Observations on Developing Pineal Organ in the African Clawed Toad, *Xenopus laevis* D.[1)]

By

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(With 2 Plates)

Since Holmgren's classical work (1918) on the "Parietal-organ" in the frog, *Rana temporaria*, the pineal system in lower vertebrates has attracted many investigators' attention. Perhaps of fundamental interest at present are studies showing a relation of the pineal organ with reception of photic stimuli (for reference, see Kelly, 1962). It is now well known that the pineal system in anurans is a double structure consisting of the frontal and the pineal organs, both of which are derived from a single pineal anlage. It is still unknown what factor initiates segregation of the frontal organ from the pineal organ proper. Furthermore, in order to investigate the differentiation of the pineal photoreceptor cell, a precise description of the morphogenetic process of the pineal system seems needed.

In anurans, morphological and neurophysiological data on the pineal organ provide evidence that the organ functions as an endocrine as well as a photoreceptive organ. For instance, recent studies by Bagnara (1965) and Charlton (1966) showed that in the African clawed toad, *Xenopus laevis*, the pineal organ is the site of metabolism of melatonin, a potent neurohormonal substance for color change. Moreover, topography of the pineal organ seemed to be closely related to morphogenesis of the subcommissural organ and of the Reissner's fibers. In view of these facts, it would be interesting to observe the development of the pineal system in the larva and young toad, *Xenopus laevis* with special respect to its photoreceptor cells and innervation.

Materials and Methods

All the animals used in this investigation were obtained through induced matings with chorionic gonadotropin (Gonatropin by Teikoku Zoki Co., Tokyo). They were reared in the aerated aquaria conditioned at 20°–22°C, and fed with alfalfa powder during larval stage. After metamorphosis, they were kept in the aquaria placed in the laboratory and fed with piece of bovine liver.

Twenty larvae in each developmental stage from St. 24 to St. 65 (according to the

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Normal Table by Nieuwkoop and Faber, 1956) were selected and fixed with Heidenhain's Susa fluid or Helly's fixative. Animals from St. 24 to St. 42 were fixed *in toto*, but in older larvae from St. 43 to St. 58 only the heads without noses and lateral eyes were dissected out and fixed by immersion. In animals from St. 59 to St. 65, the whole heads were immersed in the fixatives. The skin, cranial bone and dispensable brain parts were carefully removed by fine forceps under the binocular dissecting microscope. Heidenhain's Susa fluid was employed for Heidenhain's Azan and Heidenhain's iron hematoxylin stain. Helly's was used for aldehyde fuchsin (AF) stain with preoxidation by both iodine and permanganate.

Young animals three to four months after metamorphosis were sacrificed by decapitation and fixed with a variety of fixatives. Helly's and Susa were employed for general morphological study with Azan and iron hematoxylin stain as described above. Bouin's, Gendre's and Rossmann's for periodic acid Schiff (PAS) reactions to detect glycogen with salivary digestion. Adjacent sections from tissue fixed with fixatives described above were mounted on two slides, both of which were coated with celloidin, and one of which incubated for one hour at 37°C in filtrated saliva, and the other in distilled water before PAS reaction. Bouin's, Alcohol Bouin's (Williams, 1962) and modified Bouin's (Bodian, 1937) were for demonstration of the nerve fibers with silver impregnation techniques (Bodian, 1936; Romanes, 1950; Betchaku, 1960).

All tissues were embedded in paraffin and most of them were sectioned at a thickness of 5μ sagitally or transversely. A few tissues were sectioned at a thickness of 10μ and were examined following silver impregnation.

