from the neighbourhood of the proteoglycans, these polysaccharides do not react with the PAS reagent under standard conditions (Scott & Harbinson, 1968).

By different specific staining methods, reviewed by Rambourg (1971), some type of carbohydrate-rich cell coat or glycocalyx has been demonstrated on virtually every cell type investigated. Indeed, it appears that the glycocalyx is the medium through which cells interact with their environment. Whether the coats should be regarded as integral parts of the cells themselves or as extracellular components is therefore problematic. On the one hand they are at least closely apposed to the plasma membrane and, in certain cases, cannot be removed without affecting cell viability. Moreover, it is well established that plasma membranes contain carbohydrate components. On the other hand,
there appears to be a relatively smooth transition between the glycocalyx, the basement membrane of epithelia, and the collagen network of the extracellular matrix (Rambourg & Leblond, 1967). Our results indicate that the cell coats and basement membrane complex of the early eye rudiment follow this general pattern. Because these two structures have a different morphology, which may have functional implications, we will discuss them separately.

**Glycocalyx of the lens cells**

The patterns of histochemical reactions and $[^3]$H-glucosamine labelling suggest that the macromolecules represented by these methods are mainly associated with the cell surface. The PAS-positive sites show a superficial resemblance to the images of cells containing glycogen, in which the granules of this substance have been concentrated towards one side of the cell by fixation artifact. However, the resistance of the staining to enzymic treatment and the similarity of PAS, Alcian blue and autoradiographic grain localizations indicate that this is not the case.

Although the apparently prolonged availability of $[^3]$H-glucosamine led to incorporation by the cells for the duration of the experiment, many if not most of the autoradiographic grains eventually became concentrated at the cell periphery. This is in agreement with results obtained in other systems. The bulk of the glucosamine provided to cultured cells is used for the synthesis of cell-surface-associated glycoproteins (Bosmann & Winston, 1970; Onodera & Sheinin, 1970). The incorporation very likely occurs through the production of UDP-N-acetylglucosamine (Harris & Johnson, 1969). This compound is not only the immediate precursor of protein-bound glucosamine, but also an intermediate in the biosynthesis of N-acetyl-neuraminic acid (Simkin & Jamieson, 1967), which in turn can be utilized in the production of glycoprotein. In actuality, hexosamines as such account for virtually all of the bound radioactivity in the cell-surface glycoprotein fraction of reticulocytes (Harris & Johnson, 1969) and sarcoma cells (Muramatsu & Nathenson, 1970). In several embryonic organs, on the other hand, glucosamine is a precursor for the synthesis of glycosaminoglycans, either as such or after epimerization of UDP-N-acetylglucosamine to N-acetyl-galactosamine (Manasek et al., 1973; Meier & Hay, 1973). The glycosaminoglycans are secreted into the extracellular matrix. In any case, it seems justified to conclude that cell surfaces in the embryonic lens rudiment are composed at least in part of hexosamine-containing glycoprotein and/or proteoglycan and that the cells themselves are the source of their coats.

Light microscopy gives the impression that the cell coat is not the same all around the cell. Apical, lateral and basal parts have regional characteristics. This is confirmed by electron microscopy.

On the apical surface an amorphous 300 Å thick coat of Alcian blue reactive material is observed. Its thickness is considerably less than in the light microscopic
histrochemical study, which may be explained on the basis of the already noted instability of this surface. Apical fuzzy coats on the microvilli of intestinal cells have been described in detail by Ito (1974) and probably function in the terminal digestion and absorption of nutrients from the lumen of the gut. It is at present unknown if cells at the surface of the embryo take up materials from the amniotic fluid in a similar fashion.

At the basal surface the cell coats of the presumptive lens cells are not recognizable as entities with a separate identity. They fuse into the basement membrane complex, which will be discussed below. There is one exception to this: mitotic cells always show a distinct plaque of extracellular material at their basal poles. It has been established before that the cells of the lens rudiment are in touch with their basement membrane during most of the replicative cycle. However, they round up towards the free surface in preparation for division (Zwaan et al. 1969). A drastic decrease in surface-to-volume ratio, which occurs at this time (Hendrix & Zwaan, 1974a), may well increase plasma membrane tension and so initiate the change in cell morphology. We hypothesize that the plaques seen on mitotic cells represent material which normally forms a part of the basement membrane, but which is sufficiently strongly attached to the cell membrane to stay with the latter when the cell moves away from the base of the tissue.

