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Calcium concretions in the pineal gland of aged rats: an ultrastructural and microanalytical study of their biogenesis

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Abstract. The genesis of calcium concretions in aged rats was studied by means of transmission and scanning electron microscopy. The potassium pyroantimonate method, combined with X-ray microanalysis, allowed us to study the distribution of cations and calcium. Notable accumulations of calcium (associated with phosphorus) were localized in vesicles, vacuoles, lipid droplets, lipopigments, and mitochondria of dark pinealocytes. The results obtained in the present investigation suggest that these organelles are involved in the genesis of the concretions. The presence of sulfur indicates the existence of an organic matrix. We propose that genesis takes place in dark pinealocytes, which contain more calcium than light pinealocytes. Mineralization foci are sometimes associated with cellular debris and enlarge by further apposition of material. Two types of concretions, as determined by electron microscopy and confirmed by electron diffraction, could be observed: the "amorphous" type with concentric layers and the crystalline type with needle-shaped crystals. Once formed, the concretions reach the extracellular space and the cell breaks down. Possible extracellular calcification is suggested in the extracellular calcium-rich floculent material. The mineralization process is interpreted as being an age-related phenomenon and mainly a consequence of the degeneration of pinealocytes.

Key words: Pineal gland – Aging – X-ray microanalysis – Calcium concretions – Rat (Wistar)

Introduction

Calcium concretions have been reported in the pineal gland of several mammalian species and exhibit large individual and interspecific differences (Bargmann 1943; Wildi and Frauchiger 1965; Quay 1974; Japha et al. 1976; Vollrath 1981). Among mammals, they are especially found in Mongolian gerbils and in humans (Krstić and Golaz 1972; Japha et al. 1976; Lukaszyk and Reiter 1975; Reiter et al. 1976; Welsh and Reiter 1978; Krstić 1986; Welsh 1984, 1985). In Mongolian gerbils, the number of concretions grows according to the animal's social stress (Heinzeller 1985) and seems to be seasonally dependent (Trentini et al. 1986). These concretions, the formation of which has been reported to be related to a secretory process (Lukaszyk and Reiter 1975) are of intracellular origin in this species and reach the extracellular space after the death of the cell.

In humans, the presence of these concretions, called "brain sand" or acervuli, is apparently age-dependent only. They are present in children and their number increases with age (Heidel 1965; Daramola and Oloru 1972).

Concretions were previously thought to be absent in rats (Erdinç 1977), but they have been reported in the pineal gland of 90% of middle-aged rats (Diehl 1978). Concretions have also been found in the pineal arachnoid of 4 to 6-month-old rats (Vigh et al. 1989) and in the pineal and arachnoid of the mink (Vigh and Vigh-Teichmann 1992). Their presence in aged rats has been confirmed, and seems to be related to the calcium content of the pineal gland. This is understood as a possible sign of a degenerative and/or aging state (Humbert and Pévet 1991).

To date, little is known about the origin of pineal concretions. The present study was thus focused on the genesis and the possible mechanisms of formation and mineralization of these structures by means of electron microscopy and X-ray microanalysis.

Materials and methods

Animals

Five 12-month-old and five 28-month-old Wistar rats (Centre Depré, St. Doulchard, France) were used. They were fed and watered ad libitum under normal laboratory conditions (artificial photoperiod: 12L/12D; lights on: 7 a.m.; lights off: 7 p.m.).

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Scanning electron microscopy

Aged rats were decapitated and the pineal gland was immediately removed, placed without cryoprotectants into a mixture of liquid propane (80%) and isopentane (20%), and left in a liquid nitrogen bath (LN2) at −196° C (Jehl et al. 1981). Bulk fractures were obtained by fracturing the gland with chilled instruments on an LN2-cooled brass surface. Fractured tissues were then carried under LN2 to a freeze-drying apparatus for a period of 48 h under the following conditions: 3.10^{-5} Torr; temperature of the stage carrying the specimens, −90° C. At the end of the drying process, the stage was progressively heated to +30° C. Specimens were then mounted on carbon stubs with carbon glue, and coated with carbon or Au-Pd before examination. They were analyzed with a Philips PSEM 501B scanning electron microscope equipped for X-ray microanalysis by a Link X-ray energy–dispersive spectrometer operating under the following conditions: accelerating voltage, 20 kV; probe current, 0.5 nA; diameter of the probe, 0.5 µm.

