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Ultrastructure of the Hatching Gland Cells in the South African Clawed Toad, *Xenopus laevis*

By

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(With 3 Text-figures and 6 Plates)

It has been shown in several amphibian species that the hatching embryos secrete an enzymatic substance which is capable of digesting egg-envelopes (Cooper, 1936; Ishida, 1947; Katagiri, 1963). The properties of this substance, usually referred to as a hatching enzyme, are the subject of recent biochemical studies (Carroll and Hedrick, 1972; Katagiri, 1973), as carried out in other forms including sea urchins (Yasumasu, 1961) and teleosts (Kaighn, 1964; Yamagami, 1970). On the other hand, Yanai's extensive histological studies (1953, 1966) showed the special epidermal gland cells localized in a particular area of the embryos, suggesting their participation in the hatching process. However, there is a gap in the knowledge about the relationship between the physiologically demonstrated enzymatic substance and its production site at the cellular level.

The present study aims to fill this gap from a morphological standpoint using a South African clawed toad. In spite of the wealth of information about the morphological and biochemical events that occur during the embryonic development of this animal, little attention has been paid to its hatching process until the recent biochemical study of Carroll and Hedrick (1972). The observations presented below will demonstrate at the ultrastructural level the possible participation of special epidermal gland cells in the hatching process.

Material and Method

The material used was the developing embryo of the South African clawed toad, *Xenopus laevis*. Ovulation and mating were induced by injection of a human gonadotropic hormone, Gonatropin (Teikoku Zoki Co.). Fertilized eggs were collected, and were reared in the dechlorinated water (20–22°C) with constant aeration. The embryos at stages 22–46 (Nieuwkoop and Faber, 1956) were freed from their envelopes manually, and were subjected to fixation for both light and electron microscopical observations.

For the light microscopical observations, embryos were fixed in Bouin's solution. Five μ thick paraffin sections were stained with Heidenhain's Azan or periodic acid-Schiff (PAS) method.

For the electron microscopical observations, embryos were first fixed in 5% glutaraldehyde in phosphate buffer (pH 7.3) for 3–12 hrs. at 0–5°C (Millonig, 1961). In the fixative the embryos were trimmed so that appropriate parts for observation were isolated. The isolated tissues were rinsed several times in phosphate buffer, and postfixed in 1% osmium tetroxide in phosphate buffer (pH 7.3) for 1 hr. at 0–5°C. After dehydration through ethanol, the tissues were cleared in propylene oxide and embedded in Epon 812 (Luft, 1961). Embedding in styrene-(n-)butylmethacrylate (Kushida, 1961) was also employed in some specimens, with essentially the same results. Thin sections in the silver to gold range were cut with glass knives on a Porter-Blum MT-1 ultramicrotome, stained in saturated uranyl acetate and lead citrate (Reynolds, 1963), and examined with a Hitachi HS-7 electron microscope. Micrographs were made at initial magnifications of 3,000 to 10,000, and photographically enlarged.

Observations

Histological examination of epidermal cells

Histological observations on the superficial epidermis of the stage 32 embryos revealed large, bottle-shaped cells present in the frontal region. Their prominent height measuring 25 μ and the absence of pigment granules at the apical cytoplasm easily distinguish these cells from the adjacent cell types which are commonly found throughout the epidermis (Fig. 1). The cells having quite similar features have been observed by Yanai (1966) in several anuran species under the name "dorsal gland cells". In the present paper these cells will be referred to as "hatching gland cells" (HGCs) on the basis of their apparent function to be discussed later. As depicted in Fig. 2, HGCs are distributed mostly in the frontal region, and to a lesser degree in the anterior part along the dorsal mid-line to a level of the ear vesicles.

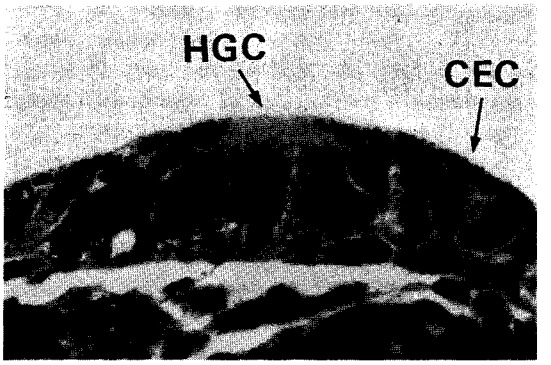

Fig. 1. Photomicrograph of a horizontal section through anterior end of stage 32 embryo, showing hatching gland cells (HGC) and common epidermal cells (CEC). Explanation in text. Heidenhain's Azan stain. × 600.

Other parts of embryonic epidermis contain 3 types of cells, namely, cilia cell (CC), common epidermal cell (CEC), and cement gland cell (CGC). Of these cells thorough ultrastructural studies have been made in *Xenopus* by Steinman (1968) for CC and by Schroeder (1970) for CEC. CGC with more specific localization has also been investigated by Eakin (1963) in *Hyla*. Accordingly, the electron microscopical observations to be described below will be concerned mostly with HGC in the frontal region.

Hatching gland cell (HGC) at stages 28–34

The typical feature of HGC in the pre-hatching embryo is shown in Fig. 4. Beneath the apical surface prominent with microvilli, there is an accumulation of the membrane-limited, electron dense granules which is most characteristic in this type of cell. Close examination of the granule region presented in Fig. 5 reveals the presence of numerous vesicles and canals, each of which peripherally possesses electron dense materials. A wide variety of the number and shape of these vesicles and electron dense granules may be related to the mode of their secretion as discussed in succeeding pages.

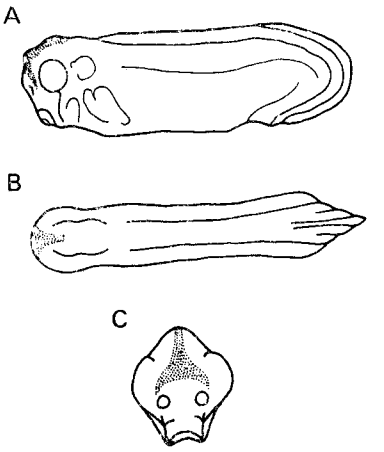

Fig. 2. Lateral (A), dorsal (B) and anterior (C) views of stage 28 embryo, showing the distribution (dots) of HGC.

