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*ORIENTED MICROTUBULES IN ELONGATING CELLS OF THE DEVELOPING LENS RUDIMENT AFTER INDUCTION**

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Experimental and histological studies of lens induction have consistently failed to reveal the mechanism of interaction between the optic vesicle and the presumptive lens ectoderm. Even recent electron microscope examination of this phenomenon in mammals by Cohen[1] and in birds by Hunt[2] and by Weiss and Jackson,[3] though effective in describing in greater detail changes in cell organelles and basement membranes, has failed to show an interpretable induction mechanism or, for that matter, any structural changes seemingly involved in such manifestation of induction as shape changes in the affected cells. To some extent these initial investigations were hampered by the inadequacies of the then available fixing reagents for preserving fine structure. Thus, with the more recent introduction of glutaraldehyde and related compounds and the demonstration of their efficacy in preserving elements of fine structure previously lost, it became important to explore again this particular phenomenon—and to look especially at the early stages of cell elongation. The results describe a prominent development of "microtubules" in the cortical regions of presumptive lens ectoderm cells subsequent to induction and especially as the cells palisade to form the lens placode. Additional observa-

tions made on the extensively elongating cells of the posterior lens epithelium have provided evidence that these microtubules appear concurrently and consistently with this type of cellular elongation.

Materials and Methods.—Eggs of White Rock domestic fowl were incubated at 39° in an upright position as described by Weiss and Jackson.[3] Fixation, rinsing, postosmification, and initial dehydration were done in the sodium-phosphate-biphosphate buffer system of Sorensen[4] containing 0.0015 *M* $CaCl_2$. After incubation, the embryo was exposed by stripping off the shell and shell membranes at the blunt end. Three per cent glutaraldehyde[5] in 0.05 *M* buffer was dropped on the embryo, which was then cut away from the yolk and placed in the same fixative for 1 hr. The embryo was then washed in 0.1 *M* buffer, postosmicated in 1 per cent osmium tetroxide in 0.1 *M* buffer, dehydrated, and embedded in epon. Sections were cut "silver-gold" thickness on a Sorvall MT2 ultramicrotome, stained with uranyl acetate and lead citrate, and viewed in a Philips EM200 at 60 kv. One-μ sections for light microscopy were stained with the methylene blue-azure II-borax mixture of Richardson *et al.*[6]

Observations.—The first gross morphological manifestation of lens induction is the elongation of the ectodermal cells to produce the columnar epithelial cells of the lens placode. As is well known, the lens placode then invaginates into the cavity of the optic cup to produce a lens vesicle, which pinches off the inner surface of the rest of the head ectoderm as a spherical shell of cells whose apical poles lie on the inner surface of the shell. Subsequently, the posterior epithelium, which is the hemisphere closest to the presumptive retina, is extensively thickened by an elongation of its component cells.

We have observed that the cellular elongation involved in the formation of the lens placode and the further elongation of some of these same cells in the thickening of the posterior lens epithelium is accompanied by the appearance in the cell cortices of "microtubules," approximately 230 A in diameter. They present the same image in transverse section, a dense ring around a less dense center, as observed in microtubules described in other cells.[7-10] The microtubules are, in this instance, oriented parallel to the axis of cellular elongation.

Lens placode formation: The arrangement of presumptive lens cells and their major organelles during lens induction and placode formation has been adequately described previously.[2, 11] Our observations have confirmed that all component cells maintain one end at the free surface of the placode tissue, where terminal bars join adjacent cells, and the other end against the basement membrane throughout placode formation, except possibly during certain stages of mitosis, when the bulk of the cell comes to lie next to the free surface of the ectoderm.

At stage 11 of Hamburger and Hamilton[12] the cells are cuboidal, about 15 μ thick, with nuclei located centrally or slightly basally (Fig. 1). Large intercellular spaces are present between the apical poles, which are bound together by terminal bars, and the basal poles, where the plasma membranes are tightly adherent. A relatively homogeneous cytoplasm contains scattered free ribosomes and rosettes of glycogen, mitochondria, and simple Golgi bodies, and an occasional rough-surfaced cisterna of the endoplasmic reticulum. A few microtubules may appear parallel to the free surface or just peripheral to the nuclear envelope.