Results

Normal Development of the Pineal Organ. Visible emergence of the pineal system began at St. 26. The first sign of development was a dorsal bulging by elongation of the cells in the center of the diencephalic roof (Fig. 1). At St. 27, this initial pineal bud evaginated outward to form a vesicle-like pineal anlage containing a wide lumen. In the following stage, the rostral and upper part of the pineal vesicle became one-cell-layered wall, while the basal and caudal part was thick and multicellularly layered (Fig. 2). The pineal vesicle was communicated with the third ventricle through a wide opening, the so-called "orifice". At St. 31 and the following stage many mitotic figures could be observed only rarely in the rostral and upper part (Fig. 3). The cells were proliferated and accumulated in the lumen of the pineal vesicle, resulting in that the lumen got smaller and finally became a slit, and that the pineal anlage changed into a massive cell-cluster. At St. 39 and St. 40, vacuolization in the cytoplasm could be observed in several cells at the rostral end of the pineal anlage (Fig. 4). This is probably the first light microscopically recognizable sign of the differentiation of so-called "photoreceptor cell", the main constituent cell type in the pineal organ.

The upper half of the cell-cluster pineal anlage began to segregated from the rest of the pineal organ proper at St. 42 (Fig. 5) to take part in formation of the frontal organ. Since the orifice remained in the same position while the frontal organ proper moved forward, the pineal organ proper became elongated and increased in size during this segregation process, which extended from St. 42 to St. 49, and the pineal photoreceptor cell had differentiated during this process. The

well differentiated photoreceptor cell with outer segment, inner segment and basal part made an appearance earlier in the frontal organ than in the pineal organ proper. Segregation of the frontal organ was followed by invasion of the meningeal melanophores into the slit between the frontal and pineal organ (Van de Kamer *et al.*, 1962). During St. 47 and St. 48, segregation of the frontal organ proceeded to some extent, and the connection between the frontal and pineal organ was a broad bridge of cells in which many well differentiated photoreceptor cells were seen (Fig. 6). The bridge became thinner until it finally diminished at St. 49. During this long segregation process a drastic change in shape was observed in the frontal organ. The frontal organ which was triangular in shape in earlier stage, became an oval shape elongated anterio-positeriorly containing a lumen in its dorsal half. On the other hand, the pineal organ became a flattened sac-like shape containing a wide lumen connecting with the third verticle by the orifice (Fig. 7).

Structure of the Pineal Organ. The pineal organ represented a flattened sac-like form containing a wide lumen or lacunae surrounded by an almost one-cell-layered wall which was named the roof or floor according to the position. Apparently four cell types could be distinguished in the pineal organ at the light microscopical level. They were the photoreceptor cell, two types of supportive cells mainly constituting the roof and floor (Figs. 8 and 9), and the free cell observed in the lumen (Fig. 10).

The photoreceptor cells were the main constituent cell of the pineal organ and they were more abundant in the floor than in the roof (Fig. 11). Definite clusters of the photoreceptor cells have been reported previously as a notable feature of the photoreceptor cells in *Rana esculenta* (Kelly and Van de Kamer, 1960). However, in *Xenopus* such a feature could be hardly seen even in a precisely sagittal section. The photoreceptor cell consisted of three parts; the outer segment, the inner segment and the basal part. Among the methods employed only AF and PAS reactions were trustworthy to demonstrate the outer segment (Fig. 13), and inconsistently positive results were obtained by acid fuchsin and iron hematoxylin stain. In PAS reaction the positive reaction remained in the outer segment after the salivary digestion. The morphology of the outer segment was very diverse as has been reported previously by Holmgren (1918).

The ellipsoid in the inner segment and the "Ersatz" ellipsoid in the basal part were positive to Altmann's mitochondria stain (Fig. 12). In the developing photoreceptor cell during St. 43 to St. 49, the inner segment contained a large vacuole surrounded by accumulation of the mitochondria which constitute the ellipsoid (Fig. 14). During the larval stage after St. 50 and adult form, the pineal photoreceptor cell was scarcely demonstrated to possess a vacuolized ellipsoid. The inner segment of the photoreceptor cell was positive to PAS reaction (Fig. 15). This reaction could be abolished by the salivary digestion, the fact indicating the existence of glycogen in this segment. The fixatives used in this investigation in order to demonstrate glycogen showed different ability to preserve glycogen in the photoreceptor cell. Alcohol flight was observed in the preparations made with

Rossmann's fixative and to some extent in those made with Gendre's fixative. Therefore, the precise distribution of glycogen in the inner segment could not be determined.

