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# Cell proliferation in condensing scleral ectomesenchyme associated with the conjunctival papillae in the chick embryo

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SUMMARY

The role of cell proliferation in the formation of scleral ectomesenchymal condensations underlying the conjunctival papillae was examined with *in vivo* tritiated thymidine labelling in chick embryos ranging in age from 8 days 0 h to 10 days 12 h. Percentages of labelled nuclei were determined in both ectomesenchyme and the deeper fibrous sclera for short-term and continuous tritiated thymidine incubations. During formation of the ectomesenchymal condensations the percentages of labelled nuclei were consistently higher within the condensations than in corresponding non-condensing ectomesenchyme between papillae. The consistent differences of labelling percentages observed within the condensing *versus* non-condensing ectomesenchyme were not found in the fibrous sclera at any stage. All areas of both the ectomesenchyme and fibrous sclera showed decreases in the percentages of labelled nuclei from 8 days 0 h to 10 days 12 h, although the decline in the ectomesenchymal condensations beneath papillae occurred more slowly than in areas between papillae. The data suggest that the conjunctival papillae directly influence the proliferation in the subjacent condensing ectomesenchyme but have no effect on the ectomesenchyme between papillae or any region of the deeper fibrous sclera. The observations of this investigation are discussed in relation to other studies of the development of the pre-ossicular mesenchyme.

## INTRODUCTION

Scleral ossicles, a ring of plate-like membrane bones surrounding the corneal margin, are present in many submammalian vertebrates, including birds. This scleral skeleton provides rigidity to the concave corneoscleral junction (sulcus) and is structurally necessary for visual accommodation in Sauropsidans (Walls, 1942). During embryonic development, these skeletal elements affect both the corneal curvature and the shaping of the posterior segment of the eye (Coulombre, 1965).

In the chick embryo this scleral skeleton normally contains 14 ossicles and develops during the second week of incubation following a prolonged and intimate association with transitory epithelial papillae in the overlying conjunctiva. The conjunctival papillae arise during the eighth day of development and correspond precisely in number and location to the areas of underlying sclera in which the

*Key words*: cell proliferation, conjunctival papillae, chick embryo, scleral ectomesenchyme, ocular development, scleral ossicles.

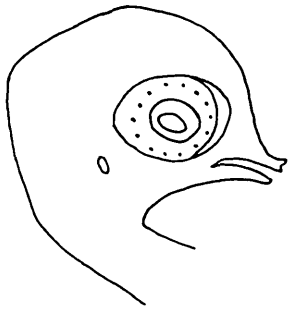


Fig. 1. Diagrammatic representation of 9 days 12 h embryonic chick head showing location of conjunctival papillae. The 14 papillae form a ring surrounding the corneal margin.

ossicles subsequently form (Fig. 1). The papillae undergo a complex morphogenesis in intimate association with subjacent scleral tissue and degenerate by the twelfth day of development as the bones begin to ossify (Murray, 1943; Coulombre, Coulombre & Mehta, 1962; van de Kamp, 1968).

Evidence from several studies suggests that the conjunctival papillae initiate the formation of the corresponding scleral ossicles (Coulombre *et al.* 1962; Puchkov, 1962, 1965; Palmoski & Goetinck, 1970; Blanck, McAleese & Sawyer, 1981; Johnston, 1973).

The morphogenesis of the conjunctival papillae in the chick embryo has been delineated as six convenient stages by Murray (1943; Fig. 2). Subsequent studies have confirmed and extended these excellent earlier observations (Coulombre *et al.* 1962; Puchkov, 1964; van de Kamp, 1968; Fyfe & Hall, 1981).

The scleral mesenchyme underlying the conjunctival papillae consists of a loosely structured outer layer derived from the embryonic neural crest and a densely structured inner layer derived from embryonic mesoderm (Noden, 1978; Johnston *et al.* 1979). The neural-crest-derived mesenchyme has been termed ectomesenchyme to distinguish that layer from the densely structured inner layers or fibrous sclera, the mesodermally derived mesenchyme (Fyfe & Hall, 1983).

The ectomesenchyme underlying the conjunctival papillae becomes markedly condensed in association with collagenous fibrils that extend inward from the papillae between stages 3 and 5. During stages 4 and 5, these condensations have a columnar appearance with their cells oriented parallel to the collagen strands. By late in stage 5 the innermost portions of the condensations begin spreading to form flattened plate-like condensations immediately above the fibrous sclera. As the papillae disintegrate during stage 6, the columnar condensations and collagen strands disappear while the flattened plates continue to enlarge. The onset of ossification occurs in these plate-like condensations as the papillae disintegrate during the 12th day of development.