During the latter part of stage 11, large numbers of long, straight microtubules of

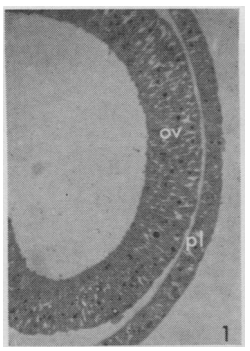

FIG. 1.—Photomicrograph of the optic vesicle (*ov*) and overlying presumptive lens ectoderm (*pl*) before lens induction. Hamburger-Hamilton stage 11.

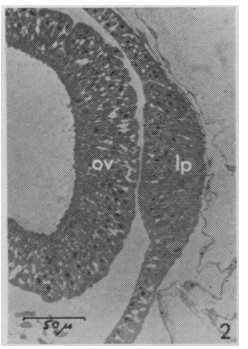

FIG. 2.—Photomicrograph of the optic vesicle and the lens placode (*lp*) after lens induction. Those cells of the presumptive lens ectoderm which have received the inductive stimulus from the optic vesicle have trebled in length with little change in cell volume, thereby drawing many cells into a small area of ectoderm. Stage 13.

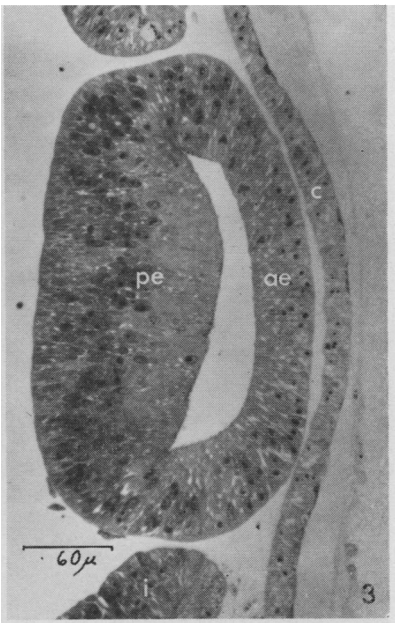

FIG. 3.—Photomicrograph of the early lens during thickening of the posterior epithelium (*pe*); stage 19; (*c*, presumptive cornea; *ae*, anterior epithelium of lens; *i*, lips of presumptive iris).

approximately 230 A diameter appear in a basoapical orientation in the cortical regions of the presumptive lens cells. With their appearance the nuclei move to a more basal position, and smaller organelles like mitochondria become oriented with their major axes parallel to the microtubules. The tubules themselves are much nearer to one another at the apical pole, and it is from this site that other organelles appear to migrate as the cells begin their elongation. In some images the microtubules seem indeed to be inserted within the dense material comprising the terminal web.

During stages 12 and 13, the placode cells continue to elongate. Just prior to placode invagination, a placode cell is about 4 μ in diameter at its base, 3 μ at its apex, and 50 μ long (Fig. 2). The smaller diameter of the apical poles of these cells accounts for the fact that the peripheral cells of the placode extend obliquely toward the center from base to apex. The nuclear portions of these columnar cells are about 7.5 μ in diameter, and are staggered in position to form a pseudostratified epithelium with very little intercellular space. Most nuclei are situated near the basal cell surface and possess an elongate form. Mitochondria, Golgi complexes, and rough-surfaced cisternae of the endoplasmic reticulum are concentrated near the basal pole, with their major axes generally parallel to the microtubules.

In oblique sections, the cell cortices display strikingly parallel segments of these microtubules, spaced about 50 mμ apart, in the apical and nuclear regions. Relatively few tubules extend into the basal poles. These microtubules (like those seen by us in various other tissues of the chick embryo) are individually

