FINE STRUCTURE OF THE PINEAL ORGANS OF THE ADULT FROG, *RANA PIPIENS*

DOUGLAS E. KELLY, Ph.D., and STUART W. SMITH, M.D.

From the Department of Biological Structure, University of Washington School of Medicine, Seattle; the Department of Biology, University of Colorado, Boulder, Colorado; and the Department of Anatomy, University of Colorado School of Medicine, Denver, Colorado. Dr. Kelly's permanent address is the Department of Biological Structure, University of Washington School of Medicine, Seattle

ABSTRACT

Frontal organs and epiphyses of the pineal system from the adult frog, *Rana pipiens*, were fixed in *s*-collidine–buffered osmium tetroxide, embedded in Epon 812, and examined by electron microscopy. Epiphyseal material was also fixed in a variety of ways and subjected to a series of cytochemical tests for light microscopy. An ultrastructure resembling that of lateral eye retina is confirmed in this species. Photoreceptor cells of the epiphysis and frontal organ display many cytological features similar to those of retinal rods and cones in the arrangement of their outer and inner segments and synaptic components. However, in these pineal organs the outer segments are disoriented relative to each other and may display a disarranged internal organization unlike normal retinal photoreceptors. Furthermore, other pineal outer segments often appear degenerate. Since immature stages in the development of new outer segments also appear to be present, adult pineal photoreceptors are probably engaged in a constant renewal of outer segment membranes. The evidence further suggests that macrophages are involved in phagocytosis of degenerated outer segments. Postulated photoreceptor activities and the possibility of secondary pineal functions, such as secretion, are discussed in view of current morphological and cytochemical findings.

The presence of structures suggesting photoreceptive activity in pineal cells of cold-blooded vertebrates, earlier suspected on the basis of light microscopical studies, has now been confirmed for many organisms at the ultrastructural level.

Eakin and Westfall (20, 21) and Steyn (51), working independently, were first to demonstrate stacks of lamellae, comparable to those of retinal rods and cones, in the outer segments of pineal sensory cells of the lizard parietal (parapineal) "eye." Later, the same investigators extended this finding to the sensory cells of the more deeply lying epiphyseal (pineal) component of the pineal system (17, 52). Both the frontal (parapineal) organ and the epiphysis have now been found to possess similar ultrastructure in tadpoles of the frog, *Hyla regilla*, (16, 17, 19, 22) and in the adult frogs, *Rana pipiens* (29), *Rana temporaria* and *Rana esculenta* (41, 42). We (31) have observed that presumed photoreceptor cells, well developed in the epiphyses of larval newts, *Taricha torosa*, are apparent to a lesser degree in adults of the same species. Moreover, since Eakin (18) has made a preliminary report of similar lamellated ciliary photoreceptors in the pineal apparatus of Ammocoetes larvae of lampreys, a concept of pineal morphology involving photoreceptive capability may well be valid for most lower vertebrates.

Fig. 1, an electron micrograph taken from the epiphysis of the adult frog, *R. pipiens*, depicts the distal portions of a cell designated as a pineal photoreceptor protruding into the lumen of the

organ. It is this type of image that has been used as the basis for interpretations of photoreceptive function for pineal organs in all the studies mentioned above. Obvious organizational similarities between these photoreceptors and known photoreceptors of lateral eye retinas include ciliary components situated at the junction of inner and outer segments, and outer segment lamellae consisting of layer upon layer of invaginated plasma membrane.

Photoreceptive pigments are presumably contained within such a lamellar membrane system in retinal rods and cones (see reference 53), but there is no evidence that similar pigments exist in pineal photoreceptors. However, recent neurophysiological findings show that the frontal organ and epiphyseal region of frogs (5, 11, 12) and the parietal eye of the lizards (39) generate impulses upon exposure to light or darkness; some of the evidence is suggestive of a capacity for wave-length discrimination. Furthermore, cytologically and histochemically detectable differences in illuminated and dark-treated pineal cells (19, 34) have been demonstrated in support of the concept of pineal photoreception.

Relatively less attention has been given, in ultrastructural studies, to the remaining cellular components of pineal systems than to the photoreceptors themselves. The recent descriptions by Oksche and von Harnack (41, 42) and Eakin *et al.* (19) of several cell types in pineal organs of frogs may provide the basis upon which connections and interrelationships among pineal cellular components will ultimately be worked out.

It is the purpose of this report to present evidence which extends the current information (19, 41, 42) on photoreceptor morphology of the pineal organs (epiphysis and frontal organ) in the frog, *R. pipiens*. Several features of the pineal system of this species that have not been reported in lateral eye photoreceptors will be outlined, and evidence will be presented which supports the hypothesis (26) of renewal and casting-off of pineal photoreceptor outer segments in adult lower vertebrates. A preliminary abstract of some of these findings (32) has already appeared.

MATERIALS AND METHODS

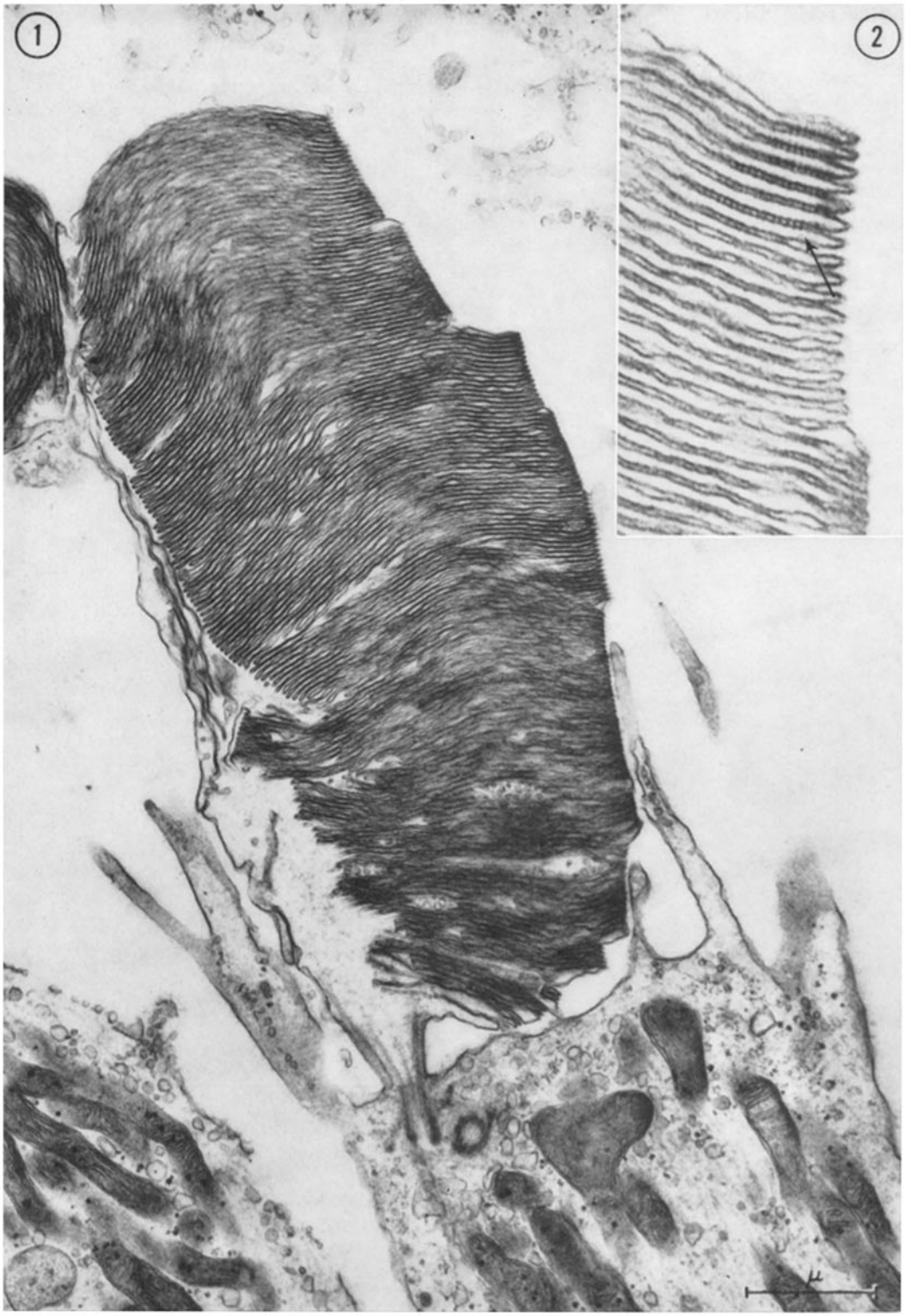
For Fine Structure

Frontal organs and epiphyses were obtained from adult *R. pipiens* and fixed in a solution of 3.0 per cent OsO_4 in 0.05 M purified *s*-collidine buffer (final pH of 7.4) (6). Frontal organs were quickly excised from the dorsal head skin of Nembutal-anesthetized frogs and immediately immersed for 1 hour in fixative chilled to 2°C. A variety of methods was used for fixation of epiphyses since this organ is located within the cranial case. Usually chilled fixative was introduced to the tissue with a hypodermic syringe inserted intrameningeally or into the third ventricle before opening the brain case, or else the fixative was dripped over the organ immediately after its exposure. In a few cases, vascular perfusion of the fixative was utilized (in this case buffered with Veronal-acetate) following a rapid rinse with Earle's balanced salt solution. In all cases the epiphysis was fixed *in situ* for 10 to 20 minutes before the brain itself was subjected to any manipulation. Further fixation followed excision of the diencephalic roof region by immersion for 45 to 60 minutes in chilled fixative. After fixation, the tissues were trimmed, dehydrated in an ethanol series, and embedded in Epon 812 according to Luft (38). Retinal tissue from the same species was similarly prepared for purposes of comparison. Thin sections were cut on a Porter-Blum or LKB microtome with glass or DuPont diamond knives. Millonig's alkaline lead staining procedure (40) was used routinely. The micrographs were taken with RCA-2A or 2C instruments or with a Siemens Elmiskop I operating at 80 kv.