The innervation of the pineal organ was demonstrated by the silver impregnation techniques of Bodian (1936), Romanes (1950) and Betchaku (1960), and the silver nitrate technique of Romanes was most useful in demonstrating the nerve fibers which distributed over the dien- and mesencephalic roof. However, the end-loop of neural fiber and fine neural plexus, the structures which have been known in *Rana esculenta* (Kelly and Van de Kamer, 1960) could not be found even in the best preparations made with this technique. A bundle of nerve fibers which entered into the anterior part of the pineal organ branched off, running through the right and left portion of this organ in the ventral, respectively. A part of the bundle of nerve fibers came down through the basement membrane, terminating in the habenular commissure (Fig. 16). The other went up to the roof and ran posteriorly. The right and left bundles were brought together in the posterior region of the pineal organ. Then, a bundle of the nerve fibers which was called *tractus pinealis* terminated in the subcommissural organ and the posterior commissure (Fig. 17). The question whether the *tractus pinealis* was derived from the photoreceptor cells or from the ganglion cells, still unsolved.

Discussion

It is not uncommon for anurans, which possess the frontal organ or "Stirnorgan" as an outpouching from the pineal organ, that the pineal system is established through four stages described in this paper. In *Xenopus* the cells in the vesicle-like stage remain less differentiated than those in the early larval pineal vesicle of the newt, as reported by Kelly (1963). The differentiation of the pineal organ in anurans takes place after the cell-cluster stage, whereas in urodeles it starts at the vesicle-like stage. Because the frontal organ is positioned in tanden with the pineal organ and the innervation shows sufficiently the bilateral structure in the pineal system in *Xenopus*, the double structure of the pineal system is concluded not to be directly derived from the bilateral fusion of the lateral edge of the neural plate.

In *Hyla regilla*, according to Eakin and Westfall (1961), the first stage in the formation of the photoreceptor cell is a bulbous outgrowth of an ependymal cell from the luminal surface in early larva. They postulate that no mitochondria have accumulated in cytoplasm near the bulbous outgrowth. But in the present study a certain evidence at the light microscopical level was obtained to show that the vacuole observed prior to the appearance of the outer segment was surrounded by mitochondria. The vacuolized ellipsoid or "Ersatz" ellipsoid have been reported in the pineal photoreceptor cells of the adult frog, *Rana esculenta* (Kelly and Van de Kamer, 1960). In *Xenopus*, however, such photoreceptor cells with the vacuolized ellipsoid are confined to the larval stage. Moreover, the vacuoliza-

tion in the inner segment occurs in the retinal photoreceptor during its morphogenesis as well. These facts seem to provide two possibilities as for the significance of the vacuoles. The first is that the pineal photoreceptor cells in *Rana esculenta* are considered to be immature even in adult. The second possibility is that the vacuoles themselves have nothing to do with the photoreceptive function.

The "free cell" described in this paper seems to correspond to those described under several different names by earlier investigators, such as a "disintegrated cell" in fish (Grunewald-Lowenstein, 1956), and "Freie Zelle" (Oksche and von Harnack, 1963) or "macrophage" (Kelly and Smith, 1964) in frogs.

That the pineal photoreceptor cell represents a basic structure similar to the retinal rod or cone in eyes has been pointed out by many investigators (Kelly, 1962; Van de Kamer, 1965). At the same time it must be emphasized that the ellipsoid and "Ersatz" ellipsoid contains a clump of mitochondria as well as of the retinal rod or cone seems to indicate the high oxidative metabolic activities in the photoreceptor cell. On the other hand, high glycogen content in the inner segment of the pineal photoreceptor cell imply another function such as secretion, as suggested earlier in the frontal organ of *Hyla regilla* by Eakin *et al.* (1963).

The innervation demonstrated in this pineal organ may support the possible relationship between the photoreception by the pineal system and the secretion in the subcommissural organ or the habenular commissure. Further studies are in progress with respect to cytological changes in the pineal organ and the subcommissural organ induced by different light conditions.