Next to the granule region are the fibrous structures, "microfilaments", which are commonly found in epidermal cells (*cf.* Karfunkel, 1971). Often the microfilaments terminate upon the desmosomes which are the only site of junction with the adjacent cells. Most of the oval or cylindrical mitochondria are distributed beneath the zone of microfilaments, although a few are present in other parts of the cell except for the apical granule region. Double membranes of cristae are clear, their interspace being paler than the matrix.

An elaborate Golgi complex localizing at the supranuclear region is another characteristic of HGC, although the degree of its development varies according to the developmental stage. It is composed of highly developed lamellae, numerous vacuoles, and vesicles (Fig. 6). The lamellae are always oriented parallel to the long axis of the cell with an accumulation of the intralamellar materials. An interesting observation of the Golgi area is the frequent occurrence of the membrane-limited granules similar to the apically localized ones. A small granule arising within the terminal bleb of the Golgi lamella as shown in Fig. 7 will give rise to the membrane-limited granule mentioned above.

A nucleus with nucleoli of an amorphous structure is located basally. A few yolk platelets are found in the basal half of the cell showing various degrees of decomposition. Near the yolk platelets, there are scattering lipid droplets of an irregular shape. Pigment granules are confined to the cytoplasm basal to the mitochondrial area.

Besides these cytoplasmic organelles, the intervening cytoplasm contains a sizable population of free ribosomes and glycogen. Particularly the ribosomes are most abundant as compared with other cell types. Rough surfaced endoplasmic reticula (rough ER) with fragmented cisternae are found in high frequency

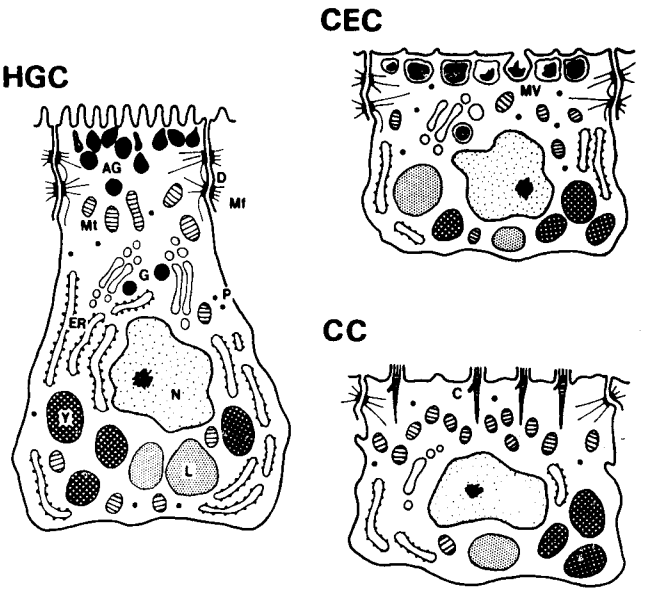

Fig. 3. Diagrammatic illustrations of 3 epidermal cells based on the electron microscopical observations, cilia cell (CC), common epidermal cell (CEC) and hatching gland cell (HGC). AG, apical granule; C, cilium; D, desmosome; ER, endoplasmic reticulum; G, Golgi complex; L, lipid droplet; Mf, microfilament; Mt, mitochondrion; MV, mucous vesicle; N, nucleus; P, pigment granule; Y, yolk platelet.

throughout the cytoplasm except for the apical granule region. Basal to the nuclear region, the long cisternae of ER are arranged parallel to the lateral and basal plasma membrane.

Two other types of cells (CEC and CC) found in the frontal region showed ultrastructures similar to those described by Schroeder (1970) and Steinman (1968). Fig. 3 is a diagrammatic illustration of HGC and the 2 other cell types. Comparison of HGC with these cell types, together with the ultrastructural features as presented above, favors the view that HGC is an active synthetic and secretory cell.

Changes of HGC during stages 22–46

The first sign of HGC differentiation is the appearance of short microvilli at the apical surface of an epidermal cell in the stage 22 embryo. In the cytoplasm of this cell the cisternae of rough ER are well developed, with occasional dilation of their sacs. However, glycogen and ribosomes in the intervening cytoplasm are not found in abundance. Other aspects of the cell are different from those found in HGC of more advanced stages, with a nucleus localizing approximately in the

center of the cell, oval mitochondria and pigment granules scattering throughout the cytoplasm including the apical zone, as well as the less developed Golgi complex. The yolk platelets and lipid droplets are distributed either apically or basally to the nucleus.

At stage 24, the electron dense granules make their first appearance in the apical region (Fig. 10). The arrays of microfilaments are also prominent. Presumably because of the progressive cytodifferentiation in the apical region, pigment granules and mitochondria take more basal positions than before. In addition, the Golgi complex and the cisternae of rough ER increase remarkably both in size and number. Concomitant with these is the presence of the membrane-limited electron opaque granules in the area of Golgi complexes. Ribosomes and glycogen are evenly distributed throughout the cytoplasm. Overall views presented above may indicate that the cell has started to synthesize the apically localized granules at stage 24.

At stages 28–34, microvilli and the apical accumulation of electron dense granules are much more prominent than in the preceding stages. Other aspects of cytoplasmic organelles have been presented in the above section (*cf.* Fig. 4). The most important feature characterizing HGC in these stages is the presence of numerous vesicles and canals found in most parts of the apical cytoplasm, besides the membrane-limited granules. Frequent occurrence of the electron dense, amorphous materials at the inner wall of the vesicles and the canals (unlabeled arrows in Fig. 5) may suggest that the latter are the limiting membrane of the emptied granules. A wide distribution of similar vesicles and canals extending a considerable distance downwards further suggests the possible mode of granule secretion in which the membrane of each granule may fuse either with those of other granules or with the indentation of the apical plasma membrane, together forming a slender canal (Fig. 8).

The supposed mode of granule secretion was tested in the following way: The isolated frontal pieces of stage 28 embryos were immersed for 15 min. in 1% pilocarpine hydrochloride in Steinberg's solution (pH 7.3) to allow an accelerated secretion, and were submitted to the electron microscopical observation. The result presented in Fig. 9 shows that the contents of granules have fairly disappeared during the 15 min. treatment. The observations stated above, together with the other aspects of the cytoplasmic organelles as presented before, are thus interpreted to show that HGC in stages 28–34 can be called "mature" with respect to the activities of both the synthesis and the secretion of the apical granules.