FIGURE 1 Outer segment of photoreceptor cell protruding into the lumen of the epiphysis in a frog. A well formed stack of lamellae derived by infolding of the plasma membrane, and a centriolar apparatus are visible components of the outer segment extending from its narrow junction with the mitochondrion-rich inner segment. Magnification, 20,500.

FIGURE 2 High magnification electron micrograph of a portion of the outer segment lamellae seen in Fig. 1. Note regular beading pattern among some of the layered membranes (arrow). Magnification, 76,000.

Fig. 1 was originally published in *American Scientist*, 1962, **50**, 597–625.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For Cytochemistry

Animals anesthetized by intraperitoneal Nembutal were perfused with 2 per cent OsO_4 in Veronal-acetate buffer containing added $CaCl_2$ or with 2 per cent glutaraldehyde in 0.1 M phosphate buffer, both at pH 7.5 to 7.6 and preceded by 5 to 10 ml of Earle's balanced salt solution. The epiphyses were excised and immersed at 2°C for 2 hours (OsO_4) or 24 hours (glutaraldehyde), washed, and dehydrated in a methanol series. Some OsO_4-fixed tissues were embedded in *n*-butyl methacrylate, sectioned with glass knives at 2.5 μ, mounted on slides, and freed of plastic in 1:1 di- and tetrachlorethanes. Other OsO_4- and the glutaraldehyde-fixed tissues were embedded in ester wax (46), sectioned at 3 or 4 μ, mounted, and dewaxed in methanol.

Other animals were similarly anesthetized and their epiphyses were fixed by initial intrameningeal injection of 1:3 acetic acid-ethanol, 4 per cent aqueous formaldehyde, or Orth's fluid. The epiphyses were excised and immersed in fixative (4 hours at 2°C and 20 and 72 hours at room temperature, respectively), washed, dehydrated, embedded in ester wax, sectioned, mounted, and dewaxed as above.

OsO_4-fixed tissues and some tissues fixed by the other procedures were stained in 0.05 per cent thionine in 1 per cent acetic acid or were subjected to periodic acid oxidation both with and without prior digestion by malt diastase according to the method of Hotchkiss as modified by Lillie (36). Glutaraldehyde- and acetic-ethanol–fixed tissues were subjected to the following reactions for protein-bound amino acid side chain groups: Deitch's (10) method for arginine; Lillie's (37) method for tyrosine, Yasuma and Itchikawa's (54; also Burstone, 7) method that demonstrates primary amino groups, mainly of lysine; Barrnett and Seligman's methods for carboxyl groups (4; also Karnovsky and Fasman, 28) and for cysteine plus cystine sulfur (3); and Glenner and Lillie's (25) method for tryptophan. In those procedures that depend on generation of aldehyde groups the aldehydes were demonstrated by the tetrazopentamethinecyanine reaction (50). Formaldehyde-fixed tissues were subjected to the diazo (35) and ninhydrin (27) methods for 5-hydroxytryptamine (both as modified by Barka and Anderson, 2).

Appropriate controls were performed for all cytochemical procedures. Since the frogs were obtained from commercial sources, their history and nutritional state were largely unknown. Care was exercised, however, to utilize only active animals showing no signs of extended starvation or infection.

OBSERVATIONS

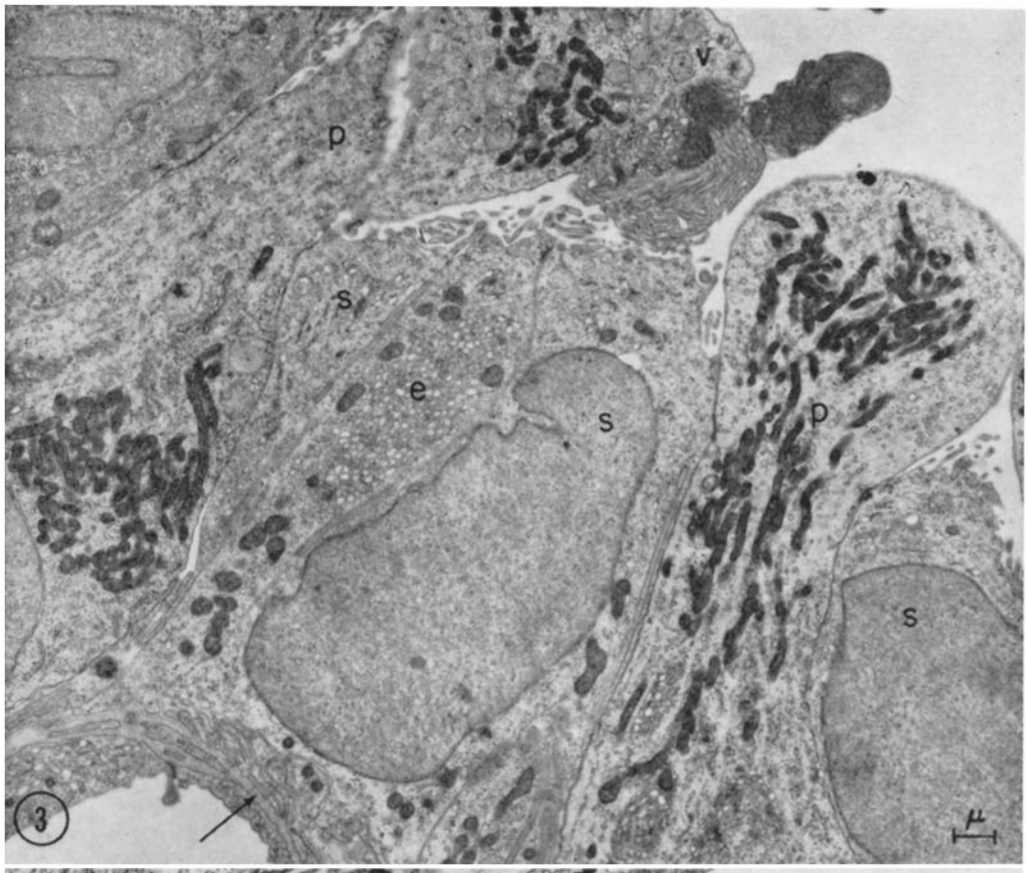
General Cytological Features

A previous study (33) has outlined the cytological features discernible at the light microscopical level of resolution in the epiphysis of the frog, *R. esculenta*. The findings presented in that report serve as a guide to the general cellular topography seen here in *R. pipiens* at the electron microscopical level. The main difference between the two species is that the epiphysis of *R. esculenta* is an elongated sac with relatively few lateral outpouchings, whereas that of *R. pipiens* is more convoluted with numerous foldings and diverticula. Otherwise, the two systems are quite similar.

THE EPIPHYSIS (PINEAL ORGAN PROPER): *A. Fine Structure:* The most prominent constituents of the epiphyseal wall are the photoreceptor cells which protrude into the wide, somewhat tortuous lumen (Fig. 1). These cells possess four basic regions which, as will be seen, vary considerably in form and extent. The distal portion of the protrusion, the outer segment, is a ciliary derivative containing a 9-0 fibrillar pattern and possessing a stack of lamellae similar to those of rod and cone outer segments (see *e.g.*, references 15, 29, and 47). Irregularity of outer segment morphology, a point to be discussed later, prevents direct analogy between pineal photoreceptors and retinal rods or cones. Most pineal outer segments are somewhat larger than typical retinal cones of the same animal, but, as

FIGURE 3 Section of the lumenward portion of the epiphyseal wall showing photoreceptor (*p*) and supportive (*s*) cells and their relation to a capillary (lower left) within the wall. Note vesiculated endoplasmic reticulum (*e*) and the incised basal portion (arrow) of supportive cells bordering the capillary. Numerous moderately dense, subspherical, membrane-bounded vesicles (*v*) are seen in one protruding inner segment. Magnification, 5,500.

FIGURE 4 Capillary coursing within the epiphyseal wall. Note basal feet (arrows) of supportive cell. These are intermingled within the perivascular connective tissue and possess fine densely stained particles. Magnification, 9,000.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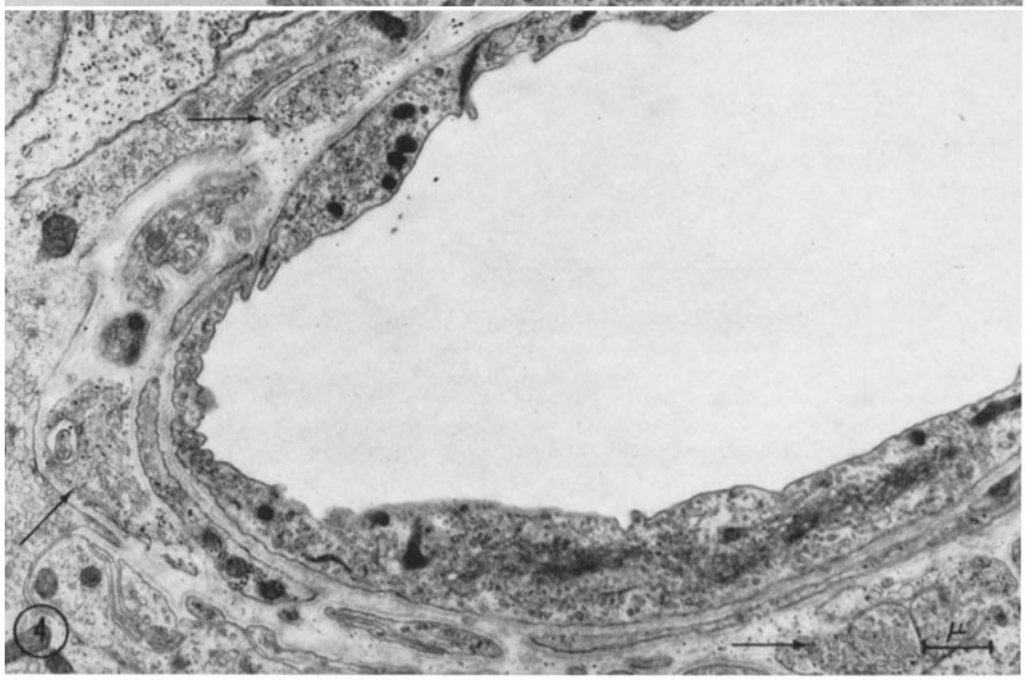
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with cones, the membranes of each lamella are traceable as infolded continuations of the plasma membrane over a wide part of the outer segment on the side opposite the ciliary fibrils. Therefore, an unfolded plasma membrane surrounding the whole stack of lamellae is seldom observed in random sections. In our osmium tetroxide-fixed material, the membrane stacks are frequently interspersed with small vesicular and tubular appendages of the sacs.