Transmission electron microscopy

Pineal glands were quickly removed from the brain after decapitation and fixed with a 3% glutaraldehyde solution in 0.1 M Na-cacodylate buffer (1 h) and postfixed with a 1% OsO_4 solution in 0.1 M Na cacodylate buffer. The potassium pyroantimonate method was used (Eisenmann et al. 1979; Mentré and Halpern 1988) for the ultrastructural localization of calcium. Pineal glands were quickly removed and placed in a fixative containing an unbuffered 5% glutaraldehyde solution (1 vol), and a 4% pyroantimonate solution (1 vol). After several rinses in the buffer, they were placed in a 2% osmium tetroxide solution (1 vol), 4% potassium pyroantimonate solution (1 vol). After dehydration in a graded series of alcohol, they were embedded in Araldite. Ultrathin gray sections were then examined after staining with uranyl acetate and lead citrate. Controls were examined unstained.

X-ray microanalysis

Unstained and carbon-coated sections were analyzed with a transmission electron microanalyser, Camebax TEM. The probe was fitted with an oblique wavelength dispersive spectrometer using a PET (Pentaerythrytol) and a TAP (Thallium acid phthalate) crystal. The conditions of analysis were as follows: accelerating voltage, 45 kV; specimen current, 150 nA; beam diameter, 100–200 nm; counting time, 50 s.

X-ray counts were measured on each side of the peak. Values obtained were subtracted from the average of two measurements of the peak according to the method of Nicholson (1974) and adapted to the CAMEBAX-TEM by Mentré and Halpern (1988). Results were expressed in the form of the ratio calcium/phosphorus, a ratio in which intensities are expressed in counts/50 s.

$$\frac{\text{Calcium}}{\text{Phosphorus}} = \frac{\text{Intensity calcium } \alpha}{\text{Intensity phosphorus K } \alpha}.$$

Variations in this ratio allow variations in the phosphorus content, according to the degree of mineralization, to be determined.

Electron diffraction

Unstained sections were observed with a JEOL 100B electron microscope working at 100 kV. Measurements of enlarged photographs were performed with the aid of a precision ruler (Siemens, type 941, Erlangen). The electron diffraction patterns were calibrated using the LiF rings by a method previously described (Voegel and Frank 1974).

Results

Different types of concretions

On the basis of SEM and TEM observations, two types of concretions were observed (Figs. 1–3, 5). The first type is located intracellularly (Fig. 1) and appears to be composed of concentric layers of radially arranged needle-shaped "crystallites" (Figs. 2–3) with an average length of about 30–40 nm and a diameter of 4–5 nm. Needle-shaped crystallites are observed in the cytoplasm close to the concretion (Fig. 3). Some of them appear to be "derived" from membranous material. The inset in Fig. 3 shows a diffraction pattern of a selected area of a concretion similar to that in Fig. 2. The diffraction pattern consists of rings, indicating the presence of hydroxyapatite in the concretion.