Development of the ectomesenchymal condensations in chick embryos has been attributed to *in situ* cell proliferation from studies of mitotic activity (Hale, 1956) and patterns of tritiated thymidine labelling in tissue explants cultured *in vitro*

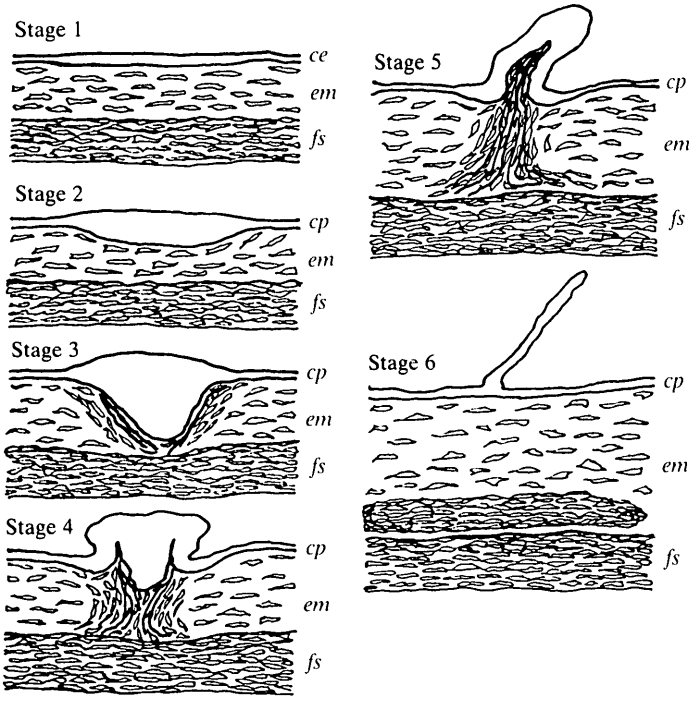



Fig. 2. Drawings illustrating the morphogenesis of conjunctival papillae according to the six stages designated by Murray (1943). Conjunctival papilla (*cp*), ectomesenchyme (*em*), fibrous sclera (*fs*). Stage 1. Conjunctival epithelium (*ce*) shows slight thickening overlying ectomesenchyme and fibrous sclera. Condensation of the ectomesenchyme is not evident at this stage. Stage 2. Conjunctival papilla is distinct as an epithelial thickening extending slightly into the ectomesenchyme. Condensation of ectomesenchyme beneath the papilla is not yet prominent. Stage 3. The conjunctival papilla is a distinct structure with a conical 'tongue' extending deep into the ectomesenchyme. In some specimens the tip of the 'tongue' appears to reach the uppermost portion of the fibrous sclera layer. Definitive condensation of the ectomesenchyme immediately surrounding the papilla appears simultaneously with fine collagenous strands beneath the basal surface of the papilla. Stage 4. The papilla extends prominently above the surrounding conjunctival surface and the 'tongue' appears to be withdrawn from the deeper ectomesenchyme. The ectomesenchymal condensation shows a columnar orientation in intimate association with numerous collagenous strands. Flattening of the innermost portion of the condensation above the fibrous sclera is not distinct at this stage. Stage 5. The papilla is filiform and elongated above the surrounding conjunctiva. The epithelium of the papilla surrounds a distinct central cavity that is continuous with the underlying ectomesenchyme. The ectomesenchymal condensation and associated collagenous strands fill the central cavity and extend inward to a distinctly flattened region spread against the fibrous sclera. The ectomesenchyme beneath the papilla is noticeably thicker than at earlier stages and the condensation is a prominent columnar feature. Stage 6. Degeneration and disappearance of the papilla characterize stage 6. The papilla is filiform but no longer surrounds a central cavity. As morphogenesis proceeds the ectomesenchymal condensation disappears from the area immediately beneath the papilla as a prominent flattened condensation forms in the innermost part of the ectomesenchyme above the fibrous sclera.

(Fyfe & Hall, 1983). The present investigation utilized labelling with tritiated thymidine to examine the *in vivo* distribution of DNA-synthesizing cells in the scleral ectomesenchyme during stages that the conjunctival papillae are present. These methods expand on earlier studies by providing a direct *in vivo* assessment of temporal and spatial aspects of DNA synthesis and cell proliferation in the pre-ossicular sclera. Our observations confirm the localization of highest percentages of DNA-synthesizing cells in developing condensations of the ectomesenchyme. Percentages of labelled cells were considerably higher than those observed with earlier *in vitro* studies for equivalent developmental stages and labelling times. The results are compared with observations from other studies in an attempt to elucidate the morphogenetic events that accompany initiation of the scleral ossicles.

## MATERIALS AND METHODS

Eggs of White Leghorn chickens were maintained at 37·5°C in a Jamesway forced draft incubator. Windows were cut from the shells during the third day of incubation, sealed with cellophane tape, and returned to the incubator until labelled thymidine was administered. To examine embryos at ages encompassing all stages of development and regression of the papillae, embryos were sacrificed at ages of either 8 days 0 h, 9 days 0 h, 9 days 12 h, or 10 days 12 h.