A periodic beading (*ca.* 140 to 150 A) has often been observed along adjacent lamellar infoldings of outer segments (Fig. 2). Since the beading is present in most outer segments studied, but seldom so precisely regular or distinct as the example depicted, it appears to represent properties in or on the membranes occasionally accentuated by lying in register.

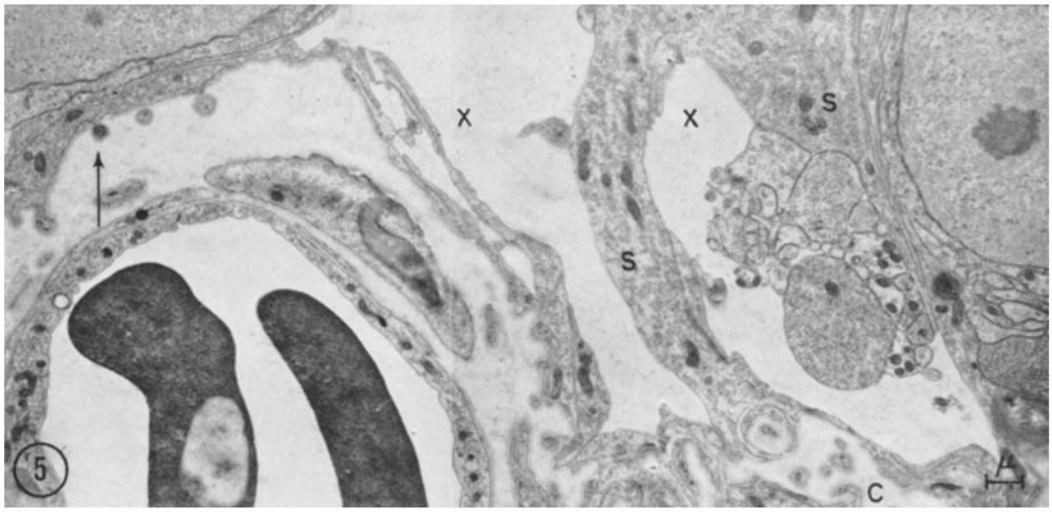
The region of the cell basal to the outer segment in pineal photoreceptors is rich in clumped mitochondria (termed the ellipsoid) and often partially separated into two parts by a constriction in the cell at the level of its protrusion into the lumen. At this point the cell membrane attaches to surrounding supportive cells by a tight junction (Fig. 3). The protruding part of this region, therefore, corresponds to the inner segment of rods and cones, although clumps of mitochondria extend far basal to the constriction. The mitochondria are more dense and elongate than those of rods or cones in the same animal. Among the mitochondria are varying amounts of predominantly smooth, vesiculate endoplasmic reticulum, Golgi complexes (usually near the nucleus), probable glycogen granules (described in greater detail by Eakin *et al.*, reference 19), and a network of fine filaments (see also Fig. 17) that appear to collect into a bundle or bundles and course around the nucleus into the basal synaptic region. Ciliary fibrils from the outer segment terminate in a basal apparatus consisting of two centrioles oriented at right angles to each other. Very infrequently, striated rootlets have been observed extending basally from the centriolar apparatus into the inner segment. Subspherical membrane-bounded vesicles of variable size containing low to moderately dense finely granular material are often numerous within the inner segment. Their abundance is apparently peculiar to pineal photoreceptors since they are not so numerous in rods and cones. Occasionally, these vesicles contain an extremely dense amorphous or granular material. Some are larger and irregular in profile, especially when located in regions near the nucleus.

Epiphyseal photoreceptors contain a large indented nucleus, basal to which the fourth and final region of the cell is a process of varying length, terminating in an intricate synaptic zone. A detailed description of the basal synaptic complex will be presented in a separate section below.

At the luminal surface, the photoreceptors are surrounded by supportive cells that are relatively undifferentiated from ependymal cells. Such supportive cells, attached to the photoreceptors by tight junctions, are characterized by their free borders with microvilli and occasional cilia, and by an abundance of smooth, vesiculate endoplasmic reticulum. The latter component is often observed virtually filling the cytoplasm with dilated vesicles (Figs. 3 and 4). Neighboring supportive cells adjoin each other by means of tortuous interdigitations of their respective plasma mem-

FIGURE 5 Low magnification montage showing peripheral pineal wall bordering the connective tissue capsule (*c*) and a blood vessel (lower left). Note intermingling of supportive cell processes (*s*) with connective tissue and the large extracellular spaces (*x*) in this area of the organ. The perikaryon of a photoreceptor cell (right) is surrounded by groups of synaptic components. One supportive cell extremity possesses a small protrusion containing a dense multivesicular body (arrow). Magnification, 4,500.

FIGURE 6 Low magnification electron micrograph showing a portion of the irregular lumen in the frontal organ and various cells and protrusions bordering it. Outer segments are seen in various planes of section in the lumen, along with other irregular membrane accumulations. Supportive cells display cilia and microvilli along their free borders and numerous "myeloid" membrane accumulations within their cytoplasm (arrows). The smaller of these accumulations characteristically are associated with the nuclear envelope. Magnification, 7,500.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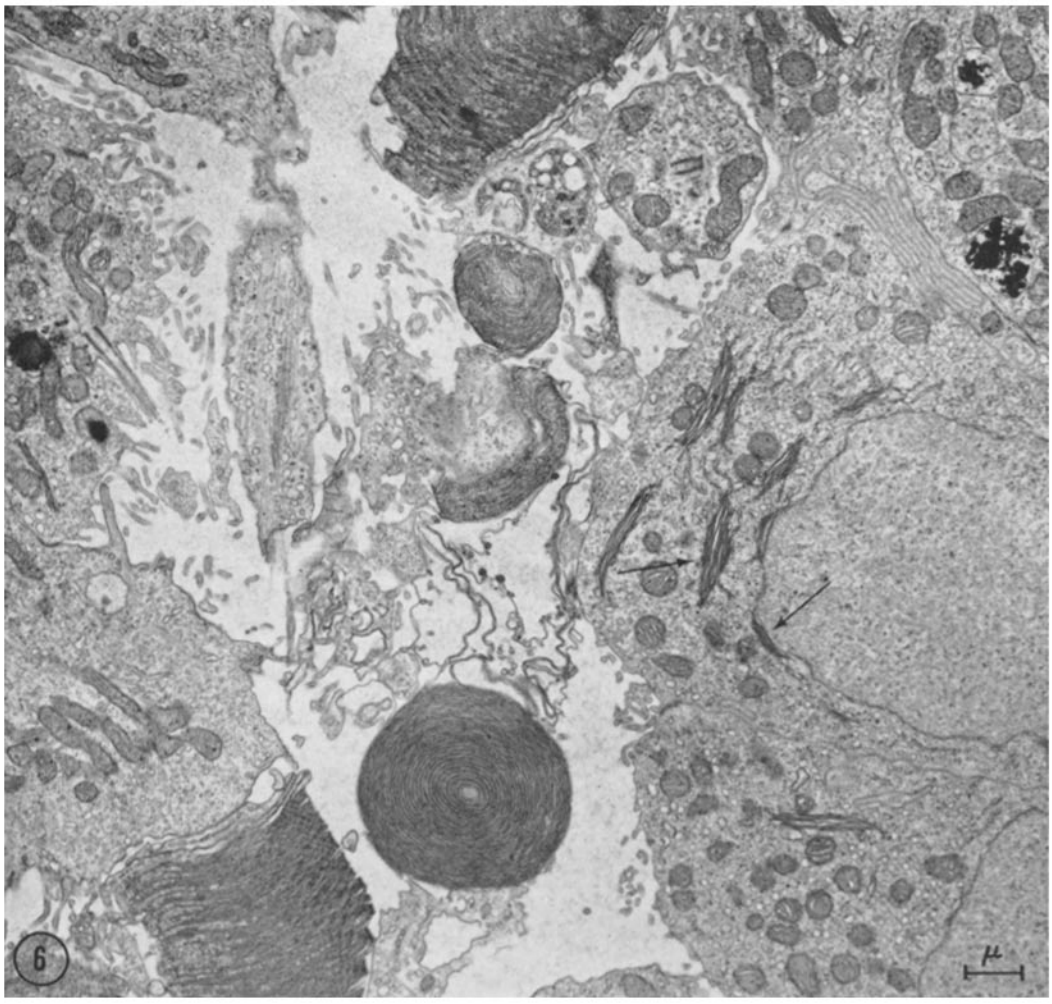
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branes, and thereby tend to enclose the perikarya and basal processes of the photoreceptors. Occasionally, packed arrays of flattened cisternae of the myeloid body type are found within these cells, but not so frequently as in the frontal organ (below). Taken together, the above features have served to identify supportive cells in nearly any pineal region or plane of section. They correspond closely to those in descriptions provided by previous authors (*e.g.*, references 19, 41, 42).

Supportive cells terminate basally in the vicinity of blood capillaries or on the connective tissue capsule of the epiphysis. Where a capillary courses within the wall of the organ (Figs. 3 and 4), the broad base of an adjacent supportive cell is divided by incisures into a number of foot processes which rest upon a relatively flat basement membrane. This, in turn, is underlaid by collagenous fibrils and the basement membrane of of the capillary endothelium. Many supportive cells are extended into longer basally directed processes coursing through much of the thickness of the epiphyseal wall. These are subsequently divided into numerous foot processes which rest upon an intricately folded basement membrane that surrounds pericapillary or capsular connective tissue enclosing the pineal wall. Some supportive cell foot processes also invade synaptic complexes (Fig. 5). Occasionally, we have observed small extensions of pericapillary supportive foot processes that contain dense multivesicular bodies. Lead-staining particles resembling glycogen are also common in cell processes and extracellular connective tissues of pericapillary regions (see Fig. 4).