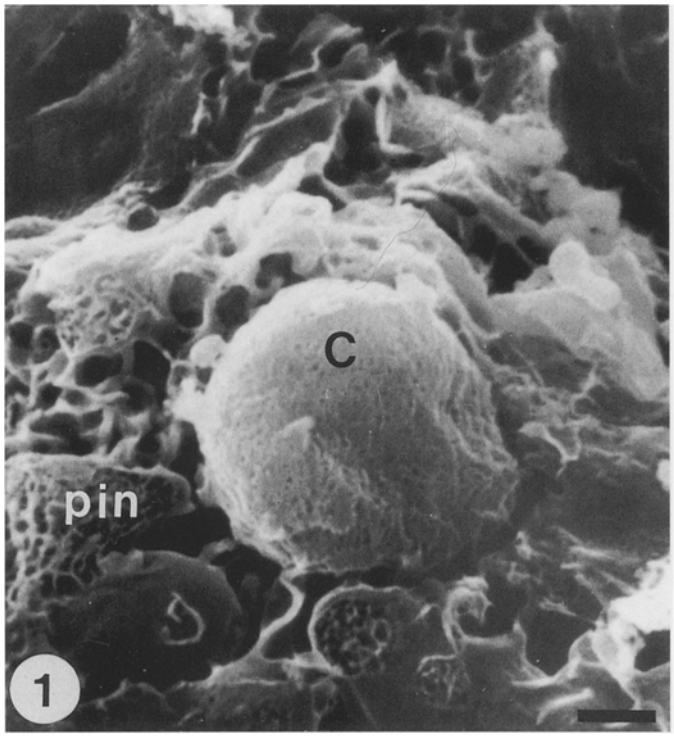
The second type of concretion consists of amorphous spherical concentric layers mostly situated in the external part or in the perivascular spaces of pineal gland; several spheres are often "clotted" together showing a mulberry structure (Fig. 5). When these concretions show "amorphous" layers, high magnifications of the external layers reveal small "crystallites" (Fig. 5). The concretions are generally oval and their size ranges between 0.5 to 15 µm. The inset in Fig. 5 shows a diffraction pattern from a selected area of a concretion similar to that in Fig. 5. The diffraction pattern consists of diffuse rings, indicating the amorphous nature of the deposits.

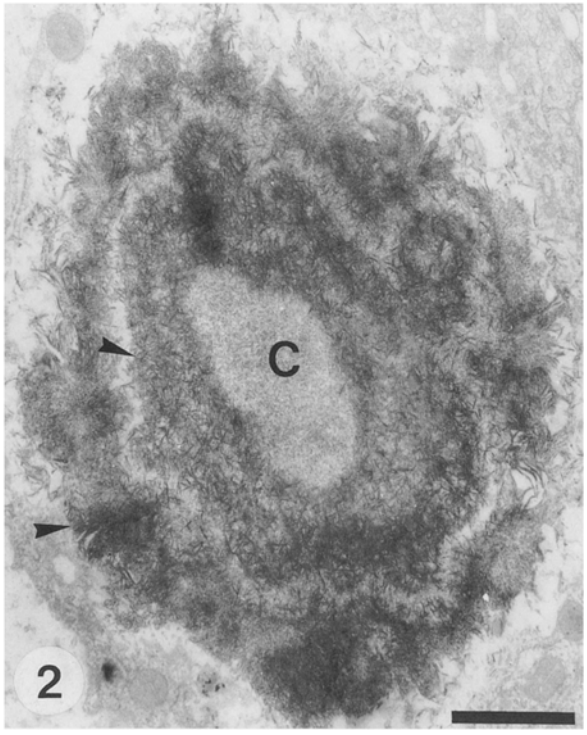
A wave-length-dispersive X-ray spectrum of P and Ca, obtained from a concretion similar to that represented in Fig. 2 is shown in Fig. 4. The osmium peak close to phosphorus is presumably related to the fixation procedure. Similar results are obtained with amorphous concretions (Fig. 5) with lower emissions of Ca and P. Semiquantitative results of X-ray microanalysis show, in addition to calcium and phosphorus, the presence of sulfur (Fig. 12).