Tritiated thymidine (activity greater than 10 Ci mM$^{-1}$; New England Nuclear Corp.) diluted to 100 $\mu$Ci ml$^{-1}$ with Hank's balanced salt solution was administered by injection into the yolk through the shell window. Preliminary tests to determine the dose necessary to provide sufficient labelling of DNA-synthesizing scleral mesenchymal cells utilized injections of 1, 5, 10, or 20 $\mu$Ci of tritiated thymidine with 8 days 0 h, 9 days 12 h, and 10 days 12 h embryos. From these tests, 10 $\mu$Ci was selected as a standard dosage for all labelling studies. It was determined that 30 min of incubation following injection of tritiated thymidine was necessary and sufficient to label DNA-synthesizing nuclei.

Continuous labelling with tritiated thymidine was carried out for periods up to 24 h. Additional injections of 5 $\mu$Ci tritiated thymidine were administered into the yolk at 4 h intervals to ensure that all proliferating cells would become labelled during continuous incubations. The observation that 100 % of neural retinal cells became labelled during incubations of 7 h or longer was taken as evidence that tritiated thymidine remained continuously available within the eye in this procedure.

Eyes were excized directly into Bouin's fixative for all histological preparations. After fixation for 12–18 h, eyes were dehydrated through a graded ethanol series. While in absolute ethanol, the outer portions of the eyes including the conjunctiva and underlying sclera were dissected by a circumferential cut near the ocular equator. These dissections provided tissues that could be embedded, oriented, and sectioned more easily than intact eyes. The tissues were cleared in dioxane and embedded in 61°C melting point Tissuemat. Tissues were oriented to permit sectioning along a radius paralleling the long temporal ciliary artery and in a plane nearly normal to the conjunctiva. Serial sections were cut at a thickness of 5 $\mu$m and mounted on glass slides.

For autoradiographic study, sections were deparaffinized with toluene and hydrated through a graded ethanol series to distilled water. The slides were dipped in Kodak NTB-3 nuclear emulsion diluted 1:1 with distilled water. Coated slides were stored for 3 weeks in light-tight boxes containing a desiccant. Following this exposure the slides were developed with Kodak D-19 developer and the sections were stained with haematin by a double-bath technique (Searls, 1967).

Autoradiographs were examined at ×500 magnification with a Wild M20 microscope equipped with a drawing tube attachment. A standard field 250 $\mu$m in width was superimposed on the section in order to score labelled and unlabelled cells. A nucleus was considered to be

labelled if the number of overlying silver grains was three or more grains higher than background.

Scoring of labelled nuclei was done only on sections cut along or closely parallel to a radius through the cornea. In this way the geometrical relationships of different conjunctival papillae and underlying scleral mesenchyme were consistent and comparable with each other. Within an individual eye, scoring of labelled nuclei was not restricted to only one papilla, but included areas beneath two or three different papillae. All determinations of labelling percentages were based on scoring of at least 500 nuclei. Three or more papillae from at least two embryos were examined at any given stage.

## RESULTS

### *Determination of synthetic index values*

The percentages of nuclei labelled during a period of 30 min following administration of tritiated thymidine were determined for scleral tissue layers with embryos aged 8 days 0 h, 9 days 12 h, and 10 days 12 h. These percentages were considered Synthetic Index (S.I.) data since they represent the portion of a cell population actually synthesizing DNA during a brief exposure to tritiated thymidine (Nuttall, 1976). The Synthetic Index data reflected differences in scleral labelling patterns with respect to: 1) association with overlying conjunctival papillae, 2) individual layers within the sclera, and 3) developmental stages of embryos and papillae.

The Synthetic Index in the ectomesenchyme declined between 8 days 0 h and 10 days 12 h in areas directly beneath as well as between papillae (Fig. 4). However, the values observed beneath papillae were consistently higher than in areas between papillae in 8 days 0 h and 9 days 12 h embryos. In 10 days 12 h embryos, the Synthetic Indices from the ectomesenchyme were lower than at earlier ages, but differences from areas either beneath or between papillae were not demonstrable at this developmental stage.

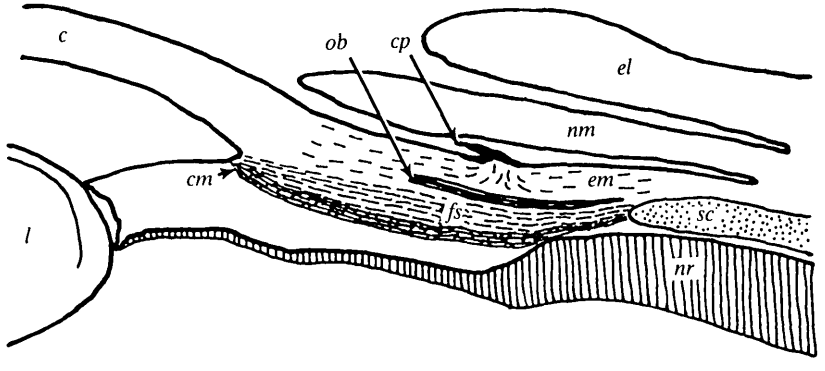



Fig. 3. Diagrammatic radial section of 10 days 12 h embryonic chick eye showing positions of conjunctival papilla and scleral ossicle primordium in relation to other eye tissues. Conjunctival papilla (*cp*), ossicular bed (*ob*), ectomesenchyme (*em*), fibrous sclera (*fs*), nictitating membrane (*nm*), eyelid (*el*), cornea (*c*), lens (*l*), scleral cartilage (*sc*), neural retinal (*nr*), ciliary musculature (*cm*).