Extracellular spaces (*x* in Fig. 5) are frequent in pineal tissue in the vicinity of the supportive foot processes adjoining the pericapillary or capsular connective tissue. Devoid of recognizable membranes and containing only a fine coagulum, these spaces do not appear to be shrinkage artifacts nor are they glial compartments. They are larger and more numerous in the postero-ventral walls of the organ.

Positive identification of ganglion cell bodies has thus far not been possible in our electron micrographs. Although a number of sections have contained portions of large cells which were suspected of being ganglion cells, other morphological features of these cells did not sufficiently differentiate them.

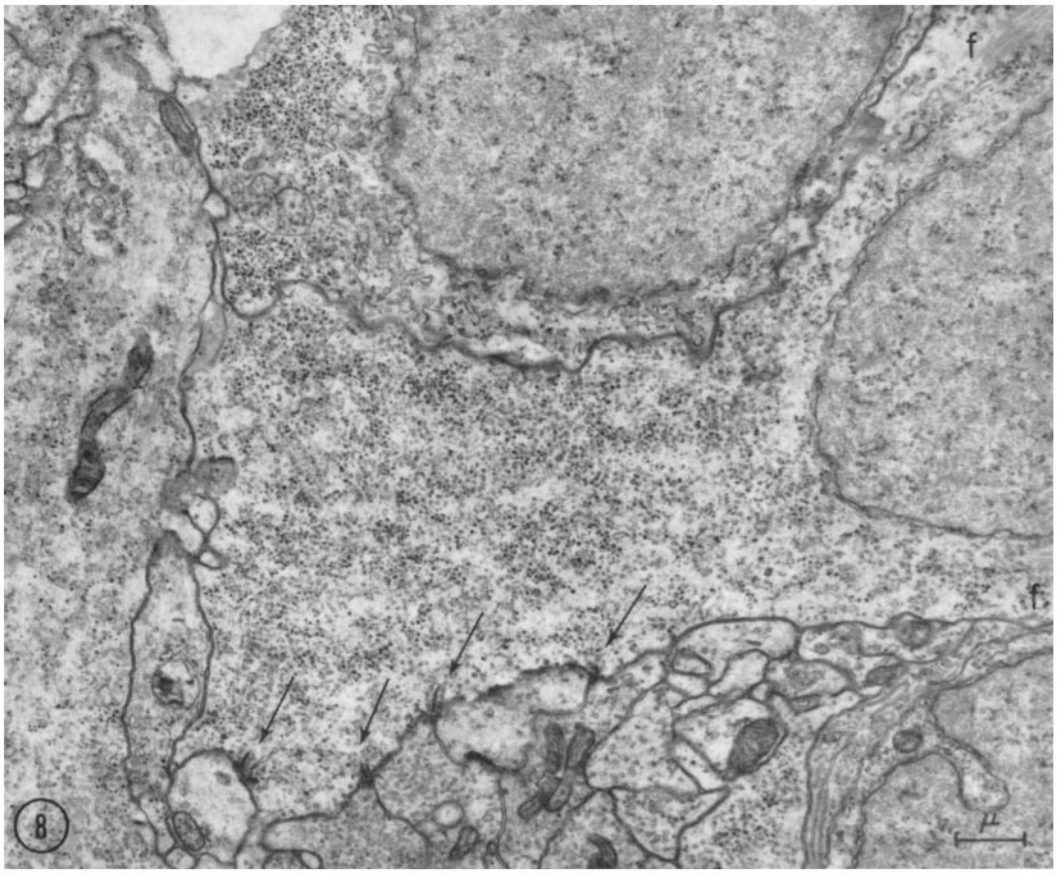
In the lateral walls of the epiphysis, and to a lesser extent in other areas bordering the connective tissue capsule, one often encounters tangentially arranged, long supportive cell processes that are less dense and contain more filaments than the supportive cell processes described above. These processes emanate from cells that probably correspond to those designated by Oksche and von Harnack (41, 42) as glial.

B. Cytochemistry. In unstained, glycerin-mounted sections of OsO_4-fixed epiphyses, structures larger than 0.2 μ seen in electron micrographs were readily discerned by phase contrast microscopy.

FIGURE 7 Higher magnification electron micrograph from the frontal organ showing a large myeloid body within the cytoplasm of a supportive cell. Smaller myeloid bodies are seen near the nuclear envelope of an adjacent cell (lower right). This figure also depicts the tortuous membrane infolding which occurs between adjacent supportive cells, and a mitochondrion displaying regular rows of cristae. Magnification, 20,000.

FIGURE 8 Basal process of a frontal organ photoreceptor cell seen as it extends from the perikaryon (right) into a bed of supportive and neural processes, and the extremities of other basal processes. Basal processes are crowded with synaptic vesicles and dense granules presumed to be glycogen. Several synaptic ribbons (arrows) are seen near the plasma membrane where it borders other processes. Two bundles of filaments (*f*) are visible as they course around the nucleus into the basal process. Magnification, 9,000.

FIGURE 9 Photographic enlargement of a portion of Fig. 8 to emphasize components in a presumed synaptic region of the basal process. A pair of synaptic ribbons on the left appear to be in proximity to a synaptic junction with a dendritic termination (*d*). It cannot be determined from the section whether the vesicle-filled processes opposite the other two synaptic ribbons are dendrites, axons, or basal extremities of other photoreceptor cells. Magnification, 18,000.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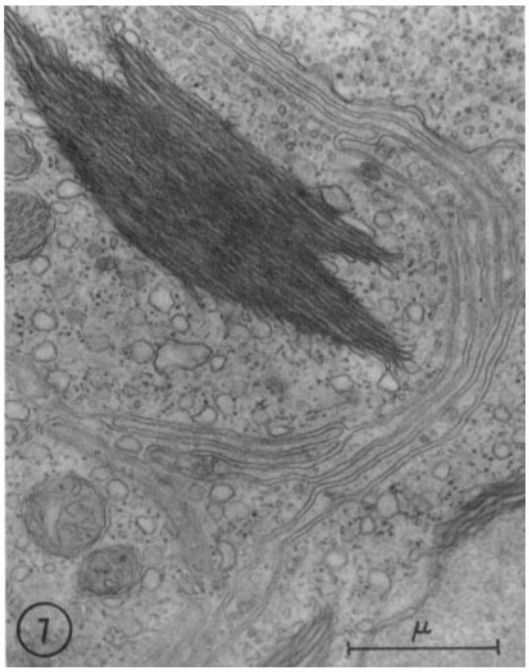
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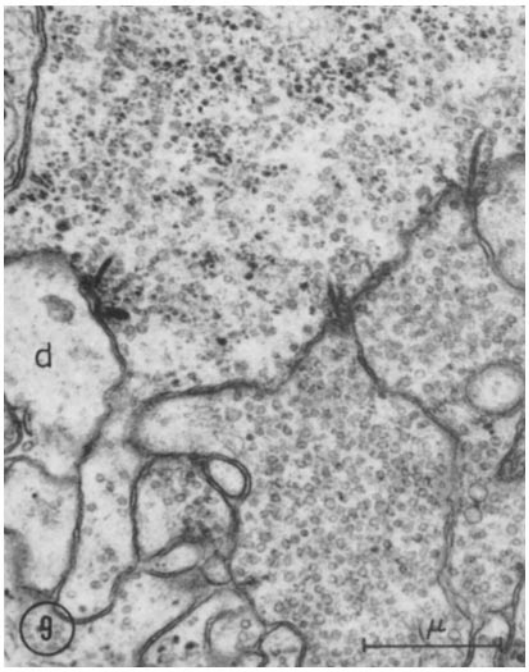
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In particular, the subspherical vesicles containing material of low density and located in the inner segments of the photoreceptors appeared as minute spherules of substantially lower contrast than the surrounding cytoplasmic matrix.

Outer segments of the photoreceptors were moderately basophilic to thionine, were weakly to moderately positive for side chain carboxyl, combined sulfhydryl and disulfide, tryptophan, and periodic acid sensitivity (malt diastase resistant), and were weakly positive for tyrosine and side chain amino groups. They were essentially unreactive in all other procedures.

Ellipsoidal regions of the photoreceptors paralleled the outer segments in all of their reactions except that their reactions for side chain carboxyls and combined sulfhydryl and disulfide were somewhat stronger than those of the outer segments.

The cytoplasm of the photoreceptors displayed scattered, diffuse areas of weak basophilia corresponding to the distribution of presumed ribosomes in electron micrographs. Periodic acid oxidation followed by chromogenic demonstration of the generated aldehydes revealed scattered patches of reactive material in inner segments and basal regions of the photoreceptors that paralleled distribution of presumed glycogen granules in electron micrographs. The latter reactions were abolished by prior digestion in malt diastase.

The subspherical vesicles of the inner segments which showed only occasional dense OsO_4-reactive material in electron micrographs were consistently devoid of any color whatever in all of the reactions for protein-bound amino acid side chains, for basophilic (acidic) materials, for periodic acid reactive materials, and most particularly for catecholamines (chromaffin reaction) and 5-hydroxytryptamine (serotonin).

It is noteworthy that no other epiphyseal area reacted for catecholamines or serotonin.

