Acta anat. *104:* 61–71 (1979)

Evolution and nature of the dense bodies in the chicken pinealocytes

J. Calvo and J. Boya

Department of Histology and General Embryology, Faculty of Medicine, University Complutense, Madrid, Spain

Key words. Pineal · Evolution · Dense bodies · Acid phosphatase · Histochemistry

Abstract. The acid phosphatase reaction, applied to light and electron microscopy, was studied in the chicken pineal gland from the moment of hatching until 2 months of age. From the moment of hatching there is a great amount of acid phosphatase, which is mainly found in the vicinity of the lumen of both the recess and large follicles. Acid phosphatase is poor in the parafollicular layer. From day 30 onwards, there is an obvious fragmentation of the recess and of large follicles. Also, the parafollicular layer differentiates to form new follicles. The dense polymorphous bodies of the B pinealocytes are ultrastructurally identified as lysosomes.

Introduction

The chicken pineal gland parenchyma presents two cellular types named A and B pinealocytes [*Boya and Zamorano,* 1975; *Boya and Calvo,* 1977a, b; *Calvo and Boya,* 1977a, b]. One of the most distinctive ultrastructural characteristics of the B pinealocytes is what we have named the 'polymorphous dense bodies' [*Boya and Calvo,* 1977a, b; *Calvo and Boya,* 1977a, b]. These structures appear very early in the embryonic development (14–15 days of incubation) [*Calvo and Boya,* 1977a, b] and they remain through post-hatching life until the most advanced ages we have studied (13 months) [*Boya and Calvo,* 1977a, b]. From an ultrastructural standpoint, polymorphous dense bodies are very similar to the lysosomes described in other cell types; however, in order to identify a cytoplasmic organelle as a lysosome, it is necessary to prove that it contains acid hydrolases.

Histochemical studies have been done in different mammal pineals [*Niemi and Ikoken,* 1960; *Miller and Palade,* 1964; *Gusek and Buss,* 1966; *Arstila,* 1967; *Bostelman,* 1969; *Vollrath and Schmidt,* 1969; *Botticcelli et al.,* 1972; *Devecerski,* 1972a, b; *Tapp et al.,* 1973]. With respect to birds, *Wight and McKenzie* [1971] have studied the chicken pineal gland with histochemical techniques using the light microscope. They demonstrated the existence of acid hydrolases in such an amount and placed in such a way that the localization of these hydrolases in the polymorphous dense bodies of the B pinealocytes is suggested.

The definite proof of the lysosomal nature of these polymorphous dense bodies may only be furnished applying the histochemical technique for acid hydrolases to the electron microscope.

During the post-hatching life, the chicken pineal gland undergoes a series of important changes in its structural pinealocytes around the cavities and the

formation of new lumens [*Boya and Calvo,* 1977a, b]. This means that, in the adult pineal, practically all the pinealocytes are in contact with a lumen. Morphologically, this may be seen as the appearance of cellular 'rosettes' in the parafollicular layer [*Boya and Calvo,* 1977a, b]. The polymorphous dense bodies are always located in the apical cytoplasm of the B pinealocytes in the vicinity of the lumen. Therefore, their histochemical demonstration may also be a morphological indication of this structural evolution since the light microscope shows a progressive number of polymorphous dense body 'rosettes' in the parafollicular layer as the animal grows older.

Materials and methods

Chicken *(Gallus gallus)* pineals have been used for our study. The chickens were maintained under natural conditions of light and feeding. The pineals were taken at intervals of 5 days from the moment of hatching until 2 months of age.

They were immediately fixed by immersion in 0.1 *M* cacodylate-buffered 3% glutaraldehyde, pH 7.4, at 4 °C. After 2 h of fixation, the samples were washed three times in cacodylate buffer at 4 °C.

Among the lysosomal acid hydrolases, the acid phosphatase was chosen for being one of the best known and for being present in all lysosomes. For the demonstration of acid phosphatase, frozen sections or thin sheets of tissue were incubated with *Miller and Palade*'s [1964] modification of the Gomori medium. The incubation was done at 37 °C during 40–45 min. Controls were done in a substrate-free medium or adding 0.01 *M* sodium fluoride as inhibitor. After the incubation, the sections were reduced in ammonium sulphite for study with the electron microscope. In part of the sections, a slight nuclear stain was applied, so that the product of reaction would be more easily localized. For the ultrastructural study, the tissues (unreduced) were washed in cacodylate buffer with 7.5% of saccharose. They were post-fixed in cacodylate-buffered 1% osmium tetroxide, then embedded in Vestopal W and sectioned with an LKB ultramicrotome. An EM 201 Philips electron microscope was used for the study.

For each time interval, unstained thin sections were studied in order to verify the specificity of the reaction, as well as sections stained with uranyl acetate and lead citrate.

Results

Light microscopy

The chicken pineal gland shows an intense acid phosphatase activity in all the age intervals studied. This activity, demonstrated with the modification of *Miller and Palade* [1964] to the Gomori technique, appears under the light microscope in the form of granules whose size and distribution vary along the period of age studied. The specificity of reaction is proven by its granular aspect as well as by the absence of activity in those sections incubated without substrate or in the presence of sodium fluoride.