Synthetic Index values in the fibrous sclera also declined during this developmental period. However, no significant difference from areas beneath or between papillae was found at any stage examined.

The ossicular bed becomes distinguishable as a discrete layer of the ectomesenchyme against the fibrous sclera after overlying conjunctival papillae have reached stage 5. At later developmental stages, the scleral ossicles differentiate within the plate-like condensations that form the ossicular bed (Fig. 2).

In contrast to either the ectomesenchyme or the fibrous sclera, the Synthetic Index values in the ossicular bed increased between 9 days 12 h and 10 days 12 h, corresponding to the period from stage 5 to stage 6 of papilla morphogenesis. Furthermore, Synthetic Index values in the ossicular bed increased in areas immediately beneath papillae as well as between papillae. This increase in Synthetic Index values of the ossicular bed from 9 days 12 h to 10 days 12 h of

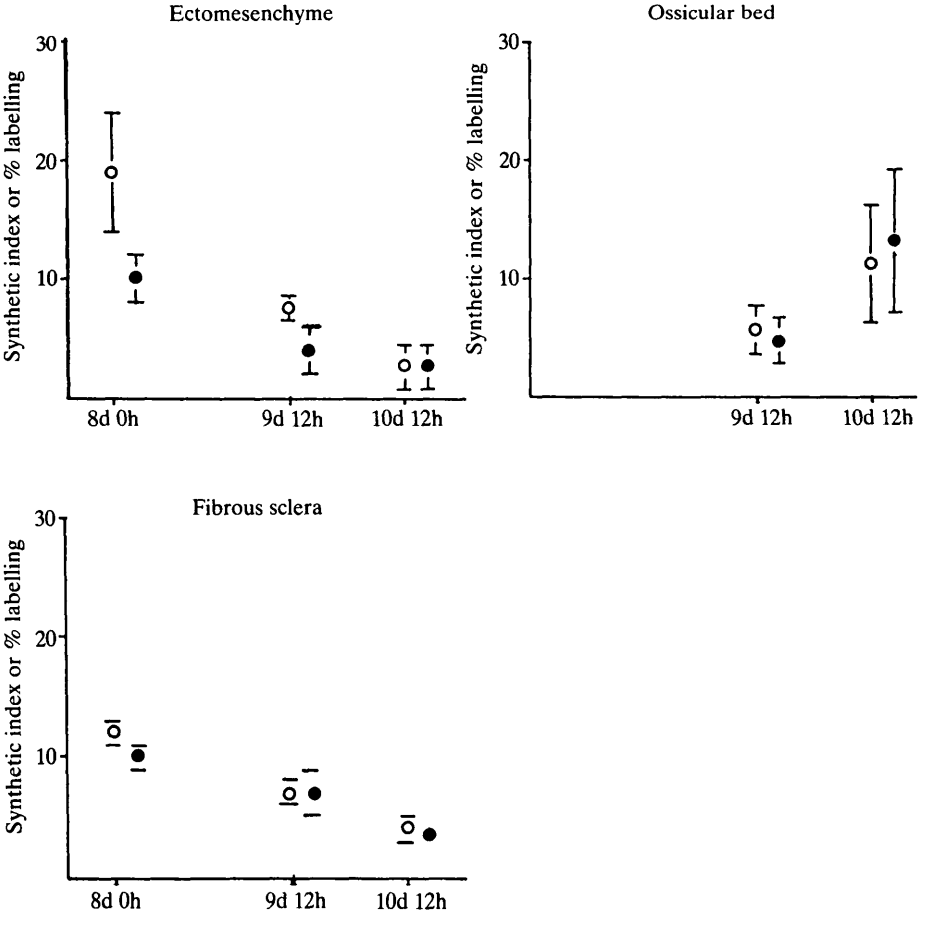



Fig. 4. Synthetic Index data (percentages of labelled nuclei ½ h after administration of tritiated thymidine) in scleral layer plotted against embryonic age and stage of overlying papilla development. Each point represents the mean of three samples of 500+ nuclei from at least two embryos and the range is the standard deviation. ○, directly beneath papillae; ●, midway between papillae.