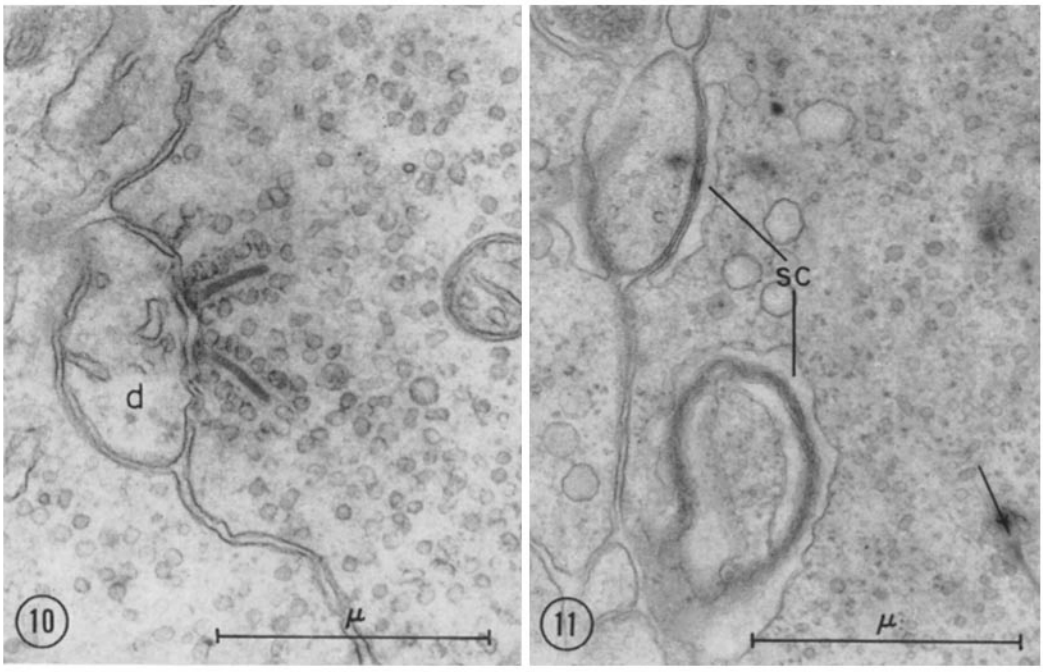
THE FRONTAL ORGAN (STIRNORGAN): With few exceptions the structure of the frontal organ is essentially similar to that of the epiphysis. The frontal organ is more compact; its cells appear more crowded and there are fewer intercellular spaces. The lumen is small and frequently irregular. Protruding outer segments tend to be impacted against or between other cells. Peripherally in the organ, the longer filamentous processes of supportive cells are numerous, forming a partial sheath within the connective tissue capsule. In general, the criteria described for the epiphysis can be used to distinguish photoreceptor and supportive types of cells in the frontal organ. Fig. 6 illustrates many of the general features of the frontal organ.

The most distinctive components of frontal organ supportive cells are numerous lamellar systems closely resembling the myeloid bodies of

FIGURE 10 One type of synaptic junction found in the basal processes and collateral spherules of pineal photoreceptor cells. This junction is characterized by synaptic ribbons and vesicles on the photoreceptor side of the junction, and a presumed dendrite termination (*d*) relatively devoid of synaptic vesicles. Magnification, 36,000.

FIGURE 11 Two synaptic junctions of a presumed second type shown on a single collateral spherule, but at two different planes of section. Opposite the adjoining neural termination, the collateral spherule (or basal process, as the case may be) characteristically displays a subsynaptic cistern (*sc*) rather than ribbons. Here this component is seen cut in cross-section (top) and tangentially (bottom). Fewer vesicles are present on the photoreceptor side of this synapse by comparison with Fig. 10, and the neural terminations may contain more vesicles than the dendrite in Fig. 10. A portion of a distant synaptic ribbon (arrow) is also seen in the spherule. Magnification, 36,000.

FIGURE 12 One section from a series showing the basal process of an epiphyseal photoreceptor cell and a nearby ribbon-containing collateral spherule (*b*). Within the basal process there is a central cavity (*cc*) containing a number of presumed dendritic processes and surrounded by synaptic ribbons and vesicles. A bundle of filaments (*f*) coursing around the nucleus (left) is directed toward the synaptic cavity. Numbers (*1*, *2*, *3*) on this micrograph correspond to points indicated on the reconstruction in Fig. 13. Magnification, 14,500.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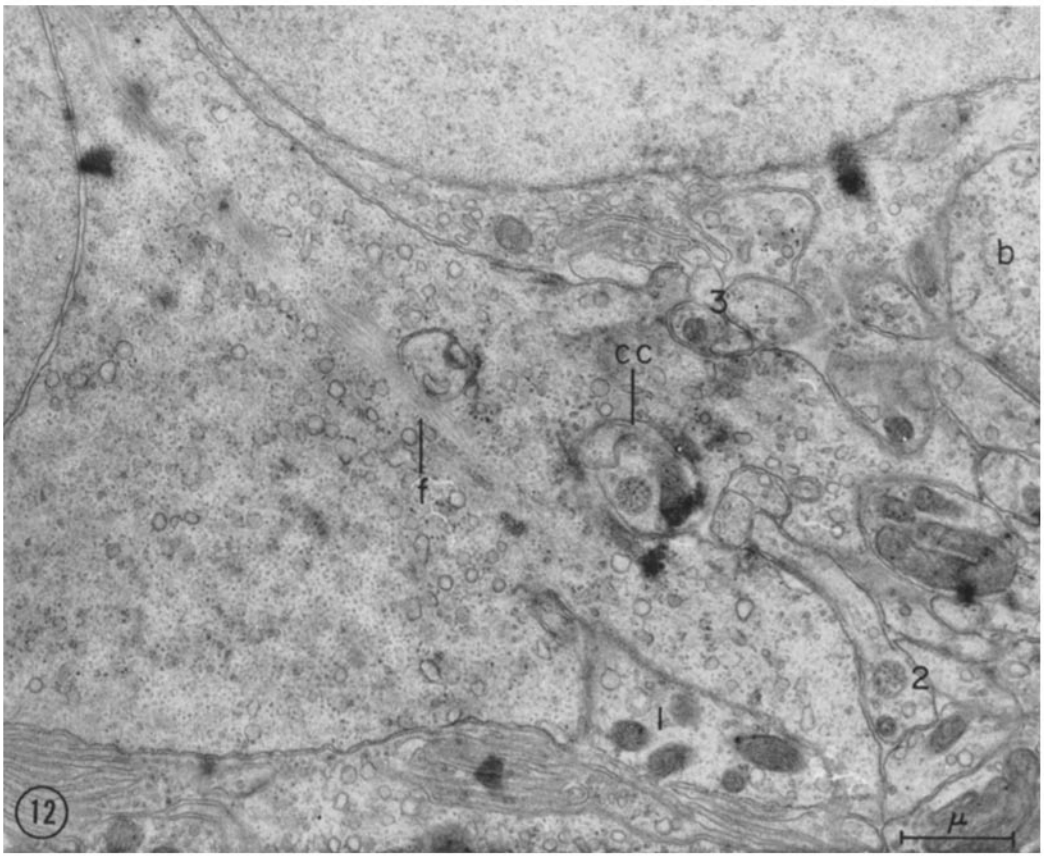
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pigment epithelial cells of the retina (Fig. 6). These myeloid bodies, also seen by previous authors (19, 41, 42), range in size from large, lenticular accumulations with 2 to 3 dozen doubled membranes, similar to those described in retinal pigment epithelial cells by Porter and Yamada (44), to very small bodies with only a few membrane systems (Fig. 7). The latter are often regularly disposed along the wavy nuclear envelope in such a way that a given nucleus appears to have stubby membranous "spokes" extending into the cytoplasm. Whether large or small, these lamellar systems have smooth vesicular enlargements of various sizes at the ends of the double, folded membrane systems. Fig. 7 also illustrates the interdigitation of plasma membranes linking supportive cells, and a mitochondrion with regularly arrayed longitudinal cristae which are occasionally seen in these cells.

Synaptic Ultrastructure

Basal processes of photoreceptors in both the epiphysis and the frontal organ extend into relatively deep-lying areas rich in small naked nerve fibers intermingled with foot processes from supportive cells (Figs. 5, 8, and 12). Some of the photoreceptor basal processes ramify into smaller branches, and it is possible that these in turn may be expanded into larger extremities which we provisionally term "collateral spherules." In the same regions bundles of unmyelinated nerve fibers are present. Presumably these bundles ultimately collect to form the heavily myelinated nervus and tractus pinealis coursing from the frontal organ through the epiphysis, and into the posterior commissure region of the brain roof (26) (see references 19, 41 and 42 for descriptions of ultrastructural details in pineal synaptic regions of closely related species).

Fig. 8 depicts the synaptic zone around a basal process in the frontal organ. Toward the basal extremity of the process there is an increasing number of presumed synaptic vesicles that average 500 A in diameter within a range of about 300 to 1000 A. In this figure the basal process is also crowded with granules which stain deeply with lead and presumably represent glycogen. Bundles of filaments (*f*, Figs. 8 and 12) within the processes are apparently continuations of the network that arises in the inner segment and sweeps around the nucleus.

Synaptic ribbons (also termed synaptic bars or lamellae) are frequently found in sections through basal processes, their extremities, or collateral spherules (see also references 19, 41, and 42). Often the ribbons are in obvious contact with or in proximity to a recognizable synaptic junction as is the case of one double ribbon in Figs. 8 and 9. Two other ribbons in the same process are seen opposite cell processes in which no obvious membrane specialization can be discerned but which contain a very high content of vesicles. Whether these latter processes are neural terminations or are cytoplasmic branches from a neighboring basal process has not been determined.

Within basal processes and collateral spherules at least two apparent types of synaptic junctions can be found. The first type, seen in cross-section in Fig. 10, shows one or more synaptic ribbons close to the plasma membrane of the basal process near its junction with a nerve termination. In this type there is usually a higher concentration of synaptic vesicles on the basal process (or spherule) side. By contrast, the other type of junction consists of a termination (about the same size as the type above) which abuts a basal process zone that contains a flattened subsynaptic cistern, but no ribbons. In this type, the termination itself commonly contains the higher concentration of synaptic vesicles. Fig. 11 shows two junctions of this second type on a single collateral spherule, one in cross-section and one cut tangentially. The spherule presumably makes both types of synaptic junctions since it also contains ribbons in other regions.