General aspects of HGC at stage 36, a hatching stage, are not noticeably different from those in the preceding stage, with still prominent microvilli and a fairly large number of apical granules. Remarkable as contrasted with the preceding stage is a considerable loss of vesicles and canals in the apical region. This indicates that the secretory activity considerably declines, although the cellular synthetic machinery is still actively working.

At stage 39, a post-hatching stage, microvilli and the indentation of apical plasma membrane are less prominent than in the preceding stages. Apical granules are still in abundance, but without indication of their active secretion (Fig. 11). More remarkable changes are found in the cytoplasmic organelles basal to the subapical region. Mitochondria take an oval shape with their extremely electron-lucent matrix. Notable in the reduced Golgi area is the occurrence of membrane-bounded, relatively large bodies containing electron dense granules of unspecified nature. The cisternae of ER are less prominent than before, accompanied by a decrease of the membrane-attached and free ribosomes. Apparently there is a reduction of both the synthetic and secretory activities in HGC at stage 39.

The disintegration of these activities is much more pronounced at stages 42 and 46 (Fig. 14). The cell height reduces to about half that in the hatching stage. Protrusions of the apical plasma membrane, formerly microvilli, are infrequent. The membrane-limited, electron dense granules, formerly concentrated more apically, are distributed in the apical half of the cell without any indication of secretion. The Golgi complex is merely residual without intralamellar materials. Both rough and smooth ER as well as free ribosomes, are increasingly less abundant. Most prominent at these stages is the feature of a fairly large, membrane-bounded body which made its first appearance at stage 39, with pronounced development from stage 42 (Fig. 13). This body, delimited from the surrounding cytoplasm by a membrane, is located laterally or basally to the nucleus and its size is sometimes even larger than that of the nucleus. It usually contains several kinds of cytoplasmic elements such as several membranous bodies of unspecified nature, pigment granules, and even the fragments of yolk platelets and mitochondria. Quite possibly this body is functioning as sequestering the cytoplasmic organelles to lyze them. HGC at stage 42 and later is thus apparently under the process of disintegration.

Other epidermal cells during stages 22–46

CEC, the major epidermal cell, contains mucous vesicles and shows their active secretion at all the stages observed. Another epidermall cell, CC, was found to make an important change after the hatching stage (Fig. 12); at stage 39 there appear small vesicles in the apical cytoplasm between the basal bodies of cilia. At stage 42 and later, the vesicles develop to a size similar to those found in CEC, whereas cilia and mitochondria show a progressive degeneration. Thus CC shows a feature quite similar to that of CEC except for the presence of a residual number of cilia at the apical surface of the former.

Discussion

Several cytological features of HGC as presented above suggest an important participation of HGC in the hatching process. Most remarkable among these is

the development of the cellular organelles which are generally known to be the cytological machinery for protein synthesis and secretion mechanism. Most, if not all, of these organelles in HGC appear to be devoted for the formation and the secretion of the apically localized granules. The maximum secretory activity of the granules just prior to hatching also favors the supposed role of HGC. Further support for this view has been obtained in another line of studies in which the hatching enzyme activity was found exclusively in the culture of dorsal anterior parts of embryos, most of which contained HGC (Katagiri and Yoshizaki, unpublished). CEC may well be ruled out in this context, because of its distribution throughout the embryonic epidermis, its continued secretory activity independent of the hatching process, and of the evidence indicating the transformation of CC to CEC. Overall ultrastructural aspects thus allow us to conclude that the hatching enzyme is localized in the apical granules of HGC.

Epidermal cell obviously corresponding to HGC in the present study has been a subject of the light microscopical observations under the name, "dorsal gland" or "frontal gland" cell, with the suggestion of its possible participation in the hatching process. In *Xenopus*, Bles (1905) stressed the functional importance of the "frontal gland" in the hatching process, without cytological demonstration of the gland cell. The supposed "frontal gland" has been substantiated as HGC in the present observation. In view of the prominent function of this cell as discussed above, the name "hatching gland cell" seems preferable to "dorsal gland" or "frontal gland" cell.

According to a review by Yanai (1966) for the distribution of "frontal gland" or "dorsal gland" cells, there is a marked difference between urodelan and anuran embryos: In the former the cells bear the name because of their restricted localization at the frontal region, whereas in the latter their localization extends toward the posterior end along the mid-dorsal line in addition to the frontal region. In this respect *Xenopus* occupies an intermediate position between urodelans and other anurans.

The highly developed ER with typical cisternae found in HGC will receive a general acceptance as one of the characteristics of proteinous gland cells, *e.g.*, thyroid gland cells, exocrine cells of the pancreas, *etc.* (for a review, see Kurosumi, 1961). It should be mentioned that this prominent feature of ER is recognizable as early as at stage 22, when no apical granules are present. Similar precocious development of rough ER before the appearance of enzyme granule has been found in the differentiating HGC of *Oryzias latipes* (Yamamoto, 1963) and pancreatic cells (Wessells and Evans, 1968). That the cisternae are the site of accumulation of the synthesized proteins has been clearly established by the work of Siekevitz and Palade (1958). If, then, a marked extension of rough ER is taken as a criterion for the active synthesis of proteins, it may be said for HGC that a considerable high synthetic activity is maintained during stages 22–36. A frequent occurrence of electron dense materials in the Golgi vesicles and of granules in the Golgi area provides support for a generally accepted view of the role of Golgi

complex in packing the materials (Kurosumi, 1961; Caro and Palade, 1964; Beams and Kessel, 1968). Thus it is likely that the limiting membrane of the apical granules in HGC is derived from the membrane of the Golgi vesicles.

Striking features in the apical cytoplasm occurring just prior to hatching (Fig. 5) may well be interpreted in terms of the elaborate indentation of the apical plasma membrane in collaboration with the fusion of the membrane of several granules. Such a mechanism seems to have an advantage for the rapid release of granules than a discharge of individual granules, as postulated also in the case of the zymogen granule secretion in pancreatic cells (Ichikawa, 1965). In this respect the present observation differs from the observed mode of the enzyme release in *Oryzias* where contents of the granules are once squeezed out into the surrounding cytoplasm and the cytoplasm as a whole liberated into the mouth cavity (Yamamoto, 1963). Around the hatching stage of the present material, a sharp decline of the secretory activity with a slow decline of the synthetic activity seems to take place, thus causing a visual increase of the granules at stage 39. The secretion or presence of the enzyme after the hatching stage has also been observed in *Oryzias* (Yokoya, personal communication). The role of the enzyme secreted at the post-hatching stages cannot be explained in the present study.