Shortly after hatching, most of the lysosomes locate themselves in the follicular layer. Their position clearly defines the placement of the recess (fig. 1) and of the large pineal follicles. The granules are small and abundant and they place themselves at the same height forming an intensely stained band in the immediate vicinity of the follicular lumen (fig. 2). In the unstained sections there remains a broad clear region without lysosomes between this periluminal band and the parafollicular layer (fig. 1, 2). This region corresponds to the nuclei of the follicular cells. This is proven by the sections in which nuclear contrast was used. Therefore, the lysosomes of the follicular layer locate themselves exclusively in the apical cytoplasm of the follicular cells. Shortly after hatching, the parafollicular layer of the chicken pineal presents small lysosomes. They are not very abundant and they appear scattered in an almost homogeneous fashion in this parafollicular layer (fig. 1, 2).

During the first month of life, there are little changes in the number and arrangement of lysosomes in the chicken pineal gland. With the increase of age, we find a progressive fragmentation of the periluminal lysosomal bands of the follicular layers. This fragmentation is

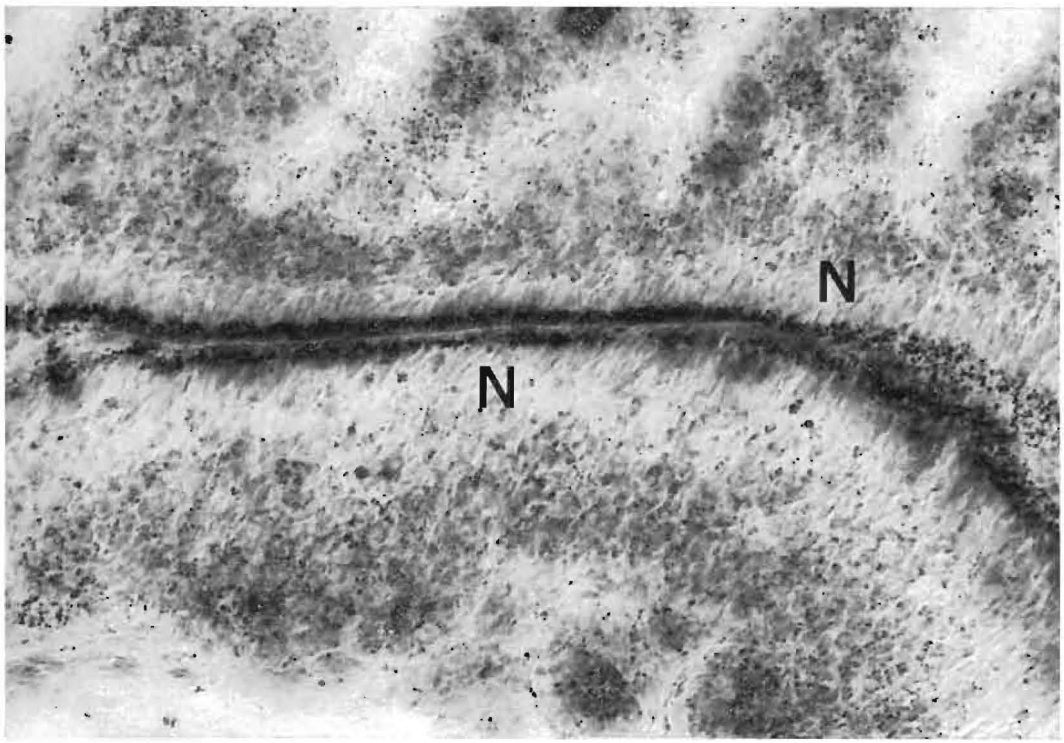


Fig. 1. 5-day chicken. Pineal recess. Note the lysosomes placed very near the lumen of the recess due to their supranuclear location in the cells of the follicular layer. The clear band (N), negative to the acid phosphatase reaction. corresponds to the position of the nuclei.

very evident in the pineal recess (fig. 3). Moreover, although the parafollicular layers become more and more broad, the number and distribution of lysosomes in them is very similar to that described shortly after hatching. However, changes do begin to appear near the end of the first month. In chickens of 20–30 days, we clearly observe localized clusters of lysosomes in the parafollicular layer (fig. 4).

From the first month after hatching onwards, we find important changes in the lysosomal pattern of the chicken pineal gland. The recess is totally fragmented by this time. However, it may still be identified in the acid-phosphatase-incubated sections, since the place is occupied in previous stages is now occupied by a series of irregular cavities. The cavities are very close to each other and they are bordered by lysosomal periluminal bands. These cavities remind us of the previous recess since they remain in a vague linear arrangement. This fragmentation and disorganization of the lysosomal periluminal bands may also be observed in many of the large follicular cavities (fig. 5). In those follicles that still show an orderly follicular layer, the lysosomes tend to locate themselves at different heights. Due to this disposition, the periluminal bands