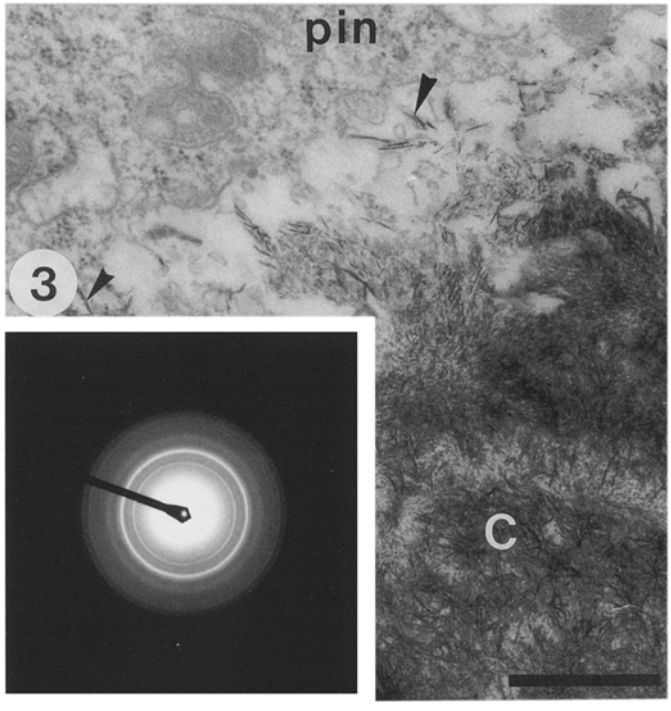
Genesis

To gain a better insight into the role that calcium ions play in the formation of concretions, the potassium pyroantimonate method was used. The complexing of pyroantimonate with intracellular calcium results in dark deposits, presumed to result from the corresponding Ca localization.

Ca-pyroantimonate deposits were found in cisternae of the rough endoplasmic reticulum, in small cytoplasmic vacuoles or vesicles (40–200 nm in diameter) (Figs. 6–8) and on cell membranes of pinealocytes (Figs. 7–8). They were also present in dense lipid bodies, in lipopigments, in mitochondria, in nuclei of dark cells, and on collagen fibers (Figs. 7–9). X-ray microanalysis confirmed the presence of Ca in these compartments. Pyroantimonate precipitates were more concentrated in the cytoplasm of dark pinealocytes than in that of light pinealocytes. These dark pinealocytes had the densest pyroantimonate precipitates (Fig. 11). Electron-probe microanalysis showed higher calcium emissions in dark pinealocytes than in light pinealocytes (Fig. 13).