The lateral cell coats have a globular appearance and stain intermittently but intensely after the Alcian blue treatment. In some cases the lateral cell surfaces border on intercellular space and are distinct; in other instances the coats of two cells are apposed and virtually indistinguishable. With regard to the latter case, such polysaccharide cell coats in combination with inorganic cations, Ca²⁺ and/or Mg²⁺, are strong candidates for playing a major role in histotypic intercellular adhesion. Properties of the cell surface are widely thought to be the base for the high specificity with which cells are organized in space to form tissues and organs (Moscona, 1968). We have not noted clear-cut changes in the structure of the cell coats during morphogenesis of the lens. Nevertheless, qualitative or quantitative changes in cell ligands, not demonstrable by our relatively crude methodology, may be involved in the shaping of the organ. An alternate possibility, that lens formation is related to changes in the extracellular matrix between ectoderm and optic vesicle, is discussed below.

The presumptive lens–optic vesicle interface

At the basal pole of both presumptive lens and optic vesicle we find two zones after staining by the method of Behnke & Zelander (1970). As is the case for other epithelia treated with glycoprotein-specific techniques (Rambourg & Leblond, 1967; Behnke & Zelander, 1970), there is an amorphous zonula interna adjacent to the plasma membrane and a more structured zonula externa, which contains globular and fibrillar materials. In regions where the two ocular rudiments are close the external zones appear to be fused. The Alcian blue
reactions indicate that the entire interfacial matrix, from lens plasma membranes to optic vesicle plasma membranes, contains glycoprotein and/or proteoglycan. This agrees with the light microscopic demonstration of a continuous PAS- and Alcian blue-positive zone between the two tissues.

With routine electron microscopy (Porte, Stoeckel & Brini, 1968; Hendrix & Zwaan, 1974c) a different subdivision of the basement membrane is apparent: as in other epithelia there is an amorphous 200–400 Å thick layer of low density next to the cell membrane, followed by a condensed 150 Å filamentous layer. Finally, a third layer of indeterminate thickness and composed of thicker fibrils is present. These layers have been termed—from the plasmalemma outward—the lamina rara interna, the lamina densa and the lamina rara externa. The first, corresponding to the cell coat, and the second, the classical basal lamina, together form the zonula interna. The third is identical to the zonula externa; preliminary ultrastructural studies show that the fibrils in this area have the cross- striations typical for collagen from early to late stages of lens–optic vesicle interaction. The various layers of the interfacial matrix are depicted diagrammatically in Fig. 20.

The lens primordium and optic vesicle themselves are probably the exclusive sources for the macromolecules of the interface. First, although there is no other tissue such as mesenchyme in the immediate area, the amounts of material at the interface increase visibly during the interaction period. This visual impression has been confirmed by microspectrophotometry at least for the glycoproteins, whose concentration peaks dramatically just prior to invagination of the lens rudiment (Hendrix & Zwaan, 1974b). Moreover, concentrations are highest at the center of the interfacial zone, which is incompatible with a diffusion of macromolecules from the outside into the area.

Secondly, autoradiography indicates that most of the [3H]glucosamine incorporated by the cells of the early eye rudiment is exported towards the interface. It is unknown at this time if the precursor is used for the synthesis of glycoproteins, glycosaminoglycans or both. Histochemical analysis shows that both compounds are present and that the bulk of the acid mucopolysaccharides is chondroitin sulfate (Hendrix & Zwaan, unpublished results).

We do not find such a specific localization for [3H]proline, but this does not preclude that the ocular epithelia produce the collagen of their interface. The rather small amounts of collagen present may explain this negative result. It is interesting that Pierce (1966) could not detect collagen in the lens–optic vesicle area of the mouse embryo by immunological methods, even though collagen is known to be present and though antisera to basement membrane glycoprotein reacted positively. Nadol & Gibbins (1970) also failed to show specific incorporation of radioactive proline into basal lamina by autoradiography.

Finally, in both lens and optic vesicle we found vacuoles adjacent to the basal cell membrane, which in other embryonic tissues are considered to represent the means for collagen secretion by the cells.
It is likely that interactions between the various components of the interfacial matrix lead to delineation of its layers. The differences between the collagen packing in the basal lamina and the spatial order of the collagen fibrils in the zonula externa may result from interactions between different quantities of glycosaminoglycans, presumably primarily chondroitin sulfate (Toole & Lowther, 1968), and collagen. Small inorganic ions may also be of influence. Thus, control of the rate and the timing for the production and excretion of the various matrix macromolecules can be expected to set up different concentration gradients for each of these compounds, which in turn would determine the mode and location of their self-assembly into larger aggregates (see Fig. 20). In an elegant study by Trelstad, Hayashi & Toole (1974) a more detailed model explaining corneal stroma morphogenesis and based on the same principles has been proposed recently.

Interfacial matrix and lens induction

The development of embryonic cell groups into organs with distinct functions involves at least two different processes. First, a cell population increases in density and is sequestered from its neighbours. This leads to the establishment of a separate three-dimensional array of cells, morphologically recognizable as an organ. In the case of the lens, for instance, ectoderm cells elongate into a placode, which invaginates and detaches as a lens vesicle from the surrounding surface layer. Secondly, individual cells within the group become specialized, which is usually notable by the onset of specific protein synthesis. Thus, presumptive lens cells one by one start the production of the lens-specific proteins or crystallins (Zwaan, 1974). In general the first group of events, collectively referred to as organogenesis, is well under way before there is any detectable sign of the second, i.e. cytodifferentiation.