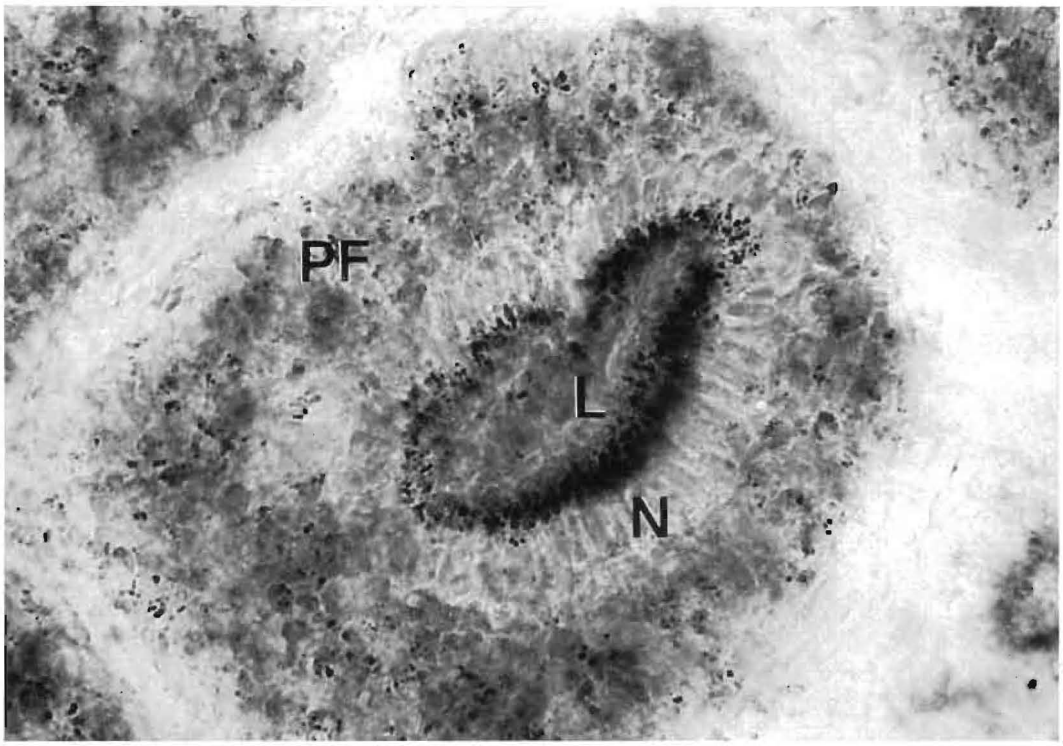


Fig. 2. 5-day chicken. Pineal follicle whose lumen (L) is limited by the lysosomal band of the pinealocytes of its follicular layer. The nuclei are located outward of it (N). Note the scarcity and disorganization of the lysosomes in the parafollicular layer (PF).

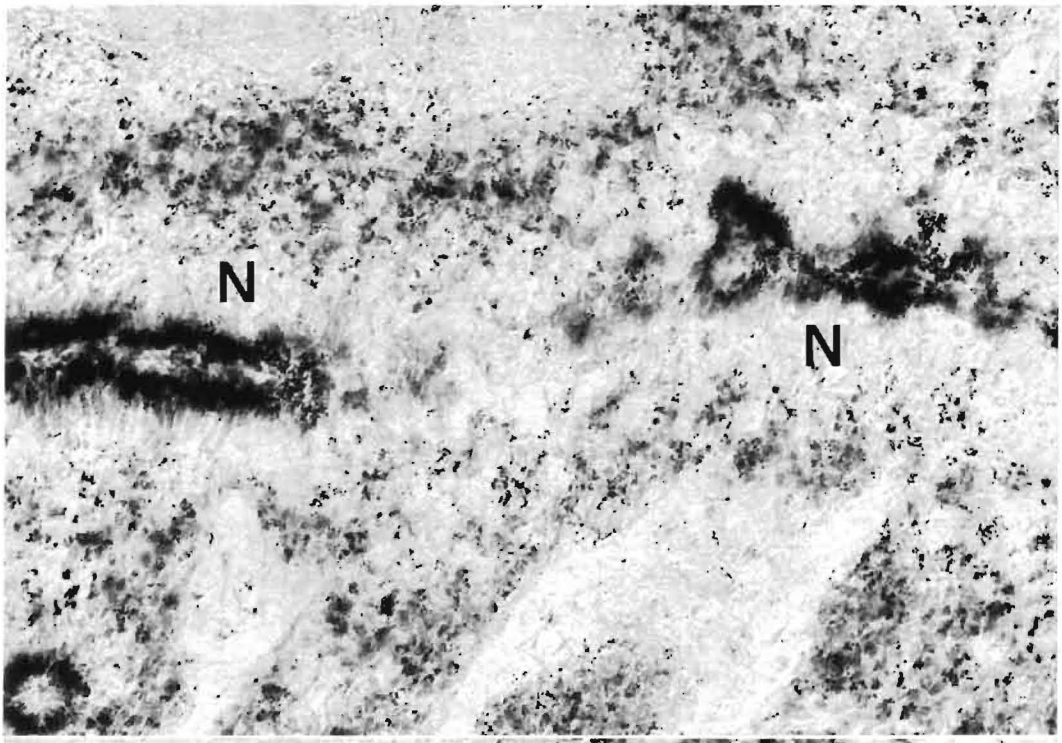
tend to be broader, having a less clear outline than in previous stages. However, in 60-day-old chicken pineals, follicles with a narrow, orderly, periluminal band may still be found.