Summary

1. The normal development of the pineal organ in the African clawed toad, *Xenopus laevis* D., was studied at the light microscopical level, and it was divided into the following four stages: the vesicle-like stage, the cell-cluster stage, the segregation process and the post-segregation stage.

2. In the pineal organ four cell types were described: the photoreceptor cell, two types of supportive cells and the free cell in the lumen.

3. The photoreceptor cell consisted of the outer segment, the inner segment and basal part, possessing a basic structure similar to the retinal rod or cone. A possible role of the vacuole present in the inner segment was discussed.

4. Observations by means of silver impregnation techniques provided a certain evidence showing that a part of the *tractus frontalis* terminated in the habenular commissure and that the *tractus pinealis* terminated in the subcommissural organ.

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Explanation of Plates VI - VII

Plate VI

Figures 1–6 represent median sagittal sections of the developing pineal organs in *Xenopus laevis* larvae. Heidenhain's iron hematoxylin stain; anterior to the left.

Fig. 1. Initial pineal bud (pb) evaginated from the diencephalic roof at St. 26. × 300.

Fig. 2. Vesicle-like stage at St. 31. The pineal vesicle contains a wide lumen (l) communicated with the third ventricle (v) by orifice (arrow). × 300.

Figs. 3–4. Two stages in cell-cluster stage St. 35 (Fig. 3) and St. 40 (Fig. 4). Note mitotic figures (Fig. 3, m) and a vacuole (Fig. 4, va). × 300.

Fig. 5. Initial appearance of the frontal organ proper (f) and the pineal organ proper (p) at St. 42. × 300.

Fig. 6. Segregation process at St. 46. The connection between the frontal organ (f) and the pineal organ proper (p) is a broad bridge of cells in which differentiated photoreceptor cells are seen. × 300.

Fig. 7. Two focus planes microphotograph showing post-segregation stage of the pineal system consisting of the frontal organ (fo) and the pineal organ (po) at St. 52. pp, paraphysis; acp, anterior choroidal plexus. Heidenhain's Azan stain. × 100.

Plate VII

Figures 8–11 are sagittal sections through the floor (Figs. 8 and 11), the roof (Fig. 9) and the lumen close to the orifice (Fig. 10) of the pineal organ. Figures 12–15 are micrographs displaying the photoreceptor cells treated with a variety of staining procedures. All the micrographs except Fig. 14 are of the specimens at least three months after metamorphosis.

Fig. 8. The first supportive cell type (s). Heidenhain's iron hematoxylin stain. × 1,500.

Fig. 9. The second type of supportive cells. Note the AF positive substance in this cell (arrow). Aldehyde fuchsin stain, × 1,500.

Fig. 10. In the lumen there are some free cells. Heidenhain's Azan stain. × 600.

Fig. 11. The photoreceptor cells are more abundant in the floor than in the roof. Acid fuchsin stain. × 800.

Fig. 12. The photoreceptor cells contain the ellipsoid (e) in the inner segment and "Ersatz" elliposid (ee) in the basal part, respectively. Altmann's mitochondria stain after Kiyono's method. × 1,250.