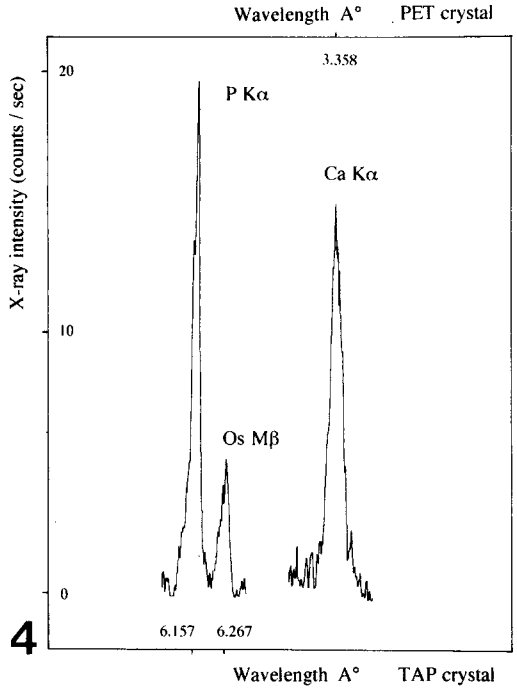
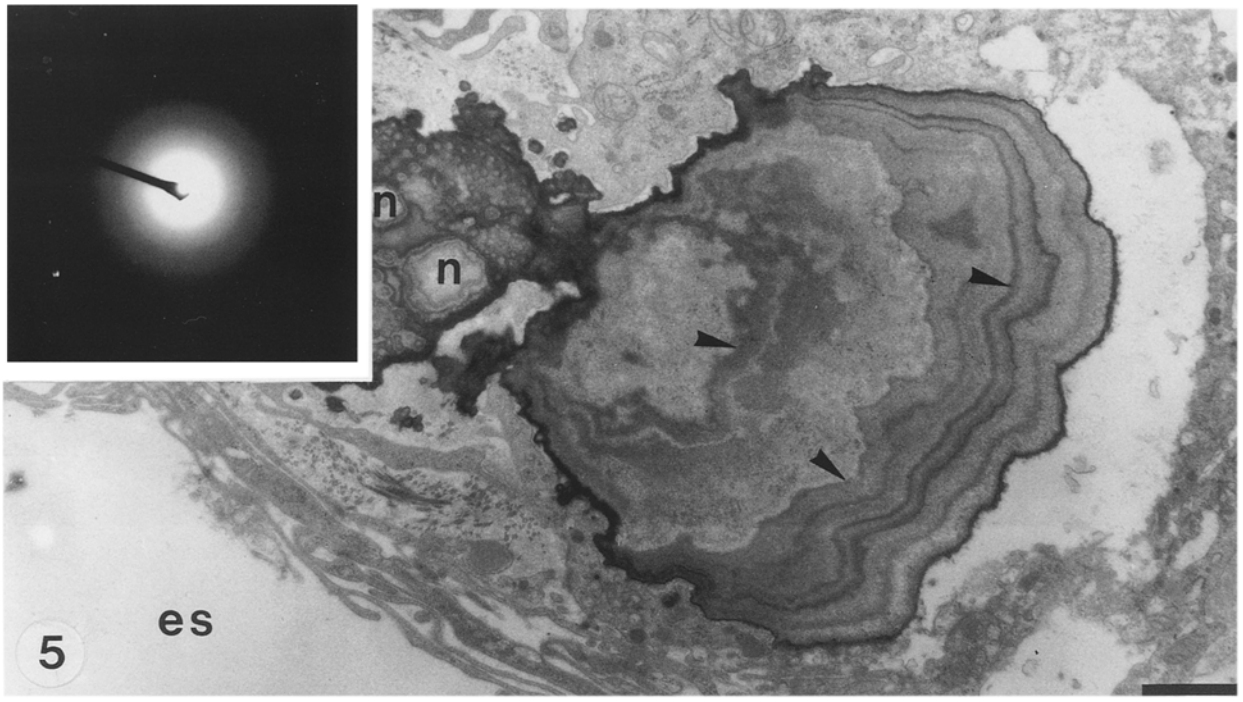


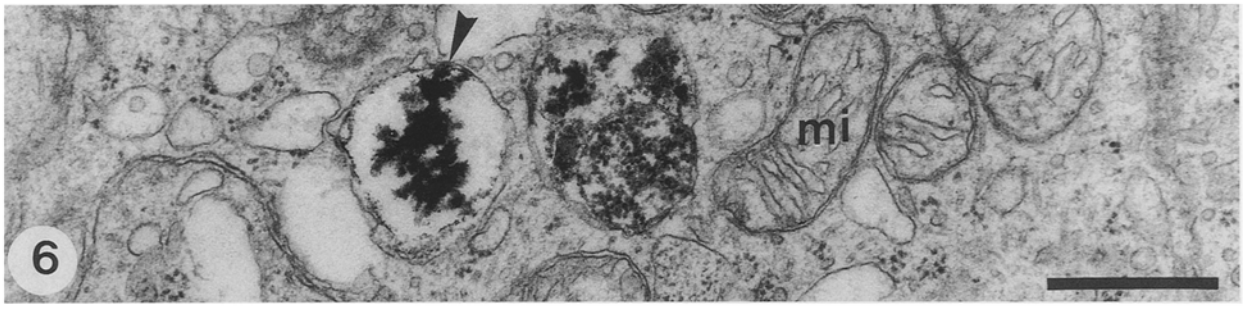
Fig. 1. Scanning electron microscopy of a frozen-fractured and frozen-dried concretion of the pineal gland of a 28-month-old rat. *pin* Pinealocytes. ×5000. *Bar:* 2 μm