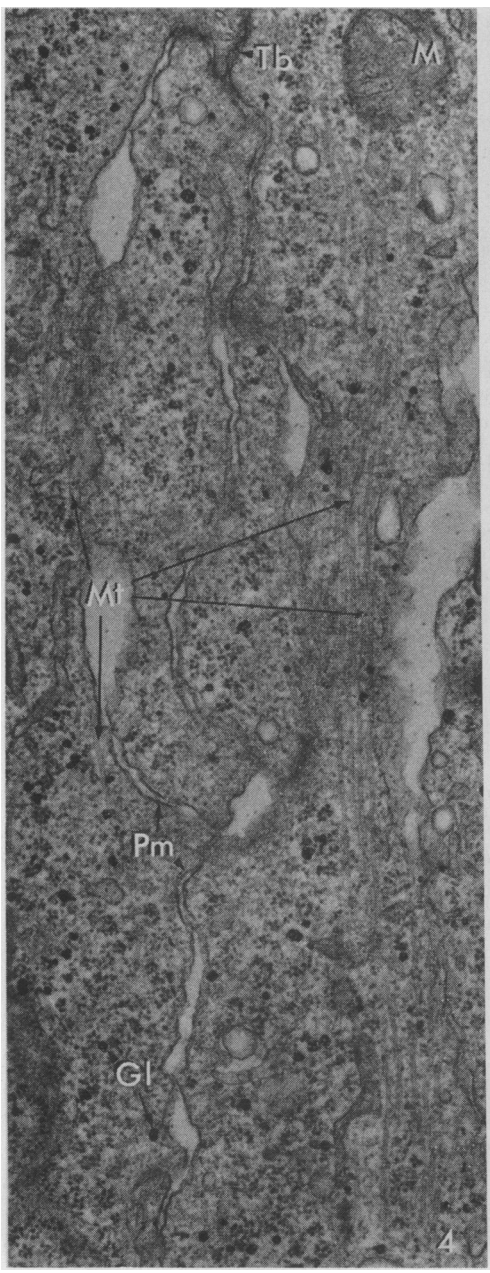

FIG. 4.—Electron micrograph of longitudinal section of the apical portions of several cells of the lens placode shown in Fig. 2. Terminal bars (*Tb*) bind cells together near the free surface. Microtubules (*Mt*) are seen along the long axes of the cells (*M*, mitochondrion; *Pm*, plasma membranes; *Gl*, glycogen).

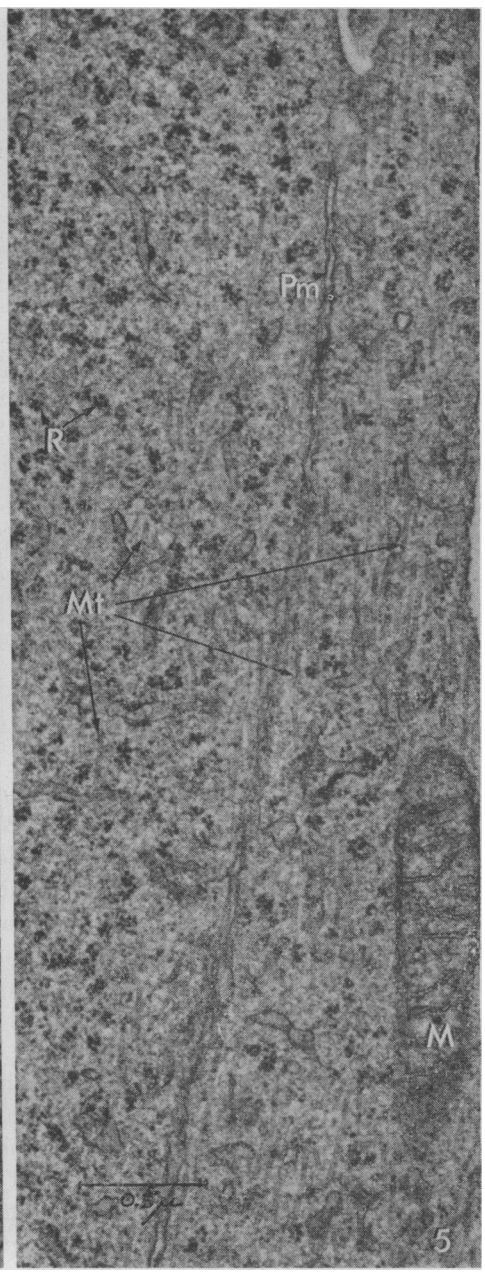

FIG. 5.—Electron micrograph of the central portion of the lens shown in Fig. 3. The section is nearly longitudinal to the cells but cuts the plasma membranes somewhat obliquely, demonstrating cortically arranged microtubules oriented in the direction of cellular elongation (*R*, clusters of ribosomes).

very long and straight (Fig. 4); they may run as far as 30 μ in a section 60 mμ thick, and rarely show any major change in direction.