Fig. 12 illustrates one section in a series of 18, through an epiphyseal photoreceptor basal process and a spherule, both of which can be demonstrated to contain synaptic ribbons and subsynaptic cisterns. Within the basal process there is apparently a central cavity (*cc*) containing presumed nerve terminations. The outline of this cavity appears similar to several pictured by Oksche and von Harnack (reference 41, pp. 257 and 272) in micrographs from frog frontal organs. Several presumed neural processes in Fig. 12 contain multivesicular bodies. A bundle of filaments (*f*) coursing around the nucleus appears to be directed toward the central cavity. When reconstructed from the serial sections (Fig. 13), the central cavity is revealed to be similar to the synaptic cavities demonstrated by Sjöstrand and coworkers (see reference 47) in the basal processes of retinal rods of the guinea pig. In the frog's pineal photo-

receptor, however, the arrangement is more complex. Several blind pockets run out from the central synaptic cavity, each carrying a cell process presumed to be a nerve terminal. Moreover, the reconstruction discloses at least three separate passages by which nerve terminals gain entrance into the central synaptic cavity. The cytoplasm of the basal process surrounding the complex of passages is richly supplied with synaptic ribbons and vesicles and is interlaced by fibrils extending from the bundle which has coursed around the nucleus.

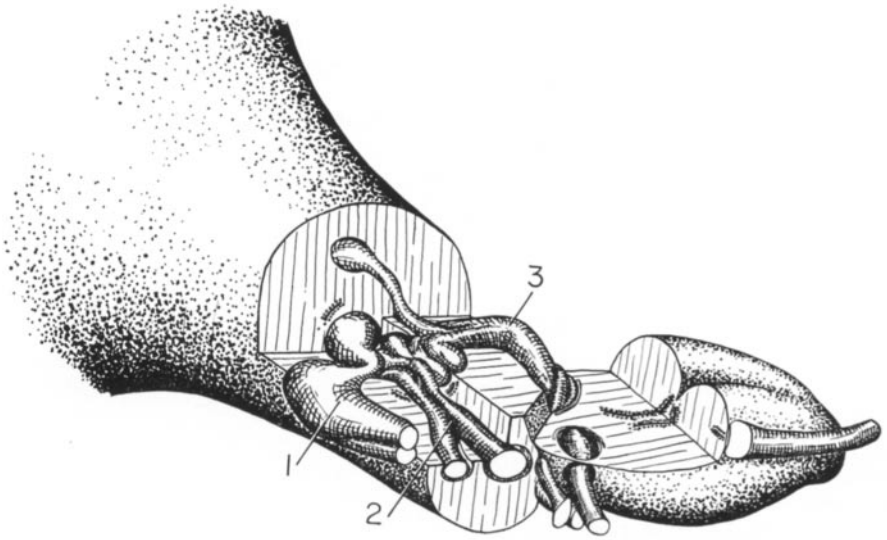


FIGURE 13 A diagrammatic reconstruction made from serial sections, one of which is shown in Fig. 12. Photoreceptor basal process (left) is depicted in contact with a collateral spherule (right). Part of the basal process has been cut away to show the contained synaptic cavity and three entrances occupied by presumed neural terminals. Numbers on the figure correspond to those on Fig. 12 for orientation. The nearby collateral spherule (right) contains both ribbons and subsynaptic cisterns.

Evidence of central cavities has been seen in other basal processes, but it cannot yet be stated that this pattern is the rule since some sections of basal processes (such as Fig. 8) disclose no central cavity.

One additional synaptic feature should be noted that further emphasizes the similarity between pineal photoreceptors and their retinal analogues. Mitochondria within pineal basal processes and collateral spherules are sometimes associated in pairs. One such pair from the frontal organ component of the pineal complex is shown in Fig. 14. Along the directly apposed membranes of the two mitochondria there is a pronounced beading with a periodicity of about 130 A. This phenomenon appears to be quite similar to the 160 A beading along adjacent mitochondrial membranes described by Pease (43) in basal spherules of the cat retina.

Outer Segment Variation and Pineal Macrophages

Although some photoreceptor outer segments, like the one shown in Fig. 1, closely resemble retinal photoreceptors, relatively few outer segments in the pineal organs are so neatly stacked and ordered. There is, rather, a wide range of variation in the size, shape, and organization of these structures (Figs. 1, 3, 6, and 15 to 17). Besides what might be considered "typical," ordered photoreceptor outer segments, the lumina of both epiphyses and frontal organs contain many objects that appear to be less highly organized, inflated, or fragmented outer segments. At the same time, one finds very small attached outer segments, with few lamellae (Fig. 16), which are, in many respects, similar to immature outer segments that have been described in the developing frontal organ of *Hyla* larvae (22) and the epiphysis of newt embryos (31). Such "young"

outer segments are not uncommon in our adult frog preparations. In one instance we have found a young outer segment sprouting from an inner segment already bearing a mature outer segment.

Macrophages are common elements within epiphyseal and frontal organ lumina, and may also be found within or along the connective tissue capsules. They are large cells displaying numerous debris-filled vesicles characteristic of macrophages in other localities. The macrophages in pineal lumina are frequently in close apposition to outer segments of photoreceptor cells (Fig. 18). Seldom have whole macrophages been found well preserved in our specimens; often they seem to be especially susceptible to preparation damage.

Regardless of their state of preservation, the majority of pineal macrophages we have examined have displayed accumulations of dense, whorled, membranous material within their cytoplasmic vesicles. Sometimes only one or a few vesicles are so occupied, the rest containing lipid, crystalline, or amorphous material (Fig. 18), but more often the macrophage is packed with large membrane-filled parcels (Fig. 19).

DISCUSSION

The observations described in this report provide added morphological support for the concept that both the epiphysis and frontal organ are photoreceptive, in agreement with the interpretations of other workers.

Pineal outer segments and their lamellae, however, are not so precisely ordered as are those of analogous retinal components. Likewise, no distinct layered arrangement of cell bodies and their processes such as exist in the retina are found in the walls of these pineal organs, and this fact hampers exact identification of cell types.

For reasons which will become apparent, we have preferred to limit our nomenclature of pineal cells to three basic types, only two of which are clearly revealed in our micrographs. These are the photoreceptors, and the supportive cells (which may or may not border the lumen, and may or may not be drawn out into basal processes).

Many workers who used silver or methylene blue in light microscopic techniques have reported the presence of ganglion cells as the third main cell type in amphibian pineal organs (see *e.g.*, references 26 and 33). Recognition of such cells in electron microscope studies is complicated by sampling problems. Oksche and von Harnack (41, 42) have pictured cells from frog pineal organs which they identified as ganglion cells primarily on the basis of the arrangement of mitochondria, Golgi apparatus, and endoplasmic reticulum. We have observed similar cells or parts of cells (although with less endoplasmic reticulum) but have been hesitant to identify these as ganglion cells until further diagnostic features such as extended axonal or dendritic processes could be demonstrated. Eakin *et al.* (19) have also indicated uncertainty in the identification of ganglion cells in *Hyla regilla* tadpoles.

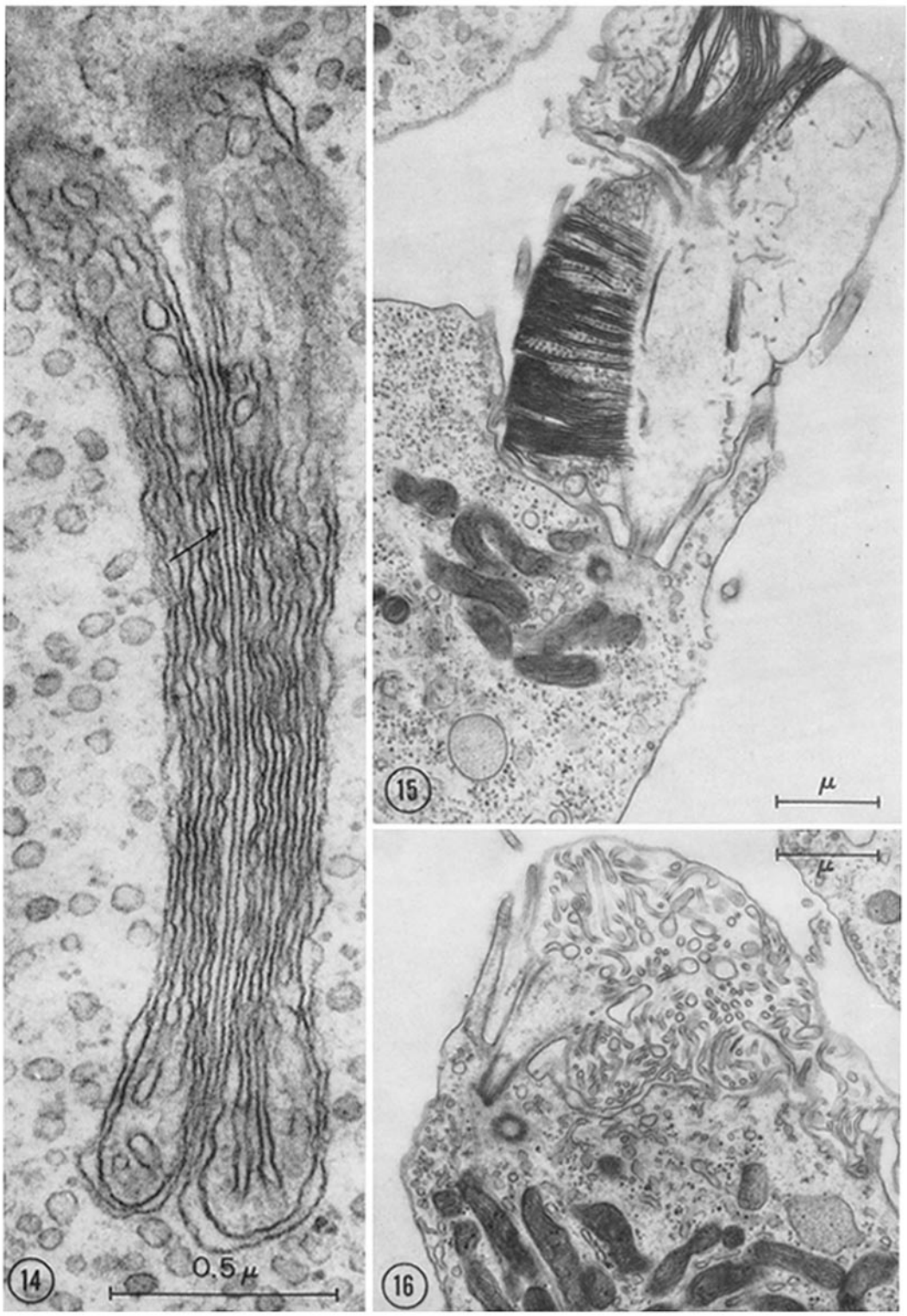
With regard to possible additonal cell categories, the "epithelial cell" proposed by Oksche and von Harnack (41) does not seem justified as a separate class on the basis of the evidence derived from our micrographs. Furthermore, a separation of the spectrum of supportive cell morphology into "ependymal" and "glial" classes appears equivocal to us.