The lysosomes of the follicular layer still situate themselves in the apical cytoplasm of the follicular cells. From the first month of age onwards, we observe a slight increase in the amount and size of lysosomes in the follicular layer.

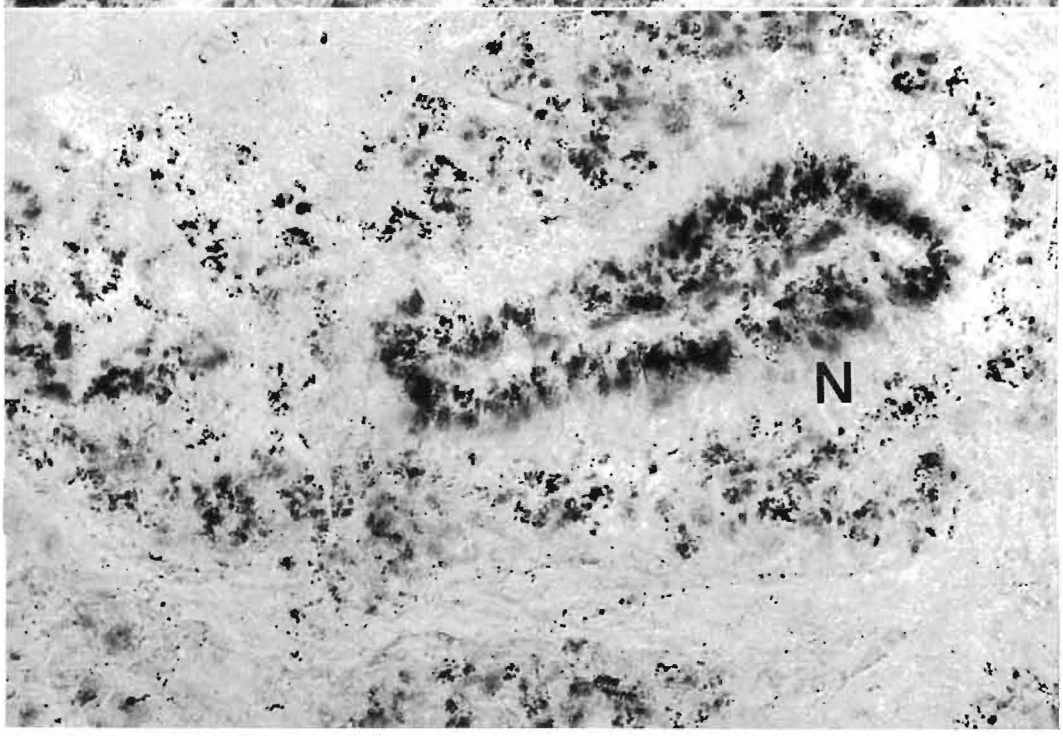
The most evident changes in the lysosomal pattern after the first month of age may be found in the parafollicular layer. With increasing age, the lysosomes in this layer regroup themselves in a circular arrangement forming lysosomal 'rosettes' (fig. 5, 6). Progressively, the lysosomes in each 'rosette' become further organized adopting an aspect that reminds us of the periluminal bands described in the follicular layer, although having a lesser degree of arrangement. In the centre of the 'rosettes', we find a badly limited area free

Fig. 3. 30-day chicken. Fragmentation of the pineal recess lumen, which is limited by the periluminal band of lysosomes. N = Nuclear band of the follicular pinealocytes.

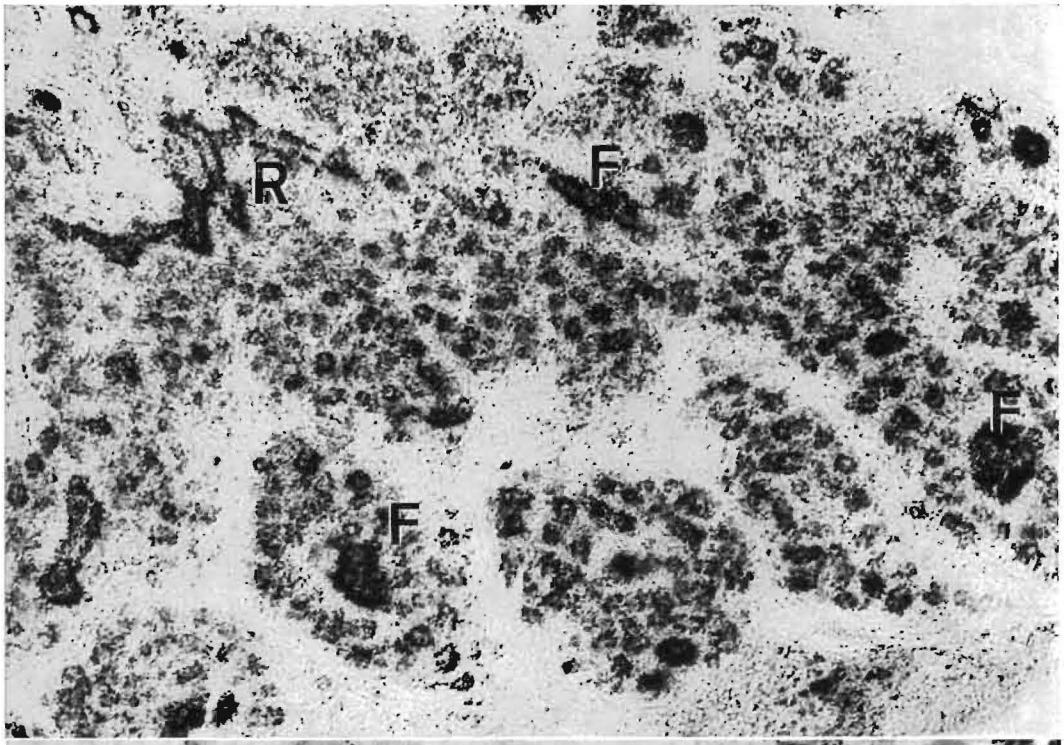
Fig. 4. 30-day chicken. Pineal foillcle that presents localized clusters of lysosomes in its parafollicular layer. N = Nuclear band of the follicular layer.