Posterior lens epithelium elongation: The lens placode subsequently invaginates and pinches off as the primary lens vesicle with a slight reduction in cell length from 50 to about 35 μ. Microtubules may still be found during this period of invagination, but apparently in smaller numbers and even more restricted to the apical pole, which now resides adjacent to the cavity of the lens vesicle.

Shortly after lens vesicle formation, the posterior lens epithelium undergoes an extensive thickening. Between stages 19 and 21 of Hamburger and Hamilton, cells in the center elongate from an original length of 35 μ to a final length of 140 μ, at

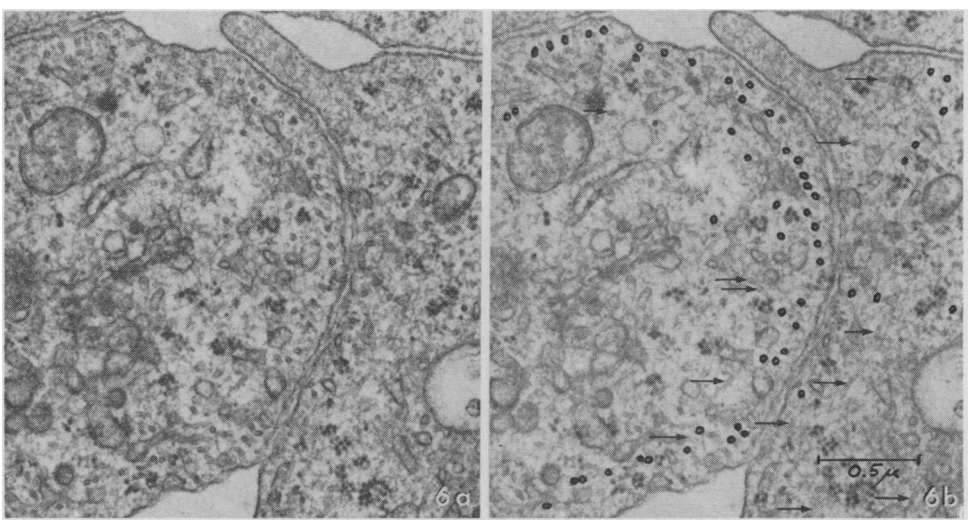

FIG. 6.—Electron micrograph of a transverse section of the same lens as in Figs. 3 and 5. (*a*) The original micrograph showing the circular cross sections of the tubules. (*b*) The microtubules in the same image have been marked to illustrate their distribution in the cortical regions of the cells. Arrows indicate some of the more oblique sections.

which stage the cavity of the lens vesicle has been completely obliterated. Figure 3 shows the profile of the vesicle during this elongation. Nuclei retain their original positions relative to the whole cell, which extends as before from the basement membrane at the outer surface of the vesicle to the apical surface against the cavity.

Microtubules, oriented parallel to this axis of elongation, become prominent again in the cortices of the cells comprising this posterior epithelium (Fig. 5). They are again well demonstrated in longitudinal sections in which the plasma membranes of adjacent cells are cut tangentially. Several instances were found where microtubules seemed to enter the dense cytoplasm adjacent to the terminal bar, which forms a ring around the cell between 1 and 2 μ from its apical surface. Shorter microtubules were found randomly oriented within this small region between the terminal bar and the apical surface.

Transverse sections of these long cells which make up the posterior epithelium show the characteristic ring-shaped cross-sectional image of the tubules and their distribution in the cell. Where the section cuts the cell near its equator, it shows an arrangement of tubules as depicted in Figure 6. Here they are present all around

the periphery of the cell in a cortical zone about 0.2 μ from the plasma membrane. Where the cell narrows toward its apex, the tubules are present in equal numbers but are brought closer to the central axis so as to occupy all parts of the cytoplasm there represented.

Cell elongation in the axial region of the posterior epithelium ceases when the cavity of the lens vesicle has been obliterated. Shortly before lens fiber formation commences, tubules disappear from these cells but persist in the equatorial cells, which continue to elongate during lens enlargement.