Fig. 2. Transmisson electron microscopy of an intracellular concretion (*C*) with concentric layers of needle-shaped crystals (*arrowheads*) 28-month-old rat. ×15000. *Bar:* 1 μm

Fig. 3. Detail of the "outer" layer of needle-shaped crystals; several crystals seem to be derived from membranous residues (*arrowhead*). *C* Concretion; *pin* pinealocyte. ×40000. *Bar:* 0.5 μm. *Inset:* Electron diffraction pattern showing typical rings indicating the presence of hydroxyapatite in the concretion

Fig. 4. Electron-probe analysis of a concretion similar to that presented on Fig. 2. Wavelength-dispersive analysis reveals characteristic Kα lines of P and Ca; the Os peak results from the fixation procedure. *Abscissa* Peak intensity (X-ray emission in counts/s). *Ordinate* Wavelength in Å (PET crystal for Ca and TAP crystal for P and Os)





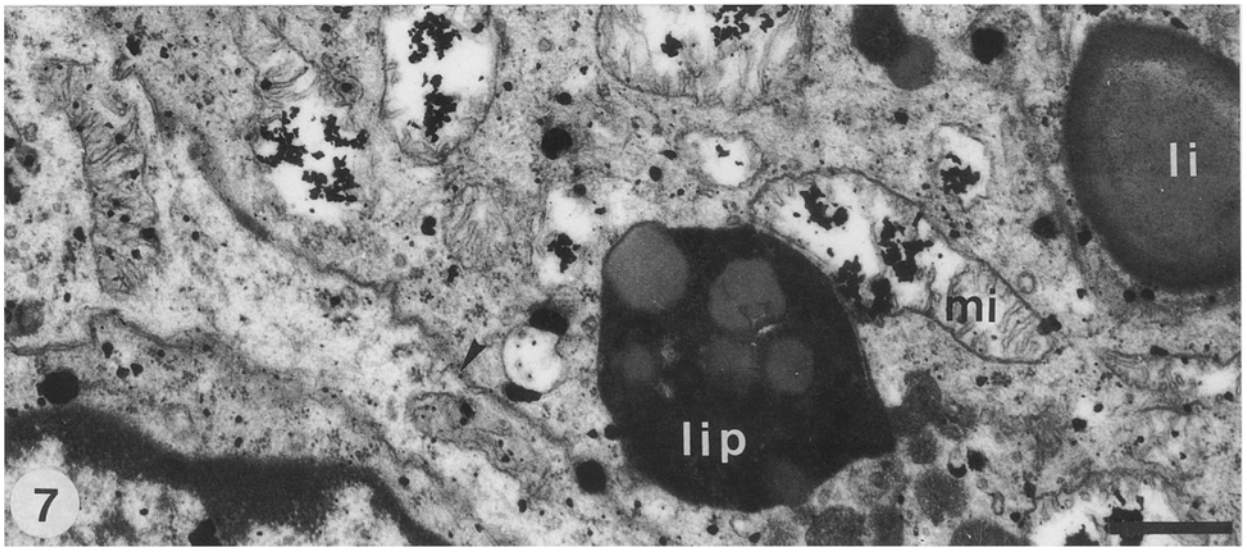
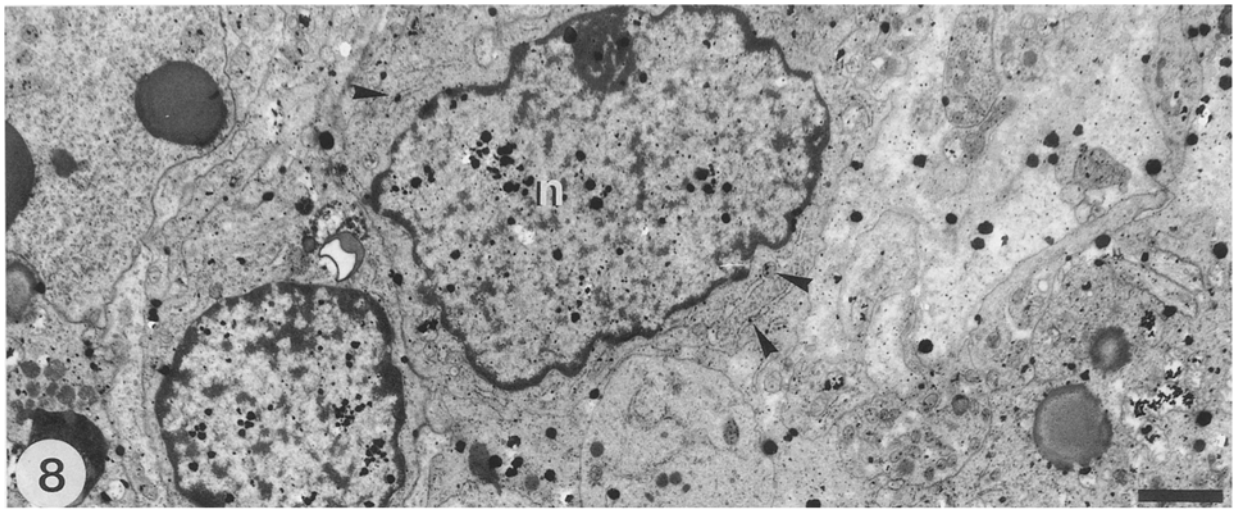