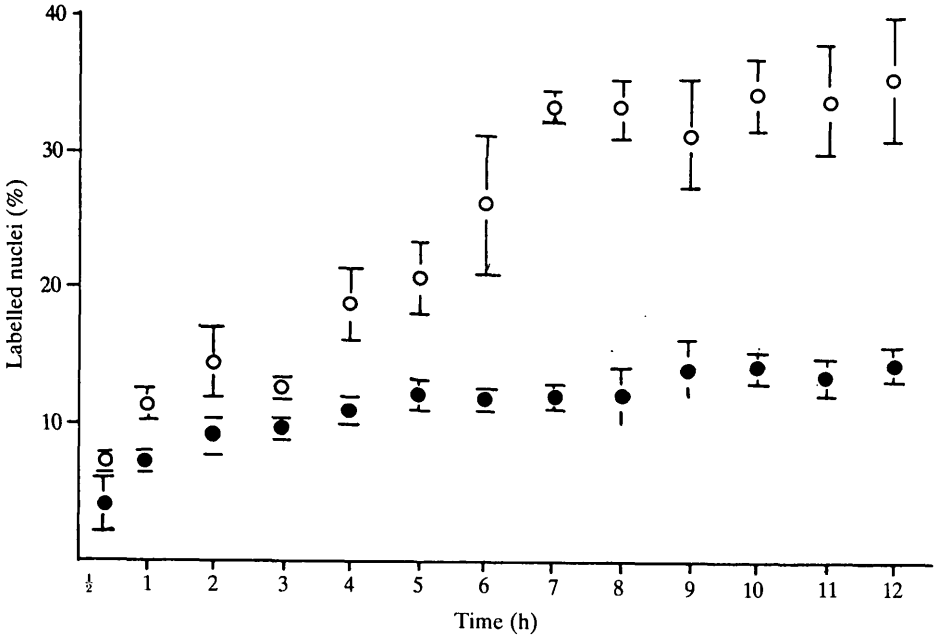



Fig. 5. Time course of thymidine labelling in ectomesenchyme beneath and between papillae in 9 days 12 h embryos. Each point represents the mean of three samples of 500+ nuclei from at least two embryos. ○, ectomesenchyme beneath papillae; ●, ectomesenchyme between papillae.

development coincided with the initiation and spreading of the flattened pre-ossicular plates within the sclera.

*Continuous labelling*

Continuous labelling with tritiated thymidine was undertaken to determine whether differences in Synthetic Index values beneath and between papillae reflected differences in percentages of dividing cells. In preliminary experiments with conjunctival papillae in stage-5 embryos (aged 9 days 12 h at time of sacrifice), percentages of labelled scleral cells were determined at hourly intervals for 12 h following initiation of labelling. The percentages of labelled cells in each layer of the scleral ectomesenchyme increased steadily during the first 7 h of labelling with lesser increases observed beyond 7 h (Fig. 5). No area of the ectomesenchyme ever attained 100 % labelled cells even with labelling periods up to 24 h. Labelling of 100 % of the nuclei of nearby retinal tissues at the optic cup margin within 7 h and at all later sampling times indicated that availability of thymidine in the eyes was not limiting in these incubations.

Percentages of labelled nuclei after continuous incubation with tritiated thymidine were consistently higher in the ectomesenchyme beneath stage-5 papillae than in corresponding areas between papillae.

Based on these observations with stage-5 papillae continuous labelling was also done with embryos aged 8 days 0 h, 9 days 0 h, and 10 days 12 h in order to compare labelling within each scleral layer during stages 1 through 6 of papilla development. Continuous thymidine labelling time was standardized with 8 h incubation periods thus providing 8 H Labelling Indices (8HLI) for these comparisons. The 8 H Labelling Index values are shown in Fig. 6.

From initiation of papilla development until stage 5 the condensations of ectomesenchyme immediately beneath papillae consistently had higher labelling indices than corresponding areas between papillae. In 8 days 0 h embryos the 8 H Labelling Index was 44 % beneath stage-1 and -2 papillae compared with 28 % between papillae. By 9 days 12 h these values decreased to 33 % (stage-5 papillae) and 12 % respectively. Under stage-6 papillae in 10 days 12 h embryos the 8 H Labelling Index was 5 % in all areas of the ectomesenchyme. This decrease in the 8 H Labelling Index coincided with the stage at which papillae were disappearing