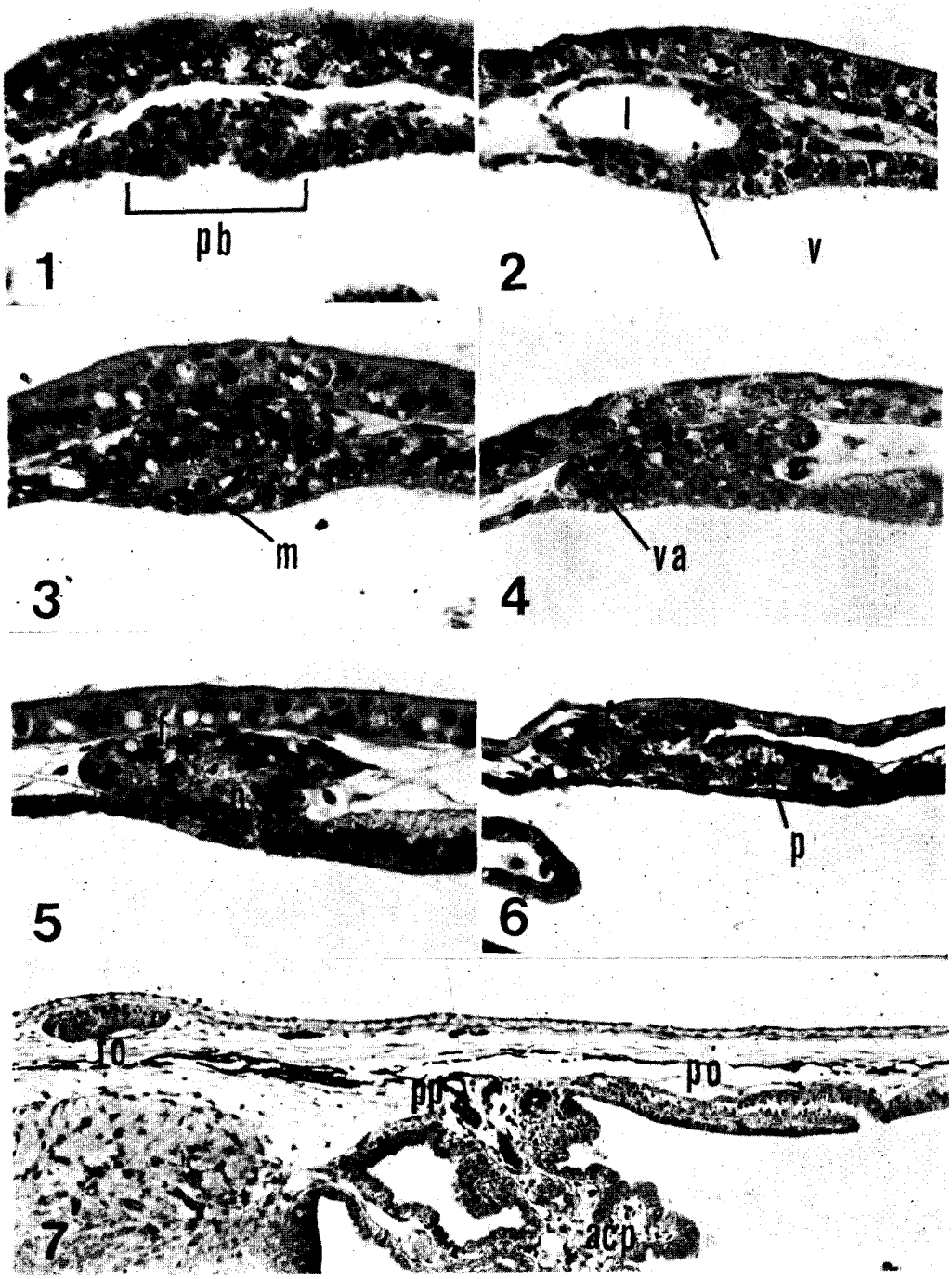
Fig. 13. The outer segment (os) is strongly positive to AF. Note that both the outer segment and the inner segment (is) protrude into the lumen. bp, basal part. Aldehyde fuchsin stain. × 1,000.

Fig. 14. Developing photoreceptor cells with vacuolized elliposids in an animal st St. 47. Heidenhain's Azan stain. × 1,000.

Fig. 15. The photoreceptor cells with the inner segment strongly positive to PAS. Note a relatively weak reaction in the ellipsoid part (arrows). PAS reaction after Gendre's fixation. × 1,500.

Fig. 16. Section through the pineal organ (po) and the habenular commissure (hc) showing the nerve fibers which run through the pineal organ and terminate in the habenular commissure. Romanes' silver stain. × 400.

Fig. 17. Section showing the nerve fibers which enter the subcommissural organ (sco) from the caudal part of the pineal organ (po). Romanes' silver stain. × 400.