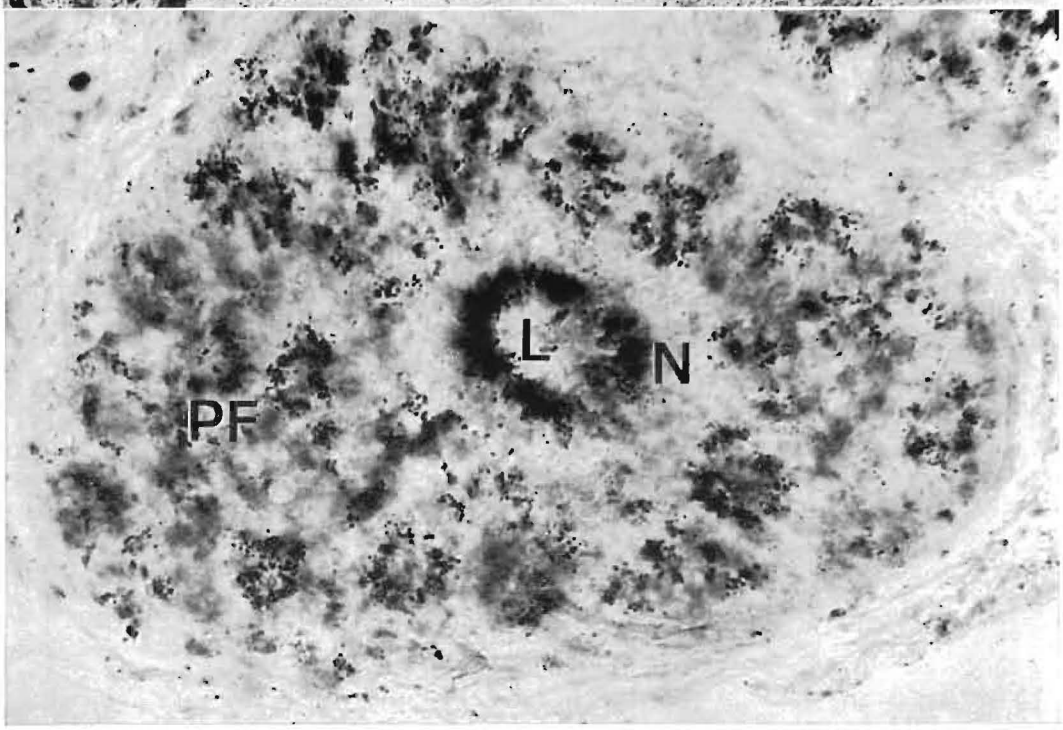
3



4



5



6

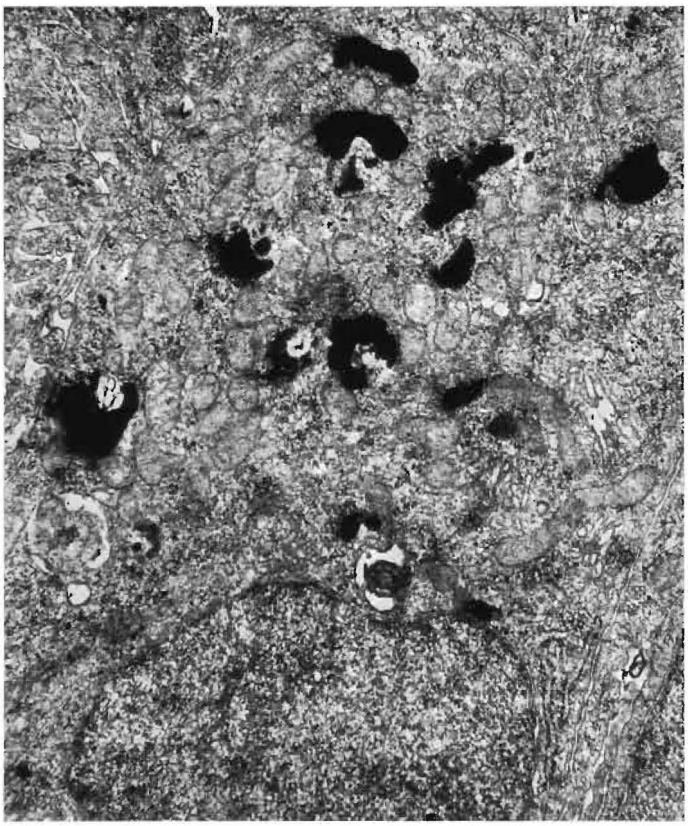


Fig. 7. 30-daychicken. B pinealocyte. Polymorphous dense bodies in supranuclear location showing positive acid phosphatase reaction.

of lysosomes (fig. 6). This region is more evident in the 'rosettes' having a larger size. In the nuclear contrasted sections we may observe the existence of nuclei situated around and outside the lysosomal 'rosette'. Finally, the increase in the size and number of lysosomes in the follicular layer is even more evident in the parafollicular layer.

Electron microscopy

The electron-microscopic study sections incubated for acid phosphatase unequivocally show the location of the reaction product on the polymorphous dense bodies of the B pinealocytes (fig. 8–10). These polymorphous dense bodies have been described previously with routine techniques [*Boya and Calvo,* 1977a, b; *Calvo and Boya,* 1977a, b]. In the unstained sections, the lead phosphate appears only on the polymorphous dense bodies and

Fig. 5. 60-day chicken. Intense fragmentation of the pineal recess (R) and of the large follicular lumens (F). Abundant lysosomal 'rosettes' in the parafollicular layers.

Fig. 6. 60-day chicken. Follicle with a small lumen limited by the periluminal lysosomal band. The parafollicular (PF) layer shows abundant lysosomes arranged in 'rosettes'. N = Nuclear band of the follicular layer.