FIGURE 14 Paired mitochondria from the basal process of a pineal photoreceptor cell. Apposed surface membranes of the two mitochondria display a regular beaded pattern (arrow). Magnification, 69,500.

FIGURE 15 Outer segment of an epiphyseal photoreceptor cell displaying a ballooned appearance. This variation of the basic lamellar pattern is considered as a possible stage in degeneration of the outer segment. Magnification, 15,000.

FIGURE 16 An example of a small photoreceptor outer segment interpreted as being a young developmental stage in a cycle of outer segment renewal. Such young outer segments are not uncommon in adult frog pineal organs. Magnification, 16,000.

FIGS. 15 and 16 were originally published in *American Scientist*, 1962, **50**, 597–625.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There is little reason based on intrinsic structure to suspect that the epiphysis and frontal organ of *R. pipiens* serve greatly different functions. Location of the frontal organ within the skin, however, might favor greater photoreceptive activities over those of the more deeply buried epiphysis. Moreover, the position occupied by the frontal organ surrounded by dense connective tissue and tough epidermis may dictate its compact arrangement of cells and the lack of prominent intercellular spaces characteristic of the epiphysis.

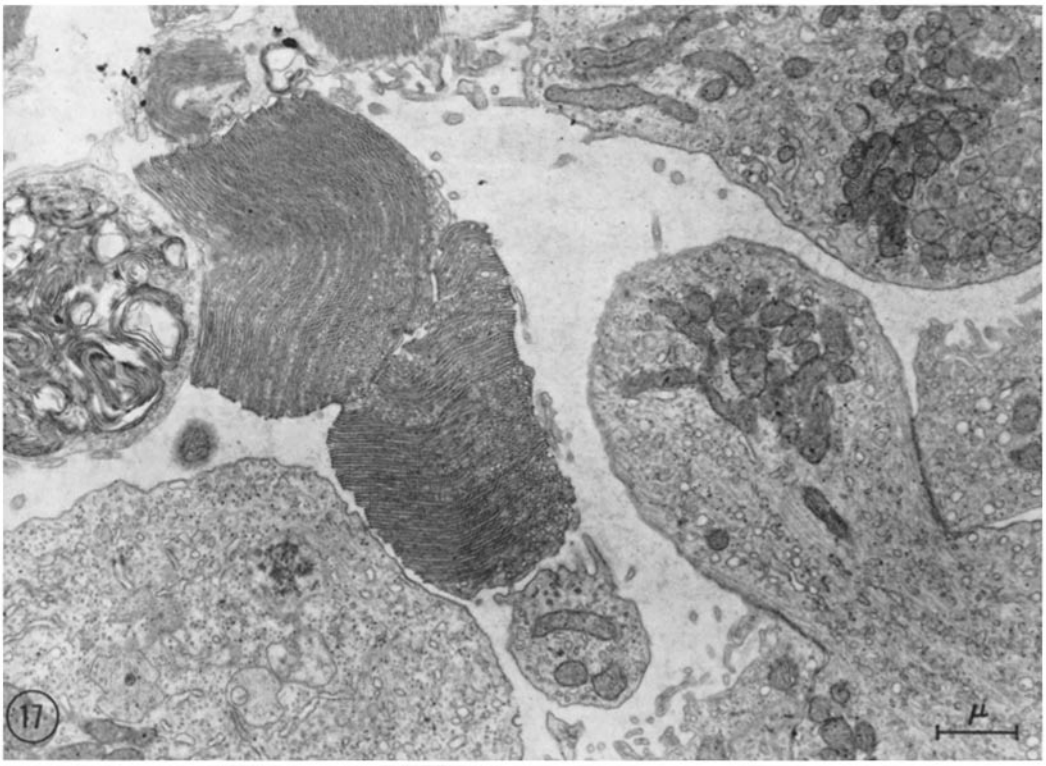
The one major distinction in fine structure between the two organs, the greater wealth of myeloid bodies in the frontal organ supportive cells, is less easily explained. Two theories have been advanced as to the function of similar myeloid bodies or their analogues in the retinal pigment epithelium: (*a*) that they are themselves photoreceptive components, perhaps related to migration of pigment granules (44); and (*b*) that they bear some relationship to membrane formation or to recycling of vitamin A in the metabolism of the visual pigments (13–15). In the pineal system of this frog there are no pigment granules and there is no evidence as yet concerning the presence of vitamin A or photoreceptive pigments in any pineal system. It seems tentatively reasonable to view the supporting cells in the epiphysis and frontal organ as equivalent in part to the cells of the pigment epithelium of the retina of the lateral eye, but different in that they have not become segregated into one territory of the wall of either of these saccular organs. (The implications of such equivalence are further discussed below.)

The observations on synaptic relationships in pineal organs described in this report are limited. Extensive serial sections are essential for determining synaptic topography accurately, but their acquisition has been limited by the random, disoriented arrangement of synaptic areas. Nevertheless, it appears that there are central synaptic cavities in the basal processes of frog pineal photoreceptors which resemble those of guinea pig rod cells (47). Basal processes of mammalian cones have been characterized as containing several scattered synaptic ribbons along shallow indented synaptic junctions, whereas rods usually display the deeply indented synaptic cavity with a single or double encircling ribbon (see recent review by Fine, 24). It is premature, however, to compare pineal photoreceptors too closely with rods or cones, and the possibility of two or more pineal photoreceptor types remains open.

Termination of neural components is complex, and may represent modulated circuitry in view of the finding of two possible types of synaptic junctions on the membranes of basal processes and collateral spherules. It is premature to assign functional roles to the two kinds of synapses apparent from this study, but a tentative proposal might be made in view of the similarity between them and terminations described in cochlear hair cells (48, 49). In that system efferent nerve terminals have been shown by degeneration studies and electron microscopy to synapse with the hair cell basal processes at a junction involving a subsynaptic cistern. On the other hand, less vesiculated afferent terminal junctions are characterized by synaptic ribbons within the basal process. Similarly, in guinea pig rods the indented synaptic cavity, encircled by ribbons, is believed to be the main transmission site for impulses from the photoreceptors to the bipolar cells, whereas presumed efferent or feedback terminations lie along outer surfaces of the process or spherule (47). Taken together, the current evidence appears to

FIGURE 17 Luminal area of a frontal organ containing several contorted portions of outer segments. Some of these (left) appear to be degenerated whorls of membranes similar to those found in dystrophic retinas. Inner segments in this micrograph display filaments and moderately dense, subspherical, membrane-bounded vesicles common to pineal photoreceptor cells. Magnification, 10,500.

FIGURE 18 Pineal macrophage in the lumen of a frontal organ. A portion of the macrophage is in close contact with a mass of outer segment membranes. Large vesicles within the macrophage cytoplasm contain dense, amorphous lipid and membranous material presumably ingested by this phagocytic cell. The extra membrane (arrow) along the margin of the macrophage is unexplained. Magnification, 14,000.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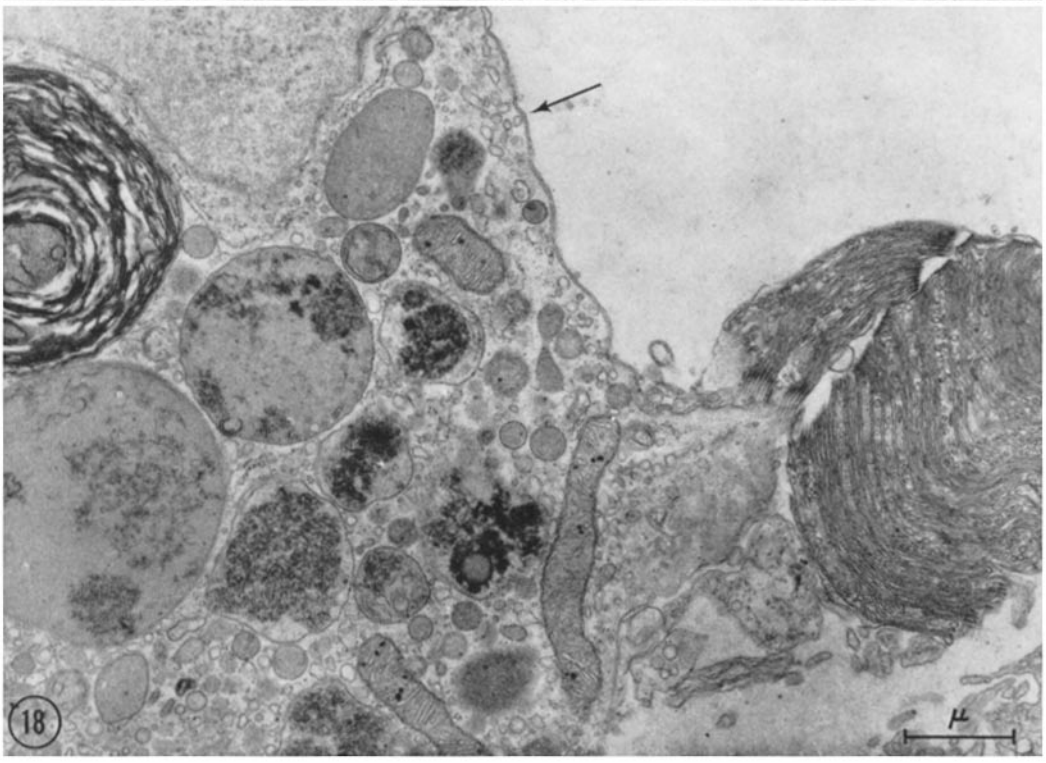
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strengthen a previous proposal (33), made on the basis of light microscopy, that efferent or feedback terminations are present on pineal photoreceptors in addition to sensory afferent synaptic sites.