The membrane-bounded body containing recognizable organelles in HGC of the post-hatching stages is similar to those found in several kinds of cells under the names of cytosegresomes (Ericsson, 1964), dense bodies (Novikoff *et al.*, 1956) and lysosomes (Farquhar, 1969). Similar structures named "inclusion bodies" occur in metamorphosing amphibian tail tissues (Weber, 1969). These bodies are generally interpreted to work in regulating the secretory process or destroying cells themselves. Though cytochemical tests for the localization of acid hydrolase or other enzymatic activities will be necessary for specifying the exact role of these bodies, it is tempting to suppose that they take part in cell degeneration by engulfing and disorganizing the cytoplasmic organelles and/or the ground cytoplasm. At any rate, the occurrence and the rapid growth of these bodies at the post-hatching stages should be regarded as an indication of a profound change in the cellular activity at these stages, and as an additional support for the proposed role of HGC in the hatching process.

The present study has given morphological evidence that the hatching enzyme is most probably produced in the particular type of cell which is cytologically and topographically unique among the epidermal cells of tailbud embryos. Of special interest in relation to the present observation is the recent success of the characterization of the hatching enzyme in *Xenopus* (Carroll and Hedrick, 1972). Thus it will be possible to ask from both morphological and biochemical sides of the question as to when and how the cytodifferentiation takes place in HGC of *Xenopus*. Studies along these lines are now in progress in other anuran, *Rana chensinensis*.

Summary

1) For the purpose of finding the source of the hatching enzyme at the cellular level in the South African clawed toad, *Xenopus laevis*, histological and electron microscopical studies were performed on the superficial epidermal cells of the stage 22–46 embryos.

2) Histological examination on the pre-hatching stage embryo revealed a large, bottle-shaped cell in the epidermis. This type of cell, referred to as the "hatching gland cell" (HGC), is distributed mostly in the frontal region, and to a lesser extent posteriorly along the dorsal mid-line to a level of the ear vesicles.

3) When observed under the electron microscope, HGC at the pre-hatching stage reveals a prominent characteristic which includes numerous microvilli in the apical surface, an apical accumulation of membrane-limited electron dense granules, highly developed Golgi complexes, an elaborate extension of rough endoplasmic reticulum (ER), and an abundance of ribosomes and glycogen.

4) HGC is first detectable at stage 22 by the presence of apical microvilli and development of rough ER. The apical granules make their first appearance at stage 24 and increase in number in succeeding stages. There are evidences to show that the granules are formed in the Golgi vesicles. The granules are mostly secreted out of the apical cell surface during stages 24–34. At stage 39 and afterwards, the cell size diminishes and there appears an extremely large, membrane-bounded structure which includes fragments of several cytoplasmic organelles, indicating the occurrence of cellular disintegration.

5) On the basis of these observations, the mode of formation and secretion of the apical granules was discussed. Discussion was further offered, emphasizing that the ultrastructural features in HGC should be regarded as a specific cytodifferentiation which enables prominent synthesis and secretion of the protein(s). It was concluded that HGC is a most reasonable candidate endowed with the production of the hatching enzyme.

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Explanation of Plates XV-XX

Abbreviations:

AG, apical granule
CEC, common epidermal cell
D, desmosome
ER, endoplasmic reticulum
G, Golgi complex
GL, Golgi lamella
HGC, hatching gland cell
L, lipid droplet
MB, membrane-bounded body
Mf, microfilament
Mt, mitochondrion
MV, mucous vesicle
N, nucleus
P, pigment granule
r, rootlet
rg, ribosome and glycogen
Y, yolk platelet

All figures except Fig. 13 are from specimens embedded in Epon 812.

Fig. 4. An electron micrograph of HGC at stage 34. Note the presence of microvilli (arrow) and apical granules (AG) in the pigment-free apical cytoplasm. Mitochondria (Mt), Golgi complex (G), and rough endoplasmic reticulum (ER) are prominent. Common epidermal cell with mucous vesicles (MV) to the left. × 8,500.

Fig. 5. Electron micrograph of an apical cytoplasm of HGC at stage 32, showing electron dense granules (AG) and various forms of vesicles and canals (unlabeled arrows) lined with electron dense materials. Note a wide variety in the shape of granules as compared with those in Fig. 11 (stage 39). × 23,700.

Fig. 6. Electron micrograph of a supranuclear region of HGC at stage 32, showing highly developed Golgi complexes (G). Note the presence of electron dense granules (arrows) and rough ER in close apposition to Golgi complexes. × 16,000.

Fig. 7. Electron micrograph of a supranuclear region of HGC at stage 32, showing the blebbing of the Golgi lamella (arrow) with electron dense materials. Rough ER takes a close juxtaposition to the Golgi lamellae (GL). × 33,700.

Fig. 8. Apical cytoplasm of HGC at stage 32. Note the protrusion of the limiting membrane of an apical granule (AG) toward the apical surface (arrow). × 40,000.

Fig. 9. Apical cytoplasm of HGC in stage 28 embryo after a short treatment with pilocarpine hydrochloride, showing the decrease in the electron density of apical granules

(AG) in the apical cytoplasm. Unlabeled arrows indicate the fusion of two granules. Part of the cilia cell with rootlets (r) is shown to the right. × 30,000.