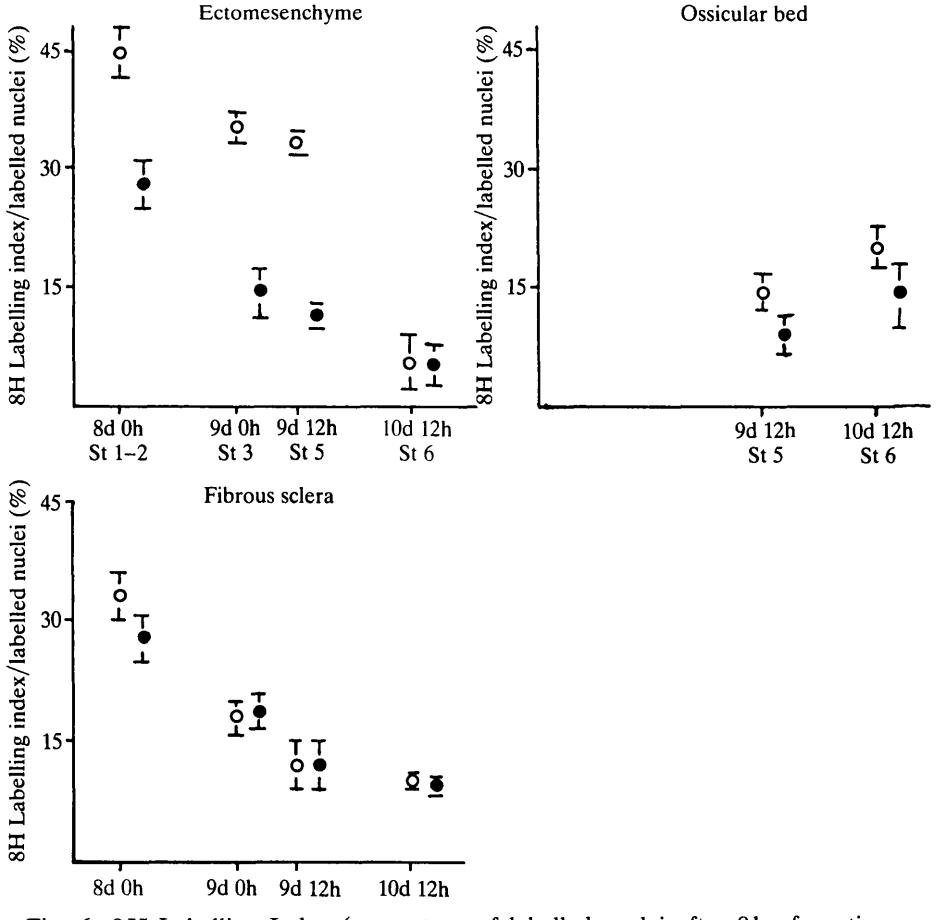



Fig. 6. 8 H Labelling Index (percentage of labelled nuclei after 8 h of continuous labelling with tritiated thymidine) of scleral layers plotted against embryonic age and stage of overlying papilla development. Each point represents the mean of five samples of 500+ nuclei from at least two embryos and the range is the standard deviation. ○, directly beneath papillae; ●, midway between papillae.

and the underlying condensations in the ectomesenchyme were no longer evident between the papilla and ossicular bed.

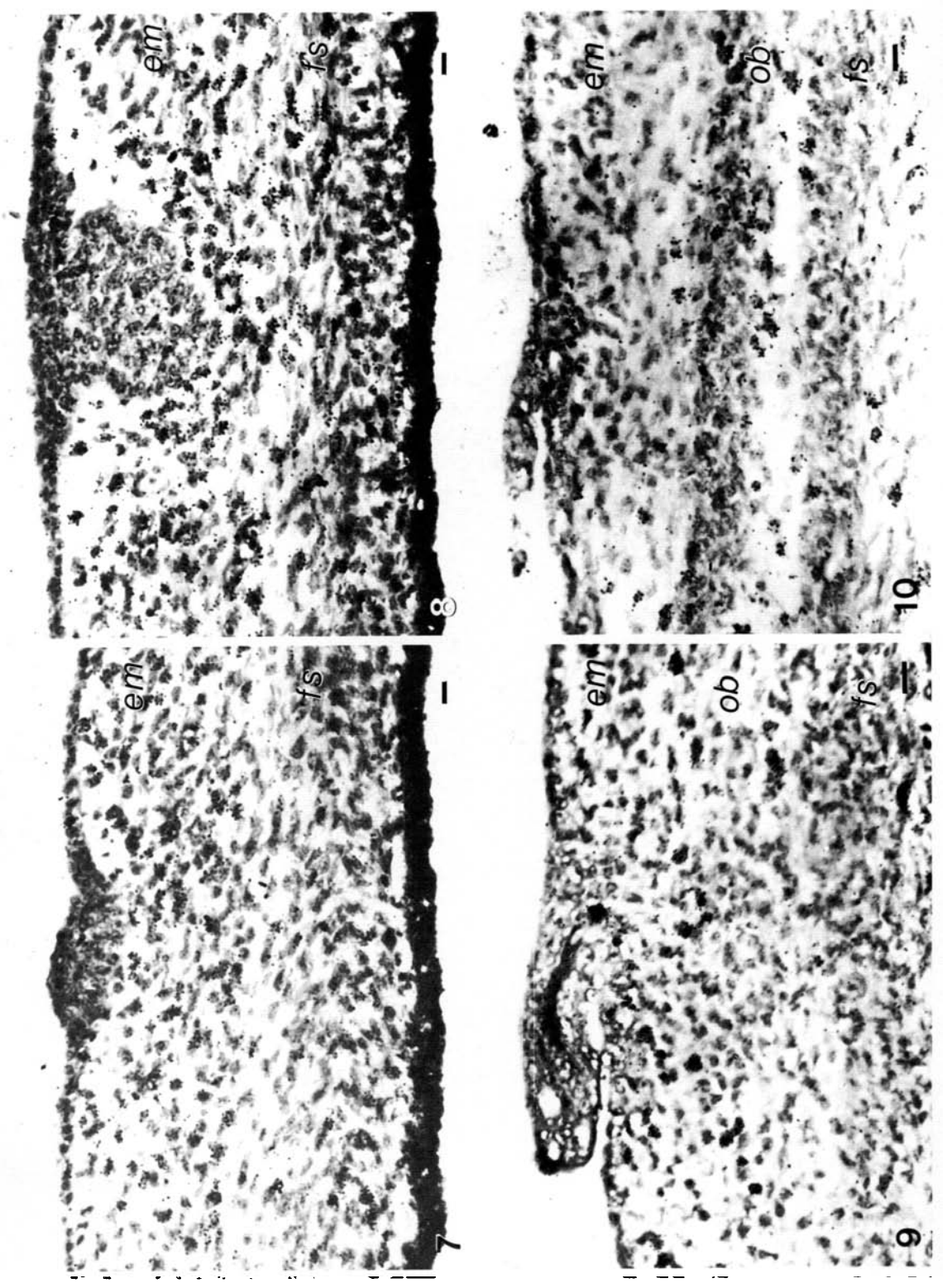
Condensation of the ossicular bed begins to appear just above the fibrous sclera under stage-5 papillae and becomes prominent at stage 6. Comparison of 8 H Labelling Index data from 9 days 12 h and 10 days 12 h embryos indicated that labelling percentages increased in this ossicular bed layer as these condensations became flattened adjacent to the fibrous sclera.