Cytodifferentiation probably requires the activation of specific portions of the genome, but this does not preclude the possibility of extracellular matrix involvement. The matrix not only occupies a strategic position with regard to the control of traffic of molecules into and out of the adjacent cells, but its components have a molecular structure ideally suited for filtering purposes on both a chemical and a physical basis. Moreover, if induction involves the transfer of activating factor(s), the extracellular matrix between inducing and responding tissue may be important in the localized and prolonged delivery of optimal quantities of such a factor or factors. Transfer of molecules may be facilitated and at the same time restricted to a relatively small area, thus enhancing spatial specificity. In the developing eye this may serve to establish a correct alignment of future lens and retina with regard to the optical axis of the eye. In summary, we propose that the extracellular matrix of the interface between presumptive lens and optic vesicle at the very least facilitates the cytodifferentiation of the presumptive lens cells. In addition, we have recently described a model of lens organogenesis, which assigns an even more im-
important and more direct role to the interfacial matrix (Zwaan & Hendrix, 1973).

The model is based on the observation that the area of contact between the two ocular primordia becomes fixed in dimensions after some hours of tissue interaction. This is followed by lens cell elongation, increase in lens cell density and decrease of the extracellular space of the lens rudiment. We hypothesize that the latter three events are related, and together are the result of cell crowding. All presumptive lens cells are still replicating (Zwaan et al. 1969) and their average volume remains the same during this time of embryonic life (Hendrix & Zwaan, 1974a). Moreover, it is likely that the constancy of the lens-optic vesicle contact area indicates a restriction of the basal surface available to the lens Anlage. Combination of these factors must automatically cause cell population pressure, which in turn leads to the described changes in cell and tissue morphology (Zwaan & Hendrix, 1973). The crucial element in this model, then, is the limitation of the basal surface of the lens primordium or the prevention of lateral expansion by the lens cell population. We now postulate that the extracellular matrix between the lens and optic vesicle is instrumental in this process and, in particular, that the glycoproteins of the interface may be involved in both lens–optic vesicle adhesion and fixation of the basal dimensions of the lens.

Glycoproteins not only play a major role in cell-to-cell adhesion (Moscona, 1968) but also in the attachment of cells to a substrate (Culp, 1974). Mouse fibroblasts in culture deposit a layer of glycoprotein on their glass or plastic substrate. These macromolecules have a relatively high carbohydrate/protein ratio, they are highly negatively charged, and [3H]glucosamine is incorporated into them. Together with Ca\(^{2+}\) ions they may cause attachment of cells to the surface of the culture dish. Perhaps more important, cells resist moving away from the edge of their colonies under their influence (Culp, 1974).

The lens–optic vesicle system may be analogous to the cultured fibroblast one in this regard. The fibrillar network in the contact area resulting from interactions between collagen and chondroitin sulfate may provide the substrate on which cells can attach. The zone between cells and substratum may be bridged by the glycocalyx, cations such as Ca\(^{2+}\) and matrix glycoproteins (Fig. 20). This would not only explain the adhesion of lens rudiment and presumptive retina during the tissue interaction phase, but also the resistance to expansion of the contact area.

Several facts fit into this model. There is an abundance of glycoproteins in the interfacial matrix; their concentration peaks just prior to the changes in cell and tissue morphology that characterize placode formation (Hendrix & Zwaan, 1974b). Although collagen is a glycoprotein, it contains relatively little carbohydrate and is present in only small amounts in the interface. Therefore, it cannot by itself account for the intense PAS reactions seen in the contact area. Secondly, trypsin in a calcium–magnesium-free medium is very effective in
dissociating the lens rudiment from the optic vesicle. Moreover, trypsin-separated lens rudiments cultured in isolation form undifferentiated flat epithelial cell sheets. The explants gradually diminish in size because of a loss of cells at their periphery (Muthukkaruppan, 1965). This suggests that removal of trypsin-sensitive molecules from the cell surface also removes previously present restrictions on cell spreading.

It is not necessary to postulate a qualitative or quantitative change in the output of glycoproteins by the cells of the developing eye. The close proximity of lens and optic vesicle during the tissue interaction phase diminishes the extent of the region into which these molecules are excreted. Thus, even in the absence of increased production, the concentration of glycoproteins in the vicinity of the cells has to go up (Hendrix & Zwaan, 1974b).

We believe that the proposed model offers a logical explanation of lens morphogenesis and agrees with available data. The principles underlying the hypothesis may also be applicable to other examples of embryonic organ formation.

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