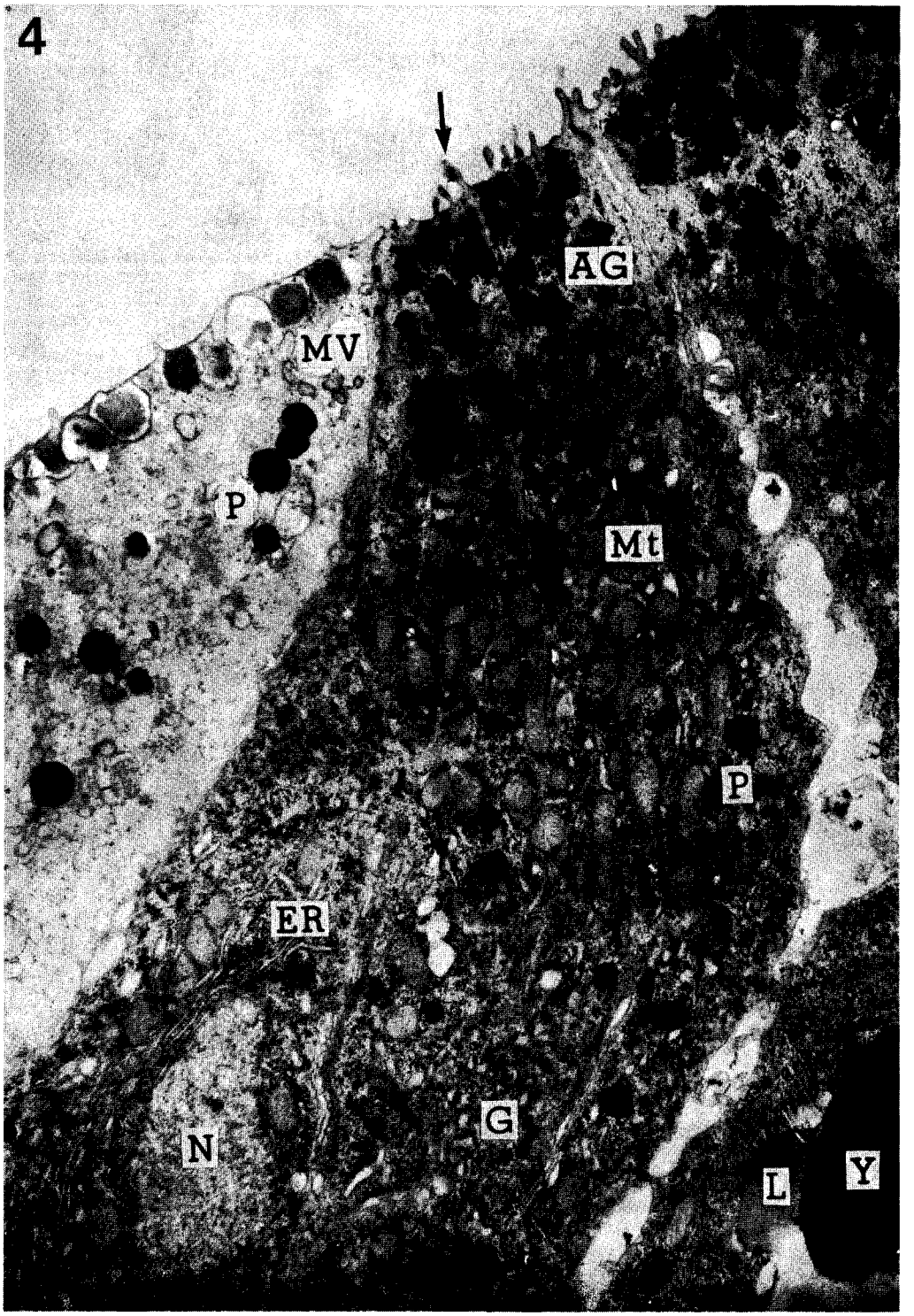
Fig. 10. Electron micrograph of HGC at stage 24, showing the first appearance of apical granules (AG) in the apical cytoplasm. × 23,000.

Fig. 11. Apical cytoplasm of HGC at stage 39, showing a good number of apical granules (AG) still present. Compare the shape of granules with those in Fig. 5 (stage 32). Note the absence of vesicles and canals which were prominent at stage 32. × 18,000.

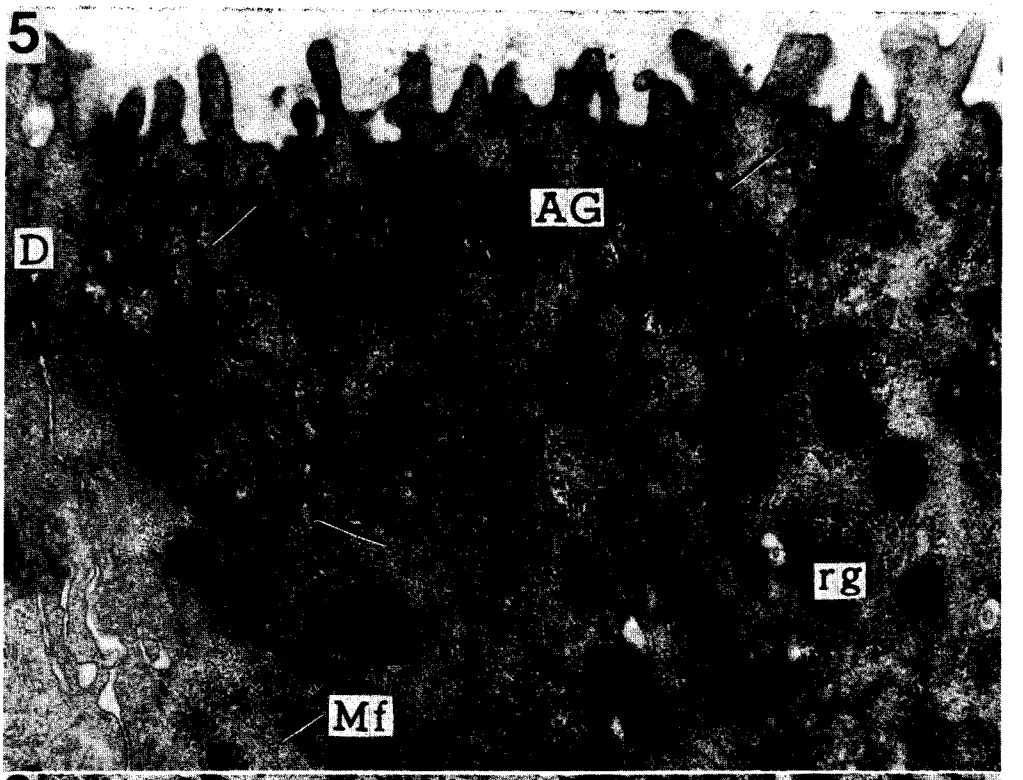
Fig. 12. Electron micrograph of an epidermal cell at stage 42 embryo, showing the transformation of cilia cell to common epidermal cell. Horizontal section of a cilium is shown to the left. Note the disintegration of mitochondria (arrows). × 16,500.