Discussion.—The observations presented above describe long, slender microtubules as prominent constitutents of the fine structure of the presumptive lens ectoderm during cell elongation following lens induction in the chick embryo. These tubular elements are oriented parallel to the long axis of the cell and are concentrated largely in the cortical regions of the cytoplasm. They seem to crowd together in the narrow apical poles of these cells as though inserted in the terminal web. In the broader basal parts of the same cells, the tubules are less numerous and seem to end freely.

These microtubules fall within a class of cytological substructures which is being identified now in a broad spectrum of cells and organisms.[8-10] They are characterized by their cross-sectional ringlike image, by their size, and by their remarkable straightness and apparent rigidity. Whether or not the units observed here have as part of the dense limiting ring the 11–13 subunits that characterize the microtubules in plant cells[13] and the filaments in flagella[14] has not as yet been determined.

Another observation that appears here as especially significant is the close coincidence in time between the appearance of the microtubules and the elongation of the cell. It is true that before induction a few microtubules are found in the cuboidal cells of the ectoderm, but these are for the most part oriented parallel to the free surface of this tissue. Only as the cells of the placode begin to palisade does a population of vertically oriented tubules appear. These diminish in prominence during the invagination of the vesicle, but come back more strikingly during the further elongation as the posterior epithelium of the lens develops.

The major questions emerging from these observations relate to the role of the microtubules. Are they involved in the elongation of the cells? Do they, as they develop, constitute a framework for the cytoplasmic movements accompanying the changes in cell shape?

The occurrence of cellular shape changes in metazoan morphogenesis has been recognized since the earliest microscopic examination of embryonic development. Davenport in 1895 described the palisading or elongation of epithelial tissues as a common accompaniment of the formation of several embryonic organ rudiments.[15] This expression of morphogenesis may be observed in the formation of the blastopore, the neural plate, the neural retina, the apical ridge of the limb bud, the placodes of lens, ear, and nose, and the very similar imaginal discs of arthropods, among other systems. As is well known, many of these epithelial thickenings are demonstrably the products of inductions mediated by close apposition to adjacent tissues.

In the particular case of the lens placode, palisading—elongation of cells extending from basement membrane to free surface with no increase in cell volume—draws ectoderm cells into the placode area. Localized augmentation of the mitotic rate

cannot account for the increase in cell numbers.[11] Nor can spreading of adjacent tissues force cellular elongation in the placode, for palisading occurs in cultured eye rudiments[16] in which adjacent tissues have no constraining elements against which to produce a force toward the placode region. Thus, the mechanism of palisading must reside within the component cells themselves.

The behavior of certain cytoplasmic organelles before the onset of palisading is indicative of the development in the cells of some organizing influence establishing polarity. For example, Weiss[17] and McKeehan[11] have reported that in the earliest phases of the inductive process the nuclei move toward the basal poles of the presumptive lens cells and assume an ovoid shape, the major axis of which parallels the direction of subsequent elongation. A similar orientation of smaller organelles such as mitochondria and cisternae of the endoplasmic reticulum as seen in electron micrographs[1, 3] likewise reflects some organizing influence in the pre-elongation phase.

These observations suggest the presence of an intercellular framework associated with cellular elongation, as was early deemed necessary by D'Arcy Thompson.[18] Weiss[19] has proposed that cellular elongation specifically associated with induction is due to "an oriented reorganization of cell components" with respect to the contact surface. The system of microtubules described here may well represent the postulated cytoskeleton. The development of this system and its presence in the cell coincides precisely with the period of elongation, and the evident straightness and implied rigidity of the microtubules as well as their distribution and orientation would provide the required direction. Whether or not the microtubules persist after the shape change is effected seems to depend on whether the change is the last the cell will make and whether the tubules may serve after differentiation in maintaining the form achieved and participating, as its elastic component, in the motion of the entire cell or of its contents.[20] Microtubules in the posterior epithelium of the lens are rapidly lost at the completion of cellular elongation.