The fibrous sclera showed a decline in LI in areas both beneath and between papillae from 8 days 0 h to 10 days 12 h. Areas of the fibrous sclera from either beneath or between papillae had similar 8 H Labelling Index data at any stage of development.

*Correlation of labelling patterns with histological changes*

Relationships between histological changes and labelling patterns were best illustrated with autoradiographs. During stages 1 and 2 (Fig. 7) the ectomesenchyme and the fibrous sclera appear equally thick. Condensations of ectomesenchyme were not evident at these stages. Labelled nuclei were randomly distributed within each layer. Condensations of the ectomesenchyme immediately surrounding the tongue at stage 3 (Fig. 8) and beneath the papillae at stage 5 (Fig. 9) were also the areas of the highest percentages of labelling. As shown previously (van de Kamp, 1968), cells within the ectomesenchymal condensations at stage 5 were generally oriented along the axes of the collagen fibrils extending from the papillae to the ossicular bed. This orientation could also be seen even though the collagen was not stained in these autoradiographs. At stage 6 (Fig. 10), the ectomesenchymal condensations were either less prominent than at stage 5 or were no longer present. Numerous capillaries were seen within the ectomesenchyme at this stage. It is also at stage 6 that the 8 H Labelling Index decreased to approximately 5 % in all parts of the ectomesenchyme. Some of the labelled nuclei within the ectomesenchyme at this stage were in endothelial cells of invading capillaries.

The most striking histological change between stages 5 and 6 occurred in the ossicular bed layer. Beneath papillae in stage 5, the ectomesenchymal condensation extended from the papilla into the ossicular bed area (Fig. 9). However, at stage 6, prominent plate-like condensations foreshadowed the prospective scleral ossicles deep beneath each papilla (Fig. 10). These pre-ossicular condensations were flattened plates oriented parallel to the fibrous sclera. As these condensations were forming, the cells in the centre became densely packed and did not become labelled with tritiated thymidine. Among the less-densely packed cells immediately surrounding the condensations, a high proportion of the cells continued to become labelled. As condensations beneath adjacent papillae grow toward one another the marginal regions of dividing cells approach each other. For these reasons, the ossicular bed between stage-6 papillae sometimes had a higher percentage of labelled nuclei than corresponding areas directly beneath papillae. The overall increase in the 8 H Labelling Index occurring in the ossicular bed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between stages 5 and 6 was indicated by the sparsity of labelled nuclei in this layer at stage 5 (Fig. 9) and their abundance at stage 6 (Fig. 10).

The appearance of the fibrous sclera in areas beneath and between papillae at each stage showed no significant histological differences within this layer.

## DISCUSSION

Observations in the present study support the view that *in situ* cell proliferation is a significant factor in the formation of ectomesenchymal condensations underlying the conjunctival papillae in the chick embryo. Labelling with tritiated thymidine revealed consistently higher percentages of labelled cells within ectomesenchymal condensations than in adjacent areas of non-condensing ectomesenchyme. Continuous labelling studies suggested that cell generation times were similar in different tissues and at the different embryonic ages that were examined. During the period from 8 days 0 h to 10 days 12 h of development, no area of scleral tissue attained 100 % nuclear labelling even with thymidine incubations up to 12 h.