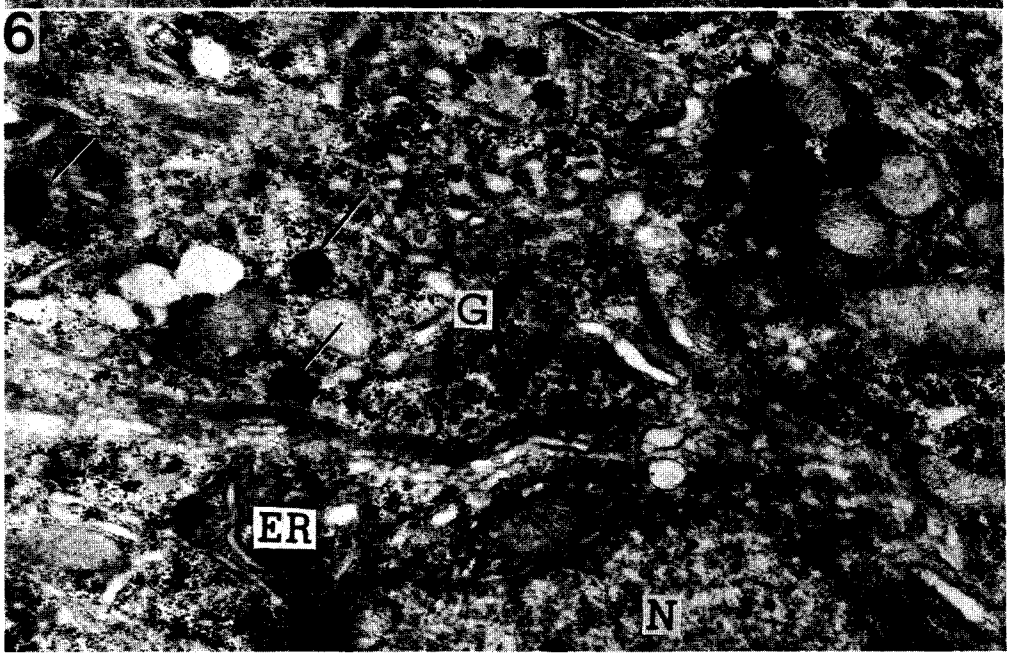
Fig. 13. Electron micrograph of a basal cytoplasm of HGC at stage 42, showing a fairly large, membrane-bounded body (MB). Note yolk platelets (Y) and pigment granule (P) in this body. Embedded in styrene-(n-)butylmethacrylate, stained with uranyl acetate and potassium permanganate. × 10,600.

Fig. 14. Electron micrograph of HGC at stage 46, showing undischarged granules (AG), residual Golgi complex (G) and ER. Unlabeled arrows indicate part of the membrane-bounded body. × 11,200.



N. Yoshizaki: Hatching Gland Cell in Xenopus





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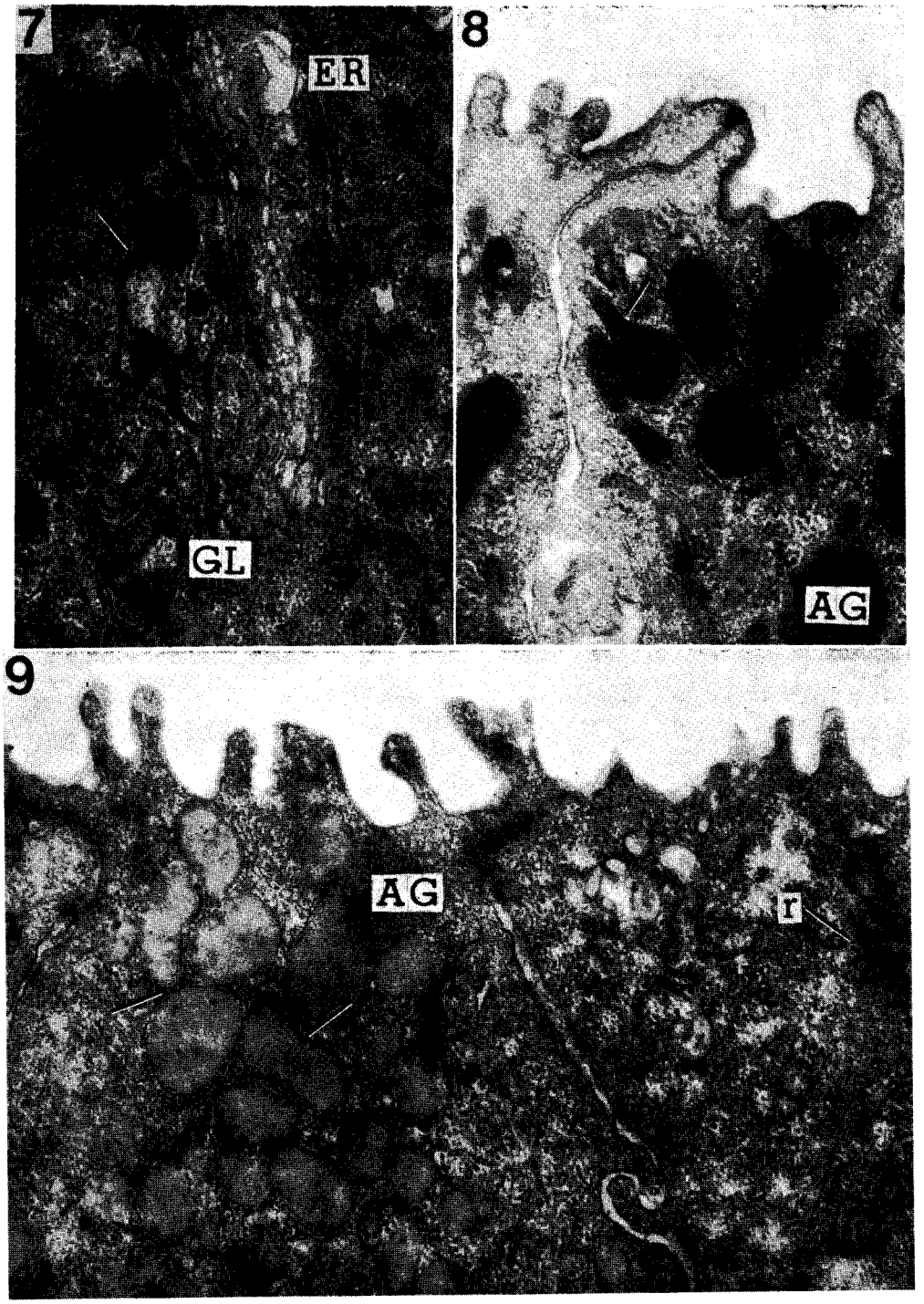