The possibility has recently been considered that synaptic ribbons common to various receptor cells (and also found in axons of the inner plexiform layer of retinas, reference 24) may take their origin from invaginated, apposed, and modified plasma membranes along which synaptic vesicles become aligned (see *e.g.*, discussion in reference 8). Although the development of synaptic ribbons in the frog pineal system has not yet been studied, it is notable that the ribbons in our sections are often located in regions of basal processes or collateral spherules where the plasma membrane is folded so as to form a groove with its apex pointing toward, but not demonstrably continuous with, the contained synaptic ribbon as diagrammed in Fig. 13. This arrangement and the fact that the ribbons display a faint dense central line might be interpreted as compatible with the view that the ribbons originate by joining and thickening of two sides of a fold in the plasma membrane. Further study is in progress on this point.

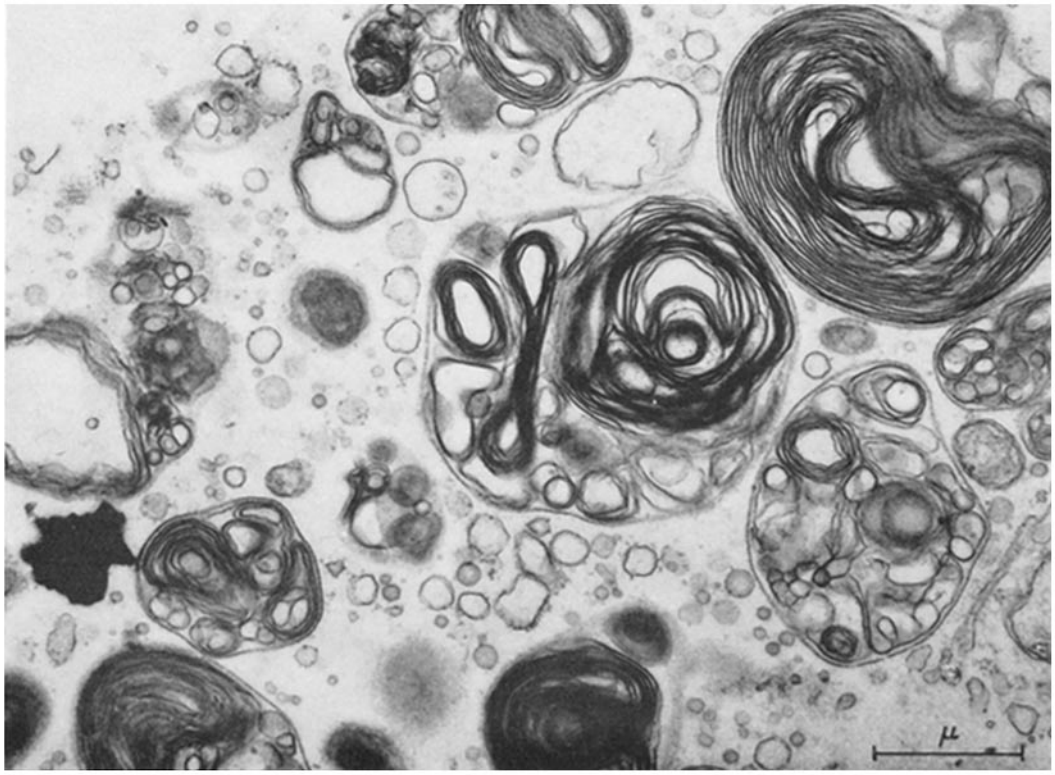

FIGURE 19 A portion of a pineal macrophage found in the lumen of the epiphysis. Note large numbers of vesicles containing whorled membranous material presumably of outer segment origin. Magnification, 19,500.

Our use of the words "young," "mature," and "degenerate" to describe the variations in pineal outer segment morphology is not unique. In 1918, Holmgren (26) expressed similar views in describing such variations at the light microscopical level. He interpreted these variations as stages in a cycle of outer segment regeneration wherein, in the adult organism, outer segments were being cast off and renewed from the inner segments. The repeated occurrence of these variations in both light (33) and electron microscopical examinations (19, 41, 42) in different species of amphibia, and with a variety of fixation methods,

tends to discount the possibility of their being due to preparation artifact. Moreover, the close ultrastructural resemblance of the "degenerate" outer segments of frog pineal photoreceptors to those in the retina of the vitamin A–deficient rat (13) (see also Eakin and Westfall's report using vitamin A–deficient lizards, reference 23) or rats with heritable retinal dystrophy (15) also supports the view that these pineal structures are, in fact, degenerating outer segments. Detection in the same adult frogs of young outer segments greatly resembling the developmental stages of pineal outer segments described in tadpoles of *Hyla regilla* (22) and newt embryos (31) leads us to the belief that outer segment development continues to occur in adult pineal organs as a normal mode of replacement for cast-off outer segments. On the basis of present evidence, we are, therefore, more inclined to regard the variations normally observed (perhaps including Oksche's and von Harnack's "epithelial cells", reference 41) as stages of a "maturation spectrum" rather than a stable population of diverse protruding cell types. A cycle of degeneration and renewal does not, however, exclude the possibility of more than one photoreceptor type in view of the diversity of synaptic structures.

There may be several explanations for the source of the membranous material found in pineal lumina and in the vesicles of pineal macrophages. Oksche and von Harnack (41, 42) have suggested that some of the membranous material in the lumen may be derived from "ependymale Begleitzellen", a possibility we can neither confirm nor deny from our micrographs. However, the position of the large phagocytic cells in close proximity to outer segment membranes (many of which appear degenerate) suggests that the membranous macrophage inclusions are derived from the outer segments (see also the discussion of "freie Zellen" by Oksche and von Harnack, reference 42, p. 605). It now becomes necessary to seek quantitative or experimental methods which will more firmly test the reality of regeneration of outer segment material and its eventual uptake by macrophages under controlled nutritional conditions.

The significance of the implied normal degeneration and renewal of outer segments in adult pineal photoreceptors is interesting in view of the fact that a similar phenomenon has been observed only in abnormal retinas of adult mammals and lizards (13, 15, 23). The normal retina has achieved its stability through several possible devices apparently not evolved in pineal systems. The frog pineal system lacks a homologue or an exact analogue of the retinal pigment epithelium to orient and support the photoreceptive elements in a manner approaching that of the retina. In view of the increasing evidence (14, 45) that retinal pigment epithelium is involved in metabolic maintenance of the supply of visual pigment in outer segments, it is appealing to speculate that a system lacking a pigment epithelium might compensate by providing an ever new supply of outer segments rather than sustaining permanent ones. In the retina of the lateral eye the pigment epithelial cells are segregated in the outer layer of the optic cup and subsequently lie in intimate contact with the outer segments of the photoreceptors facing them across the collapsed lumen of the cup. In the frontal organ and epiphysis of the frog, however, the supporting cells, which have some cytological features (the myeloid bodies) in common with pigment epithelial cells, are intermingled with photoreceptors along a lumen that is not collapsed. Accordingly, they lack intimate contact with the outer segments of the photoreceptors and seem unlikely to be able to perform the rapid transfer of materials to the photoreceptors in the fashion suggested for the lateral retina. If, in fact, they do participate in the capacity of an intermediary to the photoreceptor cells in provision of materials to the latter, the rate of transfer to the outer segments should be much less than in the lateral eye retina. The spatial arrangement of pineal supportive and photoreceptor cells appears more appropriate for supply to a growing outer segment via the cell body of the photoreceptor cell.

There is only equivocal evidence from this study that the pineal system in *R. pipiens* is involved in secretory activity. The hypothesis of Bagnara (1) that amphibian pineal organs are the source of melatonin, which is presumably responsible for the well known dark-induced blanching of pigment cells, awaits biochemical demonstration of a secretory product and its morphological correlates within amphibian pineal organs (reviewed elsewhere, 29, 30). The first attempts to identify melatonin in amphibian pineal tissue by chemical means have yielded negative results (19). The possibility of secretory activity was also suggested several years ago when it was initially suspected

that efferent neural processes might terminate on pineal photoreceptor cells (33). Our present electron microscopical evidence is not inconsistent with that possibility, but since there appears to be efferent innervation of other, purely sensory systems (*e.g.*, the cochlear hair cells, references 48, 49), the present evidence seems equally consistent with existence of pineal efferent innervation involving feedback from other sensory cells rather than any secretomotor function.

Nevertheless, pineal organs may ultimately be shown to perform a secretory activity in addition to a primary photoreceptive one. As Eakin *et al.* (19) have pointed out, even degenerated outer segment material might be viewed as a secretory product.

The wealth of subspherical vesicles containing finely granular material (described above) within the inner segments of pineal photoreceptors might also represent a secretory product. If the contents of these vesicular elements do, in fact, comprise accumulations of a secretory product, the product is a remarkable material indeed. The vesicles' content of OsO_4-reactive lipid, of protein, or periodic acid-sensitive carbohydrate or other material, or acidic materials, of catecholamines, or of 5-hydroxytryptamine is undetectably small by the available methods, in sharp contrast with other, identified, intracellular secretory products which are reactive to one or several of these tests. Their meager content of complex substances retainable by the fixatives employed is attested by their considerably lower contrast than the cytoplasmic matrix as determined by phase contrast microscopy. Although the possibility that these vesicles might contain a distinctive secretory product of special biological significance cannot be finally and categorically rejected on the basis of the cytochemical findings, such a possibility appears remote.

Finally, the multivesicular bodies observed in the foot processes of supportive cells may correspond to granules reported in greater numbers in the material of Oksche and von Harnack (41, 42) and Eakin *et al.* (19). This could represent a release of material into the perivascular space analogous to the release of biogenic amines proposed in mammalian pineal organs by De Robertis and Pellegrino de Iraldi (9). However, our cytochemical tests for catecholamines and 5-hydroxytryptamine in perivascular regions of the frog epiphysis were negative.

We feel, therefore, that to ascribe a secretory role to any of the above components in the frog pineal system would be premature. Our data, cytochemical and morphological, leave the question of pineal secretion, in the endocrine or neurosecretory sense, essentially conjectural.

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