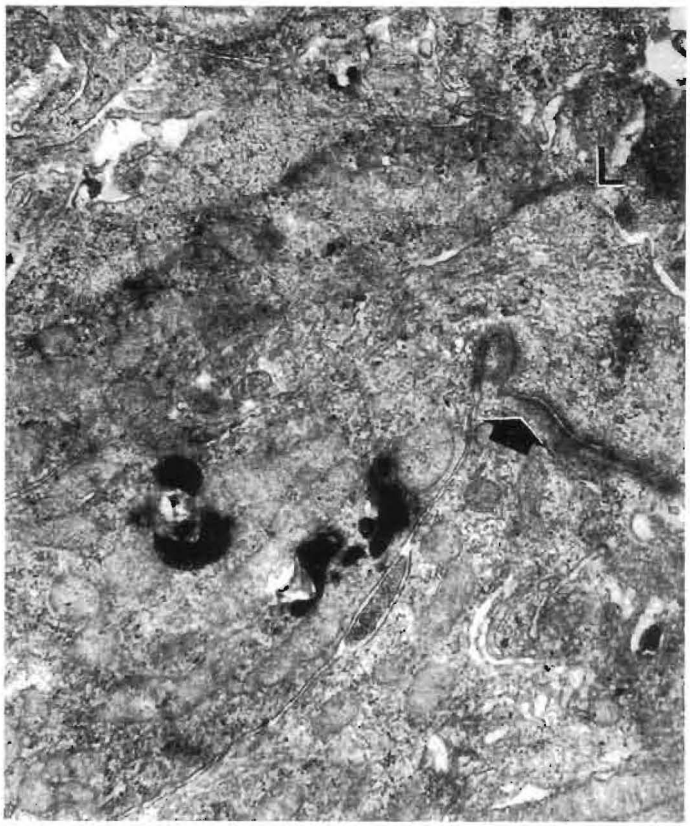


Fig. 8. 30-day chicken. Apical border of a B pinealocyte that marks the limit of a lumen (L) in formation and displays the existence of polymorphous dense bodies with a positive acid phosphatase reaction. Arrow indicates junction mechanisms.

occasionally on the Golgi system (fig. 9, 10). In most of the polymorphous dense bodies, the reaction product appears in a homogeneous granular form occupying the whole interior of the particle (fig. 7, 8, 10). In others, however, the lead phosphate appears in the form of big granules in the interior of the particle, while some areas appear unstained. The content of these polymorphous dense bodies, therefore, adopts an heterogeneous aspect. On rare occasions, we have found polymorphous dense bodies that do not present the reaction product.

In the control sections, incubated in the absence of substrate or with sodium fluoride as inhibitor, the polymorphous dense bodies lack any type of precipitate. They present a similar density to that observed with routine techniques.

In spite of the normal density of these structures, the reaction product of the acid phosphatase is perfectly visible even in the uranyl-acetate- and lead-citrate-stained sections. It appears as a granular electron-opaque precipitate located over the polymorphous dense bodies (fig. 7, 8).