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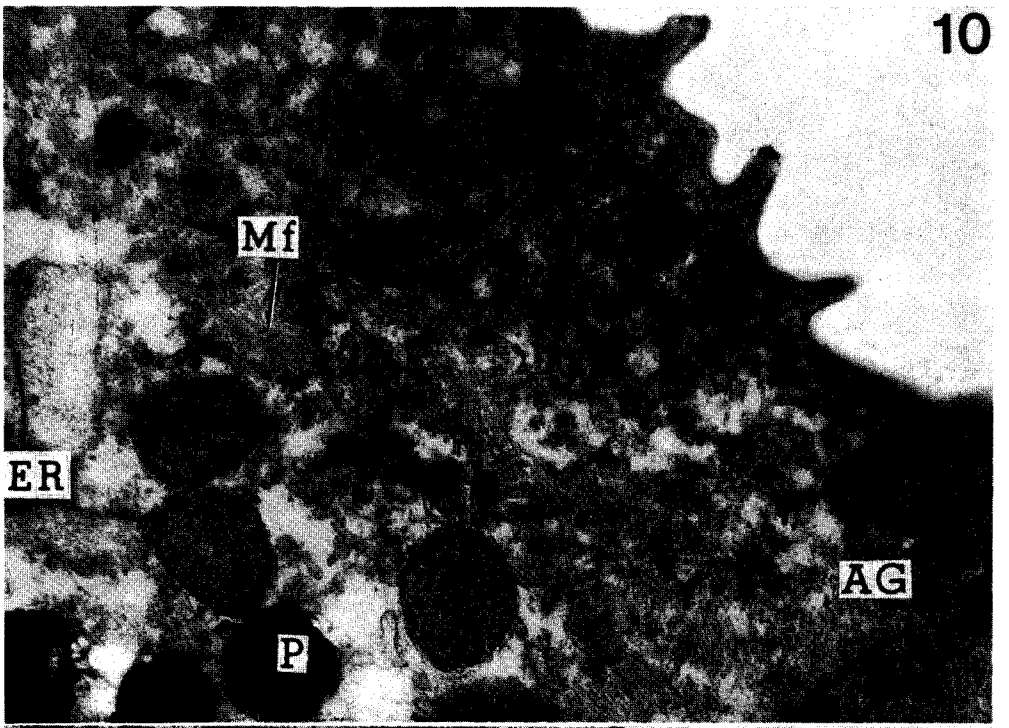