The presence in other cells and situations of similar microtubules associated in various ways with cell elongation and cytoplasmic movements[21] parallels closely the morphogenetic events displayed by the lens placode cells. The occurrence of the microtubules within plant cells has recently been observed in locations and orientations which relate them to protoplasmic streaming and wall formation.[10, 22] They seem also to be a constant structural element in slender protoplasmic processes quite apart from the cilia-flagella class.[23] A case similar to that described here is the manchette or caudal sheath present during spermiogenesis; this cylinder of parallel tubules extends caudally from the so-called nuclear ring surrounding the nucleus as the spermatid elongates and its cytoplasm moves toward the caudal pole.[7] We find it interesting that coinciding with this elongation in spermatids there are *in vitro* observations of unidirectional undulations in that part of the cell that is extending posteriorly from the nucleus.[24]

If we accept the obvious association of microtubules with changes in cell shape as a basis for assuming their involvement in producing these changes, then we are led to inquire into their mode of action. Do they simply carry the surrounding cytoplasm with them as they grow or polymerize out unidirectionally from a point of origin? Or does the cytoplasm move over them as a framework in some translational motion similar to that of actin with respect to myosin in the contracting

myofibril?[25] Cytoplasmic movement along the array of tubules, similar to the streaming of plant cells mentioned above, might well produce cellular elongation by gradual cytoplasmic translocation.

It may be significant that undulatory motion occurs frequently in cells possessing microtubules. The involvement of the 9 + 2 complex of microtubules in cilia and flagella is obvious and well known. More pertinent here is the observation by Holtfreter[26] that elongating cells isolated from amphibian gastrulae undergo wave-like peristalsis similar, perhaps, to that of spermatids described above. That these embryonic amphibian cells were endowed with microtubules is indicated by the birefringence of their slender necks[27] as well as by the widespread occurrence of these organelles in elongating cells of the primitive pit, neural plate, lens vesicle, and various placodes of chick embryos.

It does not necessarily follow, of course, that these arrays of microtubules are involved in the intracytoplasmic peristalsis, or that if involved they employ undulatory motions in molding cell shapes. Yet the coincidence between prominent developments of microtubules and these waves of cellular motion is interesting and deserving of further investigation.

Summary.—Subsequent to lens induction in the chick embryo, many microtubules, 230 A in diameter and oriented parallel to the direction of cell elongation, appear in the cells of the presumptive lens ectoderm. These long, straight structures later reappear in a similar cortical array in the elongating cells of the posterior epithelium of the lens vesicle, as well as in other palisading embryonic tissues. These relationships in the chick embryo and similar associations between microtubules and shape changes in other types of cells reported in the literature suggest a general functional significance of these organelles in the production of cell asymmetries in morphogenesis.

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*RECOVERY OF ANTIGENIC SPECIFICITY AFTER DENATURATION AND COMPLETE REDUCTION OF DISULFIDES IN A PAPAIN FRAGMENT OF ANTIBODY**

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It would now appear reasonably certain that the secondary and tertiary structure, and in some instances quaternary structure, of a number of proteins is dependent only on the information contained in the amino acid sequence.[1-8] This fits well with current concepts of protein synthesis which provide only for the colinear transfer of sequential information from the base triplets of the genetic template to the amino acids of the protein molecule without providing any mechanism for the direct transfer of conformational information.[9, 10] Antibodies are a unique group of proteins characterized by a great diversity with respect to specificity of their binding properties, but also, within any single class of antibodies, such as γ_{1A}, γ_{1M}, or γ_2, by a great similarity in charge, amino acid composition, and over-all three-dimensional structure. It has been difficult to envision a specific amino acid sequence and consequently a different genetic template determining each of the multitude of binding site conformations possible.[11] An alternative view holds that specificity is generated by three-dimensional folding more or less independent of the amino acid sequence. This is said to occur at the time of protein synthesis through the mediation of antigen. The stabilization of this structure has been postulated to be related to noncovalent interactions[12] or to a specific arrangement of disulfide bonds.[13]

A direct test of the relationship between amino acid sequence and antibody specificity has not been possible because of the chemical heterogeneity of antibody preparations available to date. Indirect tests of the role of noncovalent bonds in stabilization of the conformation of the binding site have been carried out by Karush[13] and by Buckley, Whitney, and Tanford.[14] However, in neither of these investigations could antibody specificity be regenerated after both cleavage of disulfide bonds and unfolding of secondary structure.

In the present investigation a papain-derived antibody fragment[15] containing the specific binding site is subject to full reduction of its disulfide bridges and unfolding