The histochemical technique for acid phosphatase applied to the electron microscope adds little new information about the amount, size, location and evolution of the pinealocyte lysosomes – in relation to that described with the light microscope. Furthermore, these find-

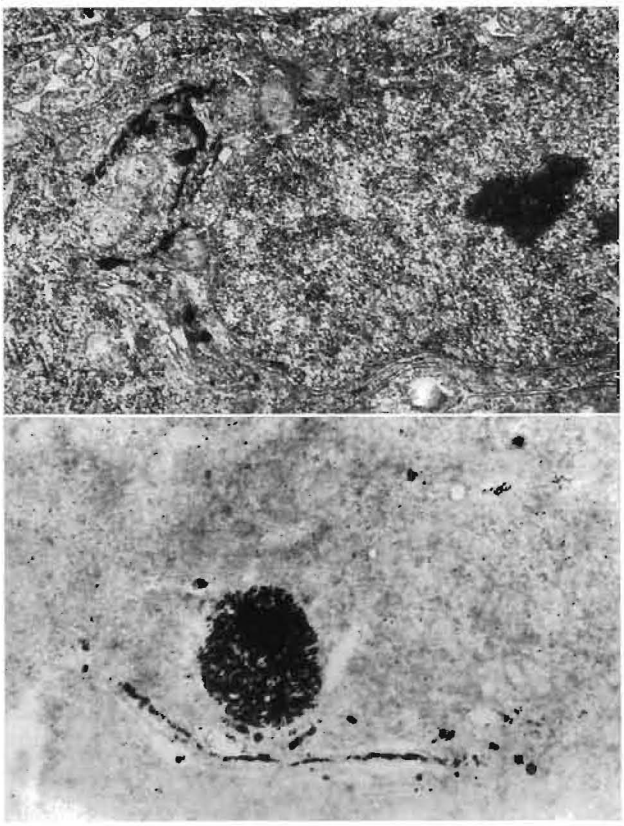


Fig. 9. 30-day chicken. B pinealocyte. Positive acid phosphatase reaction in the cisterns and in some of the vesicles of the Golgi system.

Fig. 10. 5-day chicken. Supranuclear portion of an unstained B pinealocyte. Note the granular deposit of lead phosphate over a dense body (lysosome) and over an inner cistern of a Golgi system, as well as in some of the microvesicles.

ings have already been described [*Boya and Calvo,* 1977a, b; *Calvo and Boya,* 1977a, b], since the polymorphous dense bodies are an ultrastructural component of the B pinealocytes visible with routine techniques.

The electron microscope serves to identify the lysosomes observed with the light microscope as the polymorphous dense bodies of the B pinealocytes, as well as to demonstrate that the lysosomal 'rosettes' described with the light microscope in the parafollicular layer correspond to follicular cavities in formation. These cavities in formation are surrounded by radially arranged pinealocytes A and B. The location of the polymorphous dense bodies in the apical cytoplasm of the B pinealocytes near the lumen in formation gives place to the 'rosette' image observed with the light microscope. Furthermore, the electron microscope confirms all the findings described with the light microscope about the evolution of the chicken pineal lysosomes from hatching until 2 months of age.

On many occasions we have found the reaction product located over the Golgi system of the B pinealocytes (fig. 9, 10) as well as over the small vesicle of its vicinity. In many of the cells the reaction product is located only on the innermost cistern of the Golgi system, although it is not rare to find several stained cisterns. No other pinealocyte B component presents reaction product.

Discussion

The existence of acid phosphatase activity in the chicken pineal gland has been previously described by *Wight and McKenzie* [1971] in young animals (8 weeks old) and in adults (6–9 months old). Our results confirm the existence of this enzyme in the chicken pineal from the moment of hatching until 2 months of age as well as in adults [unpublished]. The acid phosphatase histochemical technique for acid phosphatase applied to the electron microscope shows the location of this enzyme on the polymorphous dense bodies of the B pinealocytes described previously with the electron microscope [*Boya and Calvo,* 1977a, b; *Calvo and Boya,* 1977a, b]. Therefore, these dense bodies must be considered as lysosomes due to their content in acid phosphatase. The demonstration of the presence of other acid hydrolases as well as the enzymatic characterization of these dense bodies will be the object of further studies.

The polymorphous dense bodies appear during the embryonic development (14–15 days) [*Calvo and Boya,* 1977a, b] and they remain until the most advanced ages studied by us (13 months) [*Boya and Calvo,* 1977a, b]. The histochemical demonstration of these dense bodies during the embryonic development as well as in adult animals [unpublished] will be the object of further investigation.

The acid phosphatase histochemical technique enables us to show the existence of an evolution process in the amount, size, and location of the enzymes in the chicken pineal. In the study of *Wight and McKenzie* [1971] there are no variations described in the lysosomal pattern between young and adult animals. According to these authors, lysosomes are located in the apical cytoplasm of the ependymocytes (which corresponds to our follicular layer) and diffusely in the hypendemocytes (which corresponds to our parafollicular layer) in both age intervals. Our results show that, during the first 2 months of life, a progressive fragmentation in the periluminal lysosomal bands of the parafollicular layer takes place. This produces the disorganization of the recess and of the large follicular lumens. At the same time, the lysosomes of the parafollicular layer arrange themselves in circular formations (lysosomal 'rosettes'). This evolution, described previously by us using the light and electron microscope with routine techniques [*Boya and Calvo,* 1977a, b; *Calvo and Boya,* 1977a, b], is now confirmed by means of histochemical techniques.