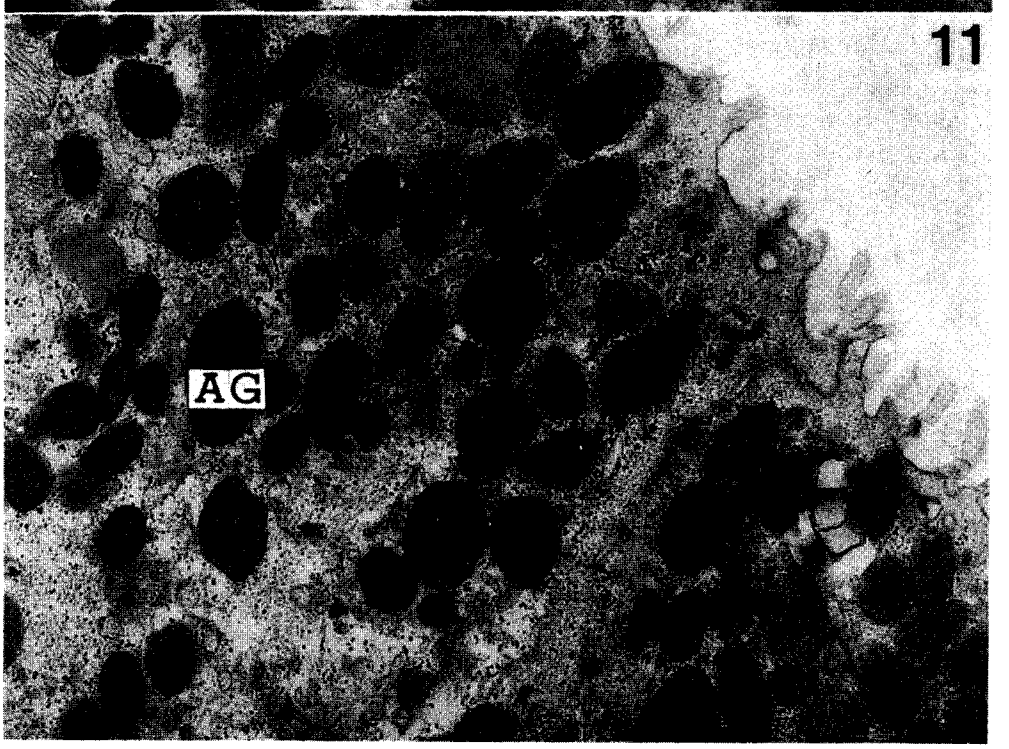

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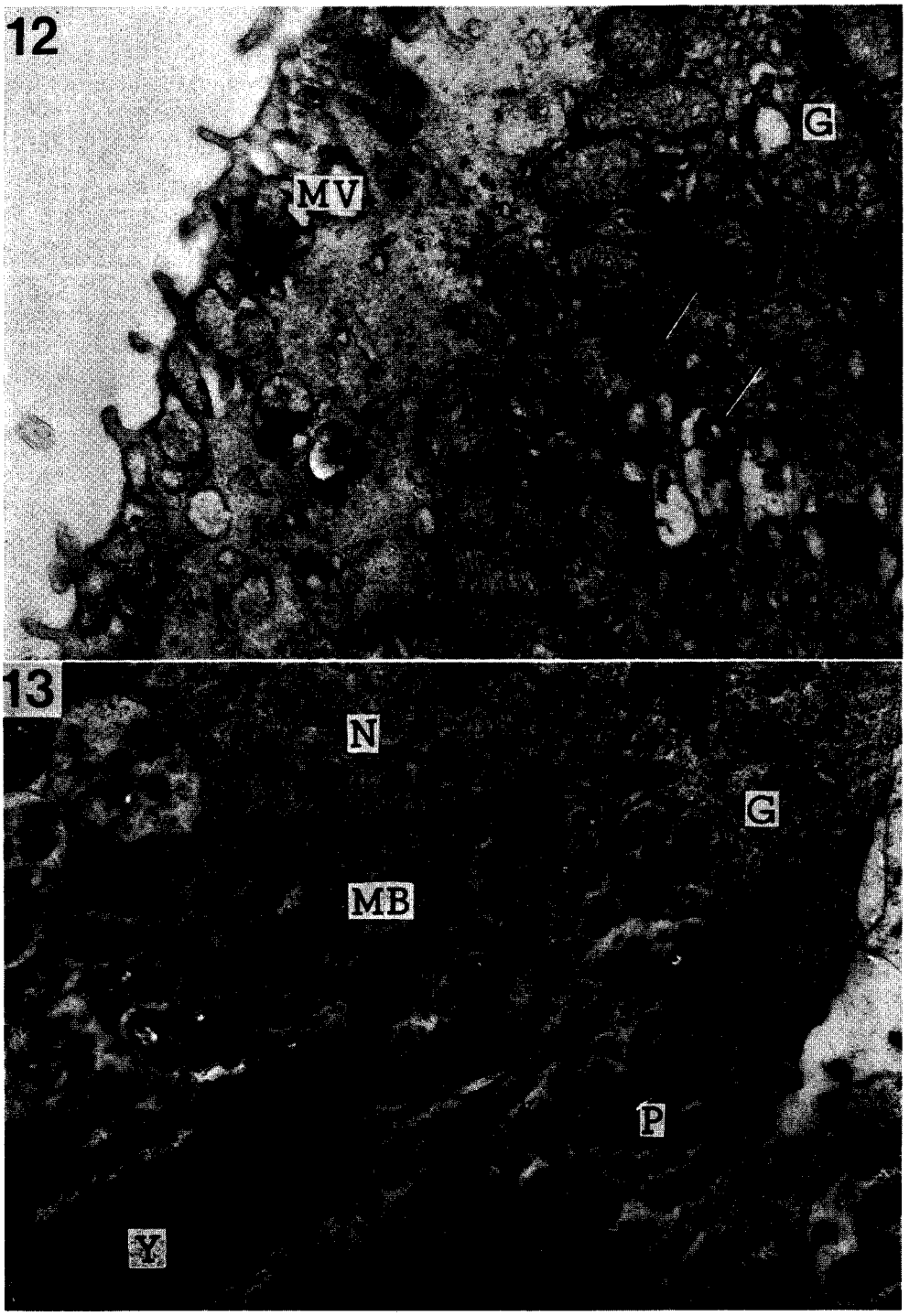

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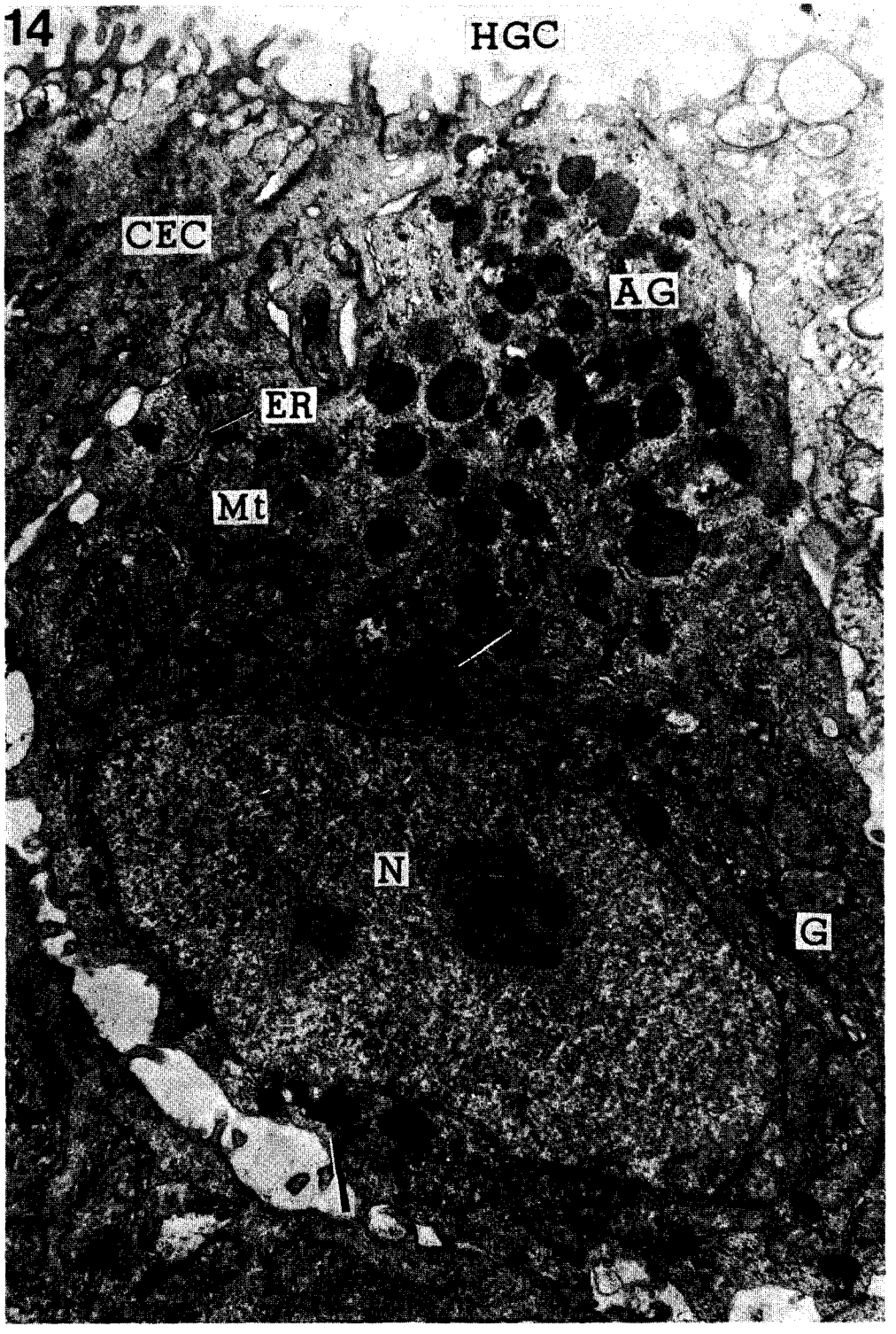

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