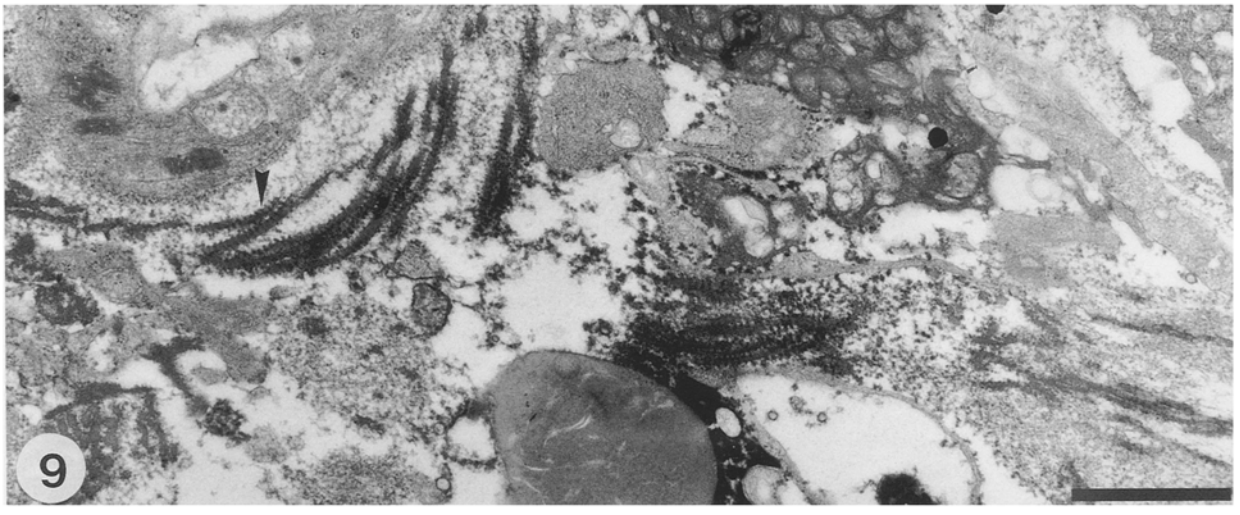