Earlier studies have also concluded that these ectomesenchymal condensations arise from *in situ* cell proliferation rather than migration from adjacent areas. Hale (1956) observed percentages of mitotic figures within ectomesenchymal condensations to be higher than in neighbouring tissue areas. Fyfe & Hall (1983) examined tritiated thymidine labelling indices in scleral explants incubated *in vitro* for a period of 4 h. The distributions of labelled ectomesenchymal cells were very similar to those observed in the present *in vivo* study although the actual

Figs 7–10. Abbreviations: *em*, ectomesenchyme; *fs*, fibrous sclera; *ob*, ossicular bed. Bars equal 10 $\mu$m.

Fig. 7. Autoradiograph of area beneath stage-1 papilla after 8 h of labelling with tritiated thymidine. Labelled nuclei are randomly distributed within both the ectomesenchyme and fibrous sclera at this stage. In the conjunctival papilla labelled nuclei are rarely found and are generally restricted to the periphery of the papilla.

Fig. 8. Autoradiograph of stage-3 papilla and underlying sclera after 8 h of labelling with tritiated thymidine. Labelled nuclei in the ectomesenchyme immediately surrounding the papilla correspond to the area of condensing ectomesenchyme. Labelled nuclei are less common in the ectomesenchyme away from the papilla and in the fibrous sclera. Labelled nuclei are not found within the epithelial papilla. In this section the long temporal ciliary artery appears within the fibrous sclera above the opaque pigmented epithelium of the retina.

Fig. 9. Autoradiograph showing a stage-5 papilla, condensed ectomesenchyme, and fibrous sclera. Labelled ectomesenchymal cells are mostly found in the condensed area directly beneath the papilla. The inner portion of the ectomesenchyme is beginning to establish a spreading, plate-like condensation against the fibrous sclera, the ossicular bed, at this stage.

Fig. 10. Autoradiograph showing 10 days 12 h stage-6 papilla and underlying sclera. The ectomesenchymal condensation appears as a plate-like layer several cells thick lying deep in the ectomesenchyme against the fibrous sclera. This inner portion of the ectomesenchyme is clearly distinguishable as the ossicular bed at this stage. Labelled nuclei in the ectomesenchyme at this stage are mostly found on the periphery of this condensation. The ectomesenchyme between the papilla and the plate-like ossicular primordium is less densely structured than at stages 3 through 5.

percentages of labelled cells were considerably lower than those found at 4 h labelling periods in the present investigation. It seems probable that the observed differences in percentages of labelled cells may be attributable to *in vitro versus in vivo* behaviour of the tissues.

The data from both short-term and continuous labelling experiments indicate that DNA synthesis and cell proliferation in the mesodermally derived fibrous sclera are not differentially influenced by the overlying conjunctival papillae. The absence of localized areas of differential labelling in the fibrous sclera contrasts with patterns observed in the scleral ectomesenchyme.

The patterns of increases in labelling percentages observed with continuous thymidine labelling demonstrated that proliferating cell populations were asynchronous in their division cycles. The inflections of the labelling curves occurring at approximately 7 h into the labelling period suggest that the non-DNA synthetic portion of the cell cycle is about 7 h in length for each layer of the scleral tissues. In the neural retina 100 % labelling was attained in 7 h, indicating that under these conditions all of the cells in a tissue can become labelled. The differences in synthetic index between and beneath papillae are related to the number of cells in the 'S' phase of the division cycle and not to the length of cycle. Although the length of the DNA synthetic phase (S) has not been measured directly in this study, other studies with chick embryos have shown that the length of the S phase ranges from approximately 4 to 6 h in different tissues and at different embryo ages (see Nuttall, 1976). The length of the cell cycle in the sclera thus appeared to be approximately 12 h long during the stages that conjunctival papillae were present.

Continuous labelling data indicated that considerably less than 100 % of the scleral cells were actively dividing during the period of 8 days 0 h to 10 days 12 h of embryonic development. The proportion of actively proliferating scleral cells also decreased markedly in each scleral layer during this developmental period. This pattern of decreasing percentages of labelled cells was strikingly similar to observations on embryonic chick corneal proliferation at comparable developmental ages (Nuttall, 1976). The marked differences observed in areas of the ectomesenchyme directly beneath or midway between papillae indicated that the proportion of proliferating ectomesenchymal cells decreased more slowly in areas associated with papillae than in other regions. The most noticeable decrease in the percentage of labelled ectomesenchymal cells directly beneath papillae occurred simultaneously with the disintegration of the papillae at stage 6. It appears that the conjunctival papillae and the associated collagenous fibrils in the underlying mesenchyme permit associated ectomesenchymal cells to maintain higher proliferative percentages than ectomesenchyme not associated with the papillae.

At a minimum the conjunctival papillae and their associated collagenous strands determine the location of the scleral ossicles by initiating these ectomesenchymal condensations. Growth of the ossicular condensations and the onset of ossification continue after the papillae disappear. The direct influences of the papillae appear to be primarily determined by ectomesenchymal association with the collagenous strands extending inward from the basal surface of the papillae. Specific

mechanisms by which the papillae or these collagenous strands affect cell division and condensation of the ectomesenchyme remain unexplored.

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