Regional differences in ultrastructure of the rabbit ciliary processes: The effect of anesthetics and fixation procedures

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Ciliary bodies were obtained from nonanesthetized rabbits slain by decapitation or air embolism as well as from animals after urethan or Diabutal (pentobarbital) anesthesia. Both light and electron microscope studies revealed distinctly different regions. A prominent feature of the cells of the nonpigmented epithelial layer in the anterior region (as defined in this paper) is a vesicle-bounded vacuole 0.4 to 1.5 μ in diameter. Regional variations occurred in different types of small vesicles as well as in the arrangement of the ciliary channels and of the functional structures found between cells. These variations have bearing on questions of how water and ions move across the epithelium. The findings suggest that the anterior epithelium is the more active region in aqueous formation. In the present experiments urethan and Diabutal—directly or indirectly—clearly altered the conditions for formation of aqueous. They provoked an edema in the anterior region of the ciliary processes, in the stroma as well as in two different locations between the epithelial cells. Further examination, with varied methods, would be required in order to establish whether these drugs may or may not be used when the physiology of the rabbit ciliary processes is to be investigated.

The anterior and posterior region of the rabbit ciliary body display prominent differences in normal histology. Several light microscopic investigations21, 32 but only one electron microscopic study84 have taken account of this peculiarity. The following report adds further evidence of differences between these two regions in their normal morphology, and in their reaction to different anesthetics and fixatives.

Methods

Albino and pigmented rabbits were put to death by intracardial air embolism or decapitation without anesthesia, unless otherwise indicated. The eyes were then removed rapidly. If an anesthetic was employed, the eyes were taken out before the animal was killed. The two drugs tested were administered via the ear vein, urethan in a 20 per cent solution, 1.5 Gm. per kilogram of body weight,6 Diabutal (pentobarbital) at a dose of 0.8 c.c. per kilogram, given in two rations.12 For prolonged anesthesia with either drug repeated injections had to be given. A circular incision was made 2 to 3 mm. behind the corneal-scleral border. The vitreous was gently pulled away, the zonule cut with scissors, and the remaining disk (cornea, iris,
ciliary body, and a little piece of sclera and retina) cut in four equal wedges which were fixed. The cornea was trimmed away only after the specimens were embedded. Pieces smaller than quadrants were not used because of curling of the ciliary body and iris during the following procedures. Fixation was started no later than five minutes after death. The four fixatives checked thoroughly were 1 per cent OsO₄ in Tyrode's solution; the same with the addition of 400 mmoles of sucrose; Dalim's chrome-osmium; and a sequence of glutaraldehyde-OsO₄. Despite other media were tested: 1 per cent OsO₄ in veronal-acetate buffer, hypo-osmolar, iso-osmolar and hyperosmolar to blood plasma; 3 per cent glutaraldehyde without postfixation; and KMnO₄ modified by using phosphate buffer in Tyrode's solution instead of water to increase the osmolarity. Usually the four quadrants of an eye were treated with different fixatives, which allowed direct comparison of the effects. Standard fixation schedule was one hour in an ice bath followed by one hour at room temperature. The tissue was embedded in epoxy resin (Araldite). An attempt was made to orient the specimens obliquely as shown in Fig. 1, so that all parts of the ciliary processes were visible within one thick section. Single and serial sections were cut on a Porter-Blum MT2 ultramicrotome, picked up on collodion- or formvar-coated 2 by 1 mm. slot grids and contrasted with uranyl acetate, lead citrate, or both. They were examined in a Siemens Elmiskop 1A. For light microscopy thick sections of plastic embedded tissue were stained with a mixture of methylene blue and azuré II.