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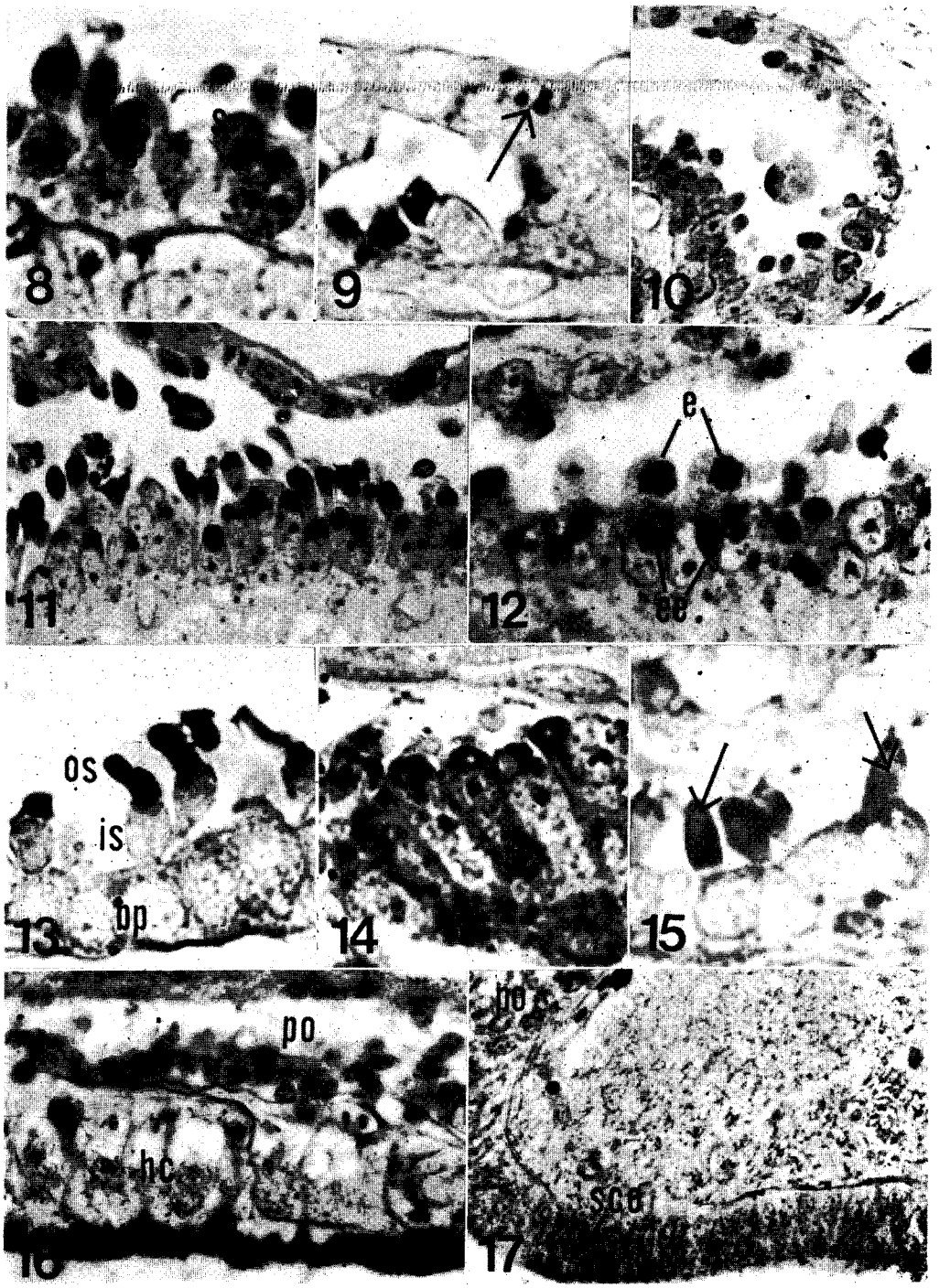

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