The appearance of lysosomal 'rosettes' in the parafollicular layer is the consequence of the new follicular lumen formation, around which the pinealocytes are radially arranged. Although the follicular lumens in formation begin to be visible with the electron microscope in late embryos [*Calvo and Boya,* 1977a, b], the lysosomal 'rosettes' are visible with the light microscope only after the first month following hatching. The lumens in formation are integrated by cellular processes of parafollicular pinealocytes that meet at a point in which they present junctional mechanisms. With increasing age, the lumen becomes more definitive and the pinealocytes orient themselves around it. This lumen is, therefore, surrounded by organoid-rich broad cytoplasm in which the polymorphous dense bodies arranged radially with respect to the lumen appear in the form of lysosomal 'rosettes' under the light microscope. In previous stages, the polymorphous dense bodies were also located in the vicinity of the lumen in a radial arrangement; however, being more scarce and less organized, they did not stand out from the rest of the lysosomes.

This explains the absence of lysosomal 'rosettes'. Study with the electron microscope of the follicular lumens in formation, using routine or acid phosphatase techniques, nevertheless shows a constant relationship between the polymorphous dense bodies or lysosomes and the lumens from the beginning of their formation.

Using the electron microscope, we have frequently found the location of the reaction product of acid phosphatase over the Golgi system. The lead phosphatase precipitate is located over the innermost cistern or cisterns of the system as well as over small vesicles associated with the inner surface. This location has often been described with the electron microscope for other enzymes. According to many authors, this location in the inner cisterns is due to the greater concentration of the secretion product in the inner most cisterns of the Golgi system. This would produce sufficient contrast as to be seen with the electron microscope. In our findings, the location of acid phosphatase in the Golgi system of the B pinealocytes becomes a proof of the origin of the polymorphous dense bodies in this system.

References

Arstila, A.U.: Electron microscopic studies on the structure and histochemistry of the pineal gland of the rat. Neuroendocrinology *2:* suppl., pp. 1–101 (1967).

Bostelman, W.: Submicroscopic and enzyme histochemical changes in rat pineal body during puberty. Acta biol. med. germ. *22:* 151–157 (1969).

Botticcelli, A.; Trentini, G.P.; Bartolini, G., and De Gaetani, C.F.: Histoenzymology of the 'circumventricular organs' of the rat. V. The oxidoreductases, the transferases and the hydrolases of the pineal gland. Acta histochem. *44:* 288–299 (1972).

Boya, J. and Calvo, J.: Post-hatching evolution of the pineal gland of the chicken. Acta anat. (in press, 1977a).

Boya, J. and Calvo, J.: Ultrastructural study of the post-hatching evolution of the pineal gland of the chicken *(Gallus gallus)*. Acta anat. (in press, 1977b).

Boya, J. and Zamorano, L.: Ultrastructural study of the pineal gland of the chicken *(Gallus gallus)*. Acta anat. *92:* 202–226 (1975).

Calvo, J. and Boya, J.: Embryonic development of the pineal gland of the chicken. Acta anat. (in press, 1977a).

Calvo, J. and Boya, J.: Ultrastructural study of the embryonic development of the pineal gland of the chicken. Acta anat. (in press, 1977b).

Devecerski, V.: Contribution histoenzymologique à l'étude du métabolisme de la glande pinéale chez la ratte gravide. C.r. Séanc. Soc. Biol. *166:* 731–732 (1972a).

Devecerski, V.: Etude histoenzymologique de la glande pinéale du rat en adaptation au froid. C.r. Séanc. Soc. Biol. *166:* 970–972 (1972b).

Gusek, W. and Buss, H.: Morphological and histochemical changes in the pineal gland under the influence of prolan and following ovariectomy. Frankf. Z. Path. *75:* 172–186 (1966).

Miller, F. and Palade, G.E.: Lytic activities in renal protein absorption droplets. An electron microscopical cytochemical study. J. Cell Biol. *23:* 519–552 (1964).

Niemi, M. and Ikoken, M.: Histochemical evidence of aminopeptidase activity in the rat pineal gland. Nature, Lond. *185:* 928 (1960).

Tapp, E.; Huxley, M., and Davies, S.: The enzyme histochemistry of the pineal gland in rats. Histochem. J. *5:* 49–56 (1973).

Vollrath, L. and Schmidt, D.S.: Enzyme histochemical studies on the pineal gland of normal and pregnant guinea pigs. Histochemie *20:* 328–337 (1969).

Wight, P.A.L. and McKenzie, G.M.: The histochemistry of the pineal gland of the domestic fowl. J. Anat. *108:* 261–273 (1971).

Received: January 11, 1978

J. Calvo, Department of Histology and General Embryology, Faculty of Medicine, University Complutense, Madrid (Spain)