Anatomical definitions

The internal limiting membrane. Since there is confusion concerning the nomenclature of different membranes, an introductory remark concerning terminology is appropriate. The basement membrane covering the outside of the optic vesicle and later the optic cup forms the internal limiting membrane of the retina and the ciliary body, facing the vitreous and the posterior chamber. By turning at the lip of the optic cup, this basement membrane is topologically continuous with the basement membrane of the pigment epithelium in both ciliary and retinal regions and faces the choroid. The latter membrane is a component of the lamina vitrea or Bruch's membrane. Thus the term "external limiting membrane" is clearly inappropriate for the basement membrane of the pigment epithelium of the ciliary body. As to the existence of morphological homologues of the external limiting membrane of the retina and Verhoeff's membrane of the pigment epithelium, the only structures to consider in the ciliary body are those at the junctions of the cells in the pigmented and nonpigmented epithelial layers. Additional cell junctions bridging the two layers follow the obliteration during development of that ventricular space of the optic cup extending from...
Figs. 2 and 3. Parts of block faces oriented as indicated in Fig. 1. Preparation: in Fig. 2, air embolism, fix. OsO₄-Tyrode-300 mmoles of sucrose; in Fig. 3, 3 hours of urethan, fix. OsO₄-Tyrode-400 mmoles sucrose. Not shown in the pictures are the retina at the ora serrata (to the left) and the membranous extensions (to the right) which are not connected by the bridge and which in edema look like the bases of the primary processes in Fig. 3, to their full extent. B, Bridge; P, pocket, S, secondary process. (x65.)
the retina to the marginal sinus. In this fusion the terminal bar systems known in the retina as external limiting membrane and Verhoeff's membrane are incorporated into a more complex system to be described.

Therefore, in the ciliary epithelium the cellular apices of both layers face each other; the bases of the nonpigmented cells face the posterior chamber, the bases of the pigmented cells face the stroma.3, 12, 16, 23

The intercellular junctions. There also is confusion in the literature about the nomenclature of intercellular junctions.1-11 In this paper the terms are used as follows: "Desmosomes" are structures which display three dark bands between cell membranes at a distance of 200 Å. Intracellular fibrils13-23 have a close relationship to this type of junction (Fig. 4). "Tight" or "occluding" junctions display "fused" inner leaflets11 of the opposing unit membranes in an intercellular separation of 100 Å. The central densification may occasionally look dotted. "Adherent" junctions display opposing dense membranes at a distance of 200 Å; sometimes a dark line can be revealed within this space. No intracellular fibrils are associated with them (Fig. 7 shows several tight and adherent junctions).

Cross anatomy and a new definition of the anterior and posterior region of the rabbit ciliary body. The pars plana is very short in the rabbit. The ciliary processes arise close to the retina and have a complex relationship with the iris. Slightly less than 50 per cent of them41, 42 are remarkably longer than the rest, and their bases continue onto the posterior surface of the iris to as much as two-thirds of its extent. Starting at the iris root towards the lens, any two long processes are joined laterally at right angles by a bridge consisting of stroma and covered by ciliary epithelium on both surfaces (Fig. 2 and 3). These bridges form pockets between the processes which open anteriorly into the posterior chamber approximately at the equator of the lens. The parts of the processes which extend even closer to the pupil than the pocket openings have been called membranous extensions41 and are hidden by the lens when viewed from the posterior side (Fig. 1). Because of their position in front of the lens they are not covered by vitreous but freely bathe in aqueous. One or more secondary processes erupt from the upper surface of each bridge; they do not connect directly to the iris (Fig. 2).

An anterior region and a posterior region is distinguished on the basis of gross and fine structure as well as different reaction to anesthetics and fixatives, as will be shown. The anterior region consists of the membranous extensions and the lateral walls and the roof of the pockets formed by the bridge as described above. The term posterior region includes that part of the ciliary body beginning at the ora serrata and running anteriorly to the anterior region.

Results

Some new findings concerning the fine structure of the ciliary body. The following description of morphology is based on observations made on tissue fixed in 1 per cent OsO4 in Tyrode's solution, unless indicated otherwise. Results of earlier electron microscopic investigations of the posterior region14, 17, 23, 26, 27, 40 apply as well to the anterior region save the exceptions to be discussed.

Some previously described structures of the ciliary epithelium showed a different distribution in the anterior and in the posterior region. This concerned the ciliary channels, the intercellular junctions and the intracellular organelles.

The ciliary channels (canaliculi ciliares2)
are a typical feature of the anterior region exclusively. Some serial sections revealed that they may protrude like a finger of a glove mostly into pigmented cells, rarely into nonpigmented ones, and end blind (CC1 in Fig. 5).

Three types of intercellular junction connect the epithelial cells of the ciliary body: desmosomes, tight junctions, and adherent junctions.

In the anterior region the connection of the two epithelial layers consisted of a close succession of tight and adherent junctions (Fig. 5). Normal intercellular space—the membranes being 200 Å apart—was rarely seen. Desmosomes were absent. Between the lateral aspects of the epithelial cells of each layer, junctional structures were found only apically. This structure was nearly always a complex of one adherent and one tight junction, the tight junction lying more apically; very infrequently the structure consisted of only a tight or only an adherent junction. (This may be compared with the reported findings in the retinal region.) These complexes "sealed" the ciliary channels completely against the extracellular space between the pigmented cells (arrow, Fig. 14) as well as against the extracellular space between the nonpigmented cells.

In the posterior region there were all three types of intercellular junctions: desmosomes, and adherent and tight junctions (Fig. 6). They connected the pigmented with the nonpigmented cell layer.

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Fig. 5. Anterior region, rabbit killed by too rapid injection of an anesthetizing dose of Diabatal, fix. OsO₄-Tyrode. Three VBV without the coating vesicles. Many adherent and two tight junctions forming the junction between the pigmented and the nonpigmented cells. Apical junctional complex between the two nonpigmented cells. CC, Ciliary channel; CC1, ciliary channel protruding into the pigmented cell; NPE, nonpigmented epithelium; PE, pigmented epithelium. (×19,300.)
and also cells within each layer; the latter, however, in contrast to the anterior region, were connected not only apically, but anywhere between apex and base. These junctions were more frequent between pigmented than between nonpigmented cells.

Exclusively in the posterior region of the ciliary body, fingerlike projections of the pigmented cells protruded into the nonpigmented epithelium; they were often seen in sections as double-membrane-bounded "vacuoles" (DMV, Fig. 6) whose continuity was established by serial sections. The examination of cross-sections of whole ciliary processes showed that these projections were characteristic of cells located on the ridges of the processes and that their number was smallest in the valleys between them. They were practically absent in the cells of the ora serrata.

Vesicle-bounded vacuoles. Only in the nonpigmented epithelium of the anterior region was there a distinctive type of large (0.4 to 1.5 μ) vacuole within the cytoplasm of the cells. These have not been previously reported. They were particularly numerous in the ridges of the membranous extensions. The number of these organelles in a cell was estimated to range from 0 to 15. Cells which displayed numerous vacuoles and cells which displayed none could be found adjacent to each other. A remarkable feature of these vacuoles was that most were surrounded by a single layer of 300 to 500 Å vesicles, spaced 300 to 500 Å apart all around the vacuole (Fig. 7), as verified by means of serial sections. Therefore, the organelle is referred to as a vesicle-bounded vacuole (VBV). Characteristically the central vacuole was almost perfectly spherical regardless of fixative or anesthetic, if the coating by the small vesicles was complete. If the vacuole had been sectioned tangentially, it sometimes appeared to have more than one layer of coating vesicles (Fig. 14). Occasionally the central vacuole exhibited in its lumen a small group of vesicles; sometimes a fluffy granular material, particles of which were strongly electron-dense. Variations of this organelle of possible functional significance were vacuoles with and without the described inclusions which were totally or partially bare of vesicles (Figs. 5 and 7). Often the VBV had a close association with the ciliary channels (Fig. 7); in other instances an association with Golgi regions or endo-

Fig. 6. Posterior region, 3 hours of urethan, fix. OsO₂-Tyrode. Vesicles budding off the finger-like projections (FP) which, when sectioned obliquely, appear as double-membrane-bounded vacuoles (DMV). CR, Ciliary rootlet; NPE, nonpigmented epithelium; PE, pigmented epithelium. (×20,000.)
Fig. 7. Anterior region, air embolism, fix. Tyrode-OsO₄. Three different stages of vesicle-bounded vacuole (VBV). CC, Ciliary channel; NPE, nonpigmented epithelium; PE, pigmented epithelium. ($\times$47,500.)
plasmic reticulum was suggested. For an unknown reason in one specimen these vacuoles had a gigantic size (up to 6 μ) and were easily observed with the light microscope. They were almost bare of vesicles but the remaining few served to identify the vacuoles.

Again exclusively in the cytoplasm of the nonpigmented cells in the anterior region a special type of small "vesicle" was found regularly. They varied in size, shape and number per cell (size approximately 50 to 1,500 Å). They were sometimes so numerous that only three components were apparent within the cytoplasm: the vesicles, the basal interdigitations, and the mitochondria (Fig. 8). Small granules of ribosomal size were dispersed among them, suggesting that these "vesicles" could in fact have been sections of a complex and highly tortuous endoplasmic reticulum. It is emphasized that these "vesicles" did not represent broken down interdigitational sheets, since the latter kind of vesicles

Fig. 8. Anterior region, same rabbit as Fig. 5. Abundance of small vesicles of irregular size and shape. No breakdown of the interdigitational membrane systems. NPE, Nonpigmented epithelium; PC, posterior chamber; PE, pigmented epithelium. (x11,400.)
are regularly ovoid and maintain the spatial arrangement of the membranes from which they are derived. The small "vesicles" had no obvious relationship with the posterior chamber.

In the anterior region of the ciliary body of pigmented rabbits the "nonpigmented" cells contained some pigment deposits (Fig. 8), whereas the albinos did not have analogous pigment-free granules. In the posterior region, pigment was not observed in this layer.

Intracellular fibrils were found only in the posterior region both in pigmented and in nonpigmented cells; they were completely absent in the anterior region.

Ciliary rootlets, centrioles, and rudimentary cilia were frequently encountered in both epithelial layers in the posterior region; a single cilium in which the 9 + 2 pattern was not maintained was found in a canalculus of the anterior region.

Naked and coated vesicles. These two types of vesicles, already well known in other cells, were also found in the ciliary epithelium, both in the anterior and in the posterior region; the small "naked" vesicles (400 to 1,000 Å in diameter), similar to the ones in the endothelial cells of muscle and lung, and the "coated" vesicles (with a similar inner diameter). The two types of vesicles showed a different pattern of distribution.

The coated vesicles were seen throughout the cytoplasm of all cells; "coated pits," presumably coated vesicles opening into the extracellular space (including the ciliary channels) could be found at all
locations of cell membranes (Figs. 9 and 10). In addition, coated vesicles were observed associated with Golgi complexes and were especially conspicuous after double staining (Fig. 11).

The naked vesicles were similarly seen throughout the cytoplasm. However, they fused only with the apical membrane of pigmented as well as nonpigmented epithelial cells.

Both types of vesicles sometimes budded off the apical membrane of the nonpigmented cells in groups or rows, often side by side.

The mitochondria in the nonpigmented cells of the anterior region are mostly round and often clearly lined up along the basal interdigitations, whereas in the posterior region they are elongated, sometimes branched and more uniformly dispersed all over the cell. In a number of cases mitochondria of opposite nonpigmented cells in any region of the ciliary body were observed in close juxtaposition. These corresponding mitochondria were seen associated with a certain number of structures resembling adherent junctions that seemed to link the mitochondria. One series of sections revealed that such a site of close apposition may be as large as the whole width of the mitochondrion (Fig. 12).

Membrane-surrounded electron-dense granules (Fig. 11) were common throughout the nonpigmented epithelium. They often featured cytoplasm-like inclusions which were or were not continuous with the surrounding cytoplasm (both proved by serial sections).
The effects of urethan and Diabutal anesthesia on the morphology of the rabbit ciliary body. The main changes by anesthesia concerned the stroma, the spaces between pigmented epithelial cells in the anterior region, and the ciliary channels.

The effects of urethan and Diabutal on the stroma of the ciliary body followed a well-defined topographical pattern. The changes were marked in the anterior region and almost absent in the posterior region. In addition, the bases of the ciliary processes in the anterior region were affected earlier and more markedly than the ridges.

Administration of urethan resulted in dilatation of the capillaries to many times their original diameter (up to 50 μ), and in considerable edema (Figs. 3 and 13). Capillary dilatation went along with an increase of the number of the capillary pores and occasionally with complete disruptions up to 0.05 μ of the capillary wall.

Compared with the findings immediately after the application of the anesthetic there was less dilatation of arterioles and capillaries but considerably more edema after three hours of anesthesia.

Diabutal caused less dilatation of the capillaries, although the edema was similar.

To a certain extent the basal portions of the ciliary processes in the anterior region displayed edema even in rabbits killed by air embolism, if they had shown marked excitation.

Administration of anesthetics produced—in analogy to paracentesis15, 25, 34, 35—saccular dilatations filled with finely granular material between pigmented epithelial cells of the anterior region. In early stages only small dilatations appeared close to Bruch’s membrane; under prolonged anesthesia their size increased up to a point where they could be seen easily by light microscopy (Fig. 13). Between the apices of the cells the dilatations were limited by the junctional complexes which never disrupted and this way sealed the dilatations from the ciliary channels (Fig. 14); toward the stroma they were limited by the thinned-out basal processes of the pigmented cells on Bruch’s membrane, with a mutual distance of no more than 200 A (Fig. 14).

In the posterior region—in contrast to the anterior region—very small dilatations could occasionally be seen anywhere between the different types of junction both between the pigmented and between the nonpigmented epithelial cells.

Concomitantly with the appearance of the saccular dilatations, the ciliary channels were enlarged (Fig. 14).

The effects of different fixatives on the structure of the ciliary body. In analogy to the effect of anesthetics the structural changes by the use of different fixatives followed again a well-defined topographical pattern. The posterior region—both epithelium and stroma—was well preserved by all of the fixatives used, regardless of osmolarity, except by the glutaraldehyde fixative given above. In the anterior region, however, the fixatives hyper-osmolar to blood plasma (e.g., 3 per cent glutaraldehyde + 50 mmoles of buffer, OsO₄-Tyrode-sucrose, and Dalton’s chrome-
osmium) caused a shrinkage, fixatives hypo-osmolar to plasma a swelling mainly of the nonpigmented epithelial cells. Shrinkage and swelling of the stroma were particularly conspicuous in edematous tissue.

Regardless of the osmolarity of the fixative, OsO₄ generally yielded excellent preservation of the cellular organelles. For the preservation of the anterior region, the choice of the vehicle seemed less important than the final osmolarity of the fixative. The well-known artifactual breakdown of the interdigitational membrane systems in rabbits after the use of OsO₄-fixative of any osmolarity⁴⁵,⁴⁹ (not in man⁴⁶,¹⁹) occurred in the nonpigmented epithelium both anteriorly and posteriorly. Many cells showed this phenomenon while other adjacent ones were perfectly preserved.

Glutaraldehyde did not preserve the anterior part sufficiently well; the tissue tore, and clefts and artifactual vacuoles appeared. The vesicle-bounded vacuoles persisted. In the posterior region glutaraldehyde caused selective shrinking of some nonpigmented cells, affecting the nucleus to the same extent as the cytoplasm (Fig. 15) (after straight fixation with OsO₄ all cells had equal size). When the material was not post-fixed in OsO₄ membranes did not stain; when it was post-fixed, the presence of shrunken cells was easily overlooked because the staining characteristics of the cytoplasm were the same in both kinds of cells.
As in early experiments, KMnO₄ did preserve the interdigitational membranes without breakdown. The cytoplasmic organelles were clustered around the nucleus, leaving cellular apices and bases looking empty. It was not feasible to cut a smooth section of this material with glass knives.

Discussion

Edema. It seems attractive to ascribe the vasodilatation and edema seen after urethan and Diabutal anesthesia to the direct action of these drugs on the smooth-muscle cells of the blood vessels. However, one should consider the possibility that, during anesthesia, blockage of the trachea may have caused an anoxia producing the vasodilatation. Control experiments with intubation during anesthesia are required to exclude this possibility.

In the anterior region the ciliary processes of some nonanesthetized rabbits killed by air embolism also exhibited edema in the basal portion. The variable status of excitement the animal has reached at the moment of the air embolism may influence the tonus of the blood vessels. The excitability and vascular lability of rabbits and especially of albino rabbits has been noted.

Fig. 15. Posterior region, 3 hours of Diabutal, fix. glutaraldehyde without OsO₄ postfixation. Some nonpigmented cells are shrunken (with their nucleus), while others are not. NPE, Nonpigmented epithelium; PC, posterior chamber; PE, pigmented epithelium. (x3,600.)

As in early experiments, KMnO₄ did preserve the interdigitational membranes without breakdown. The cytoplasmic organelles were clustered around the nucleus, leaving cellular apices and bases looking empty. It was not feasible to cut a smooth section of this material with glass knives.

Fine structure. Desmosomes, adherent junctions, and tight junctions are known to form focal patches (maculae) in many tissues; in addition, the tight and the adherent junction are also known to form continuous girdles (zonulae) around cells in certain tissues (the terminal bars of light microscopy). The extensiveness of the junctional complexes seen between the apices of both the pigmented and the nonpigmented cells of the anterior region suggests that they be interpreted as part of terminal bar systems. These systems would thus isolate the ciliary channels from the intercellular spaces between the pigmented as well as between the nonpigmented cells.

In the posterior region there is less regularity in the apical placement of the junctions, a fact that complicates interpretation. It is possible but not certain that we are here dealing with a variant of a terminal bar system where the "terminal" placement of the complexes is irregular.

The presence of terminal bar systems would have some physiologic implications for the transport of fluid. Since in the anterior region between the two cell layers there is only very little intercellular space of 200 Å besides the junctional structures, the ciliary channels may be of importance for the transport of fluid. The microvilli represent a considerable enlargement of the functional area within the canaliculi, just as do the interdigitations on the basal side of all epithelial cells in the ciliary body.

Tight junctions are assumed to be sites...
of low electrical resistance between cells. The great number of tight junctions between the pigmented and the nonpigmented cell layer would suggest that these layers should have similar resting potentials. This is in conflict with reports where considerably different resting potentials have been measured.6,22

Pinocytotic vesicles in mesothelium may aggregate to large membrane-surrounded entities.37 The VBV do not seem to be identical with these, for isolated small "naked" pinocytic vesicles are scarce in the cytoplasm of nonpigmented epithelial cells in the anterior region, whereas they coat the central vacuole of the VBV in a regular manner. So far nothing is suggested as to the function of the VBV. Preliminary experiments on incubation of the ciliary body for one hour in Tyrode's solution at 37° C. proved that the VBV may maintain their intact structure much longer than mitochondria. The VBV bear a superficial resemblance to the contractile vacuole of some protozoa.25

The function of the "naked" and "coated" small vesicles in the ciliary epithelium is at the present time unknown. In other cells "naked" vesicles are known to be related to pinocytosis. "Cell drinking" (pinocytosis) and conceivably "transmission by cell" of substances in vacuoles (cytoplasm)24 as a mechanism of formation of aqueous humor would be challenging concepts.

To recapitulate, the new definition of the anterior and the posterior region as presented here seems to be justified by the fact that on this basis clear-cut patterns of distribution have been found for the ciliary channels, the arrangement of junctional structures and organelles such as VBV, intracellular fibrils, and vesicular profiles. Furthermore, the following observations suggest that the anterior region is the more active region in the secretion of aqueous humor: viz. the more extensive capillaries (as shown in the experiments with anesthetics), the special sensitivity to changes in osmolarity (as revealed by studies with different fixatives), the abundance of ciliary channels in the anterior region, and the possible existence of terminal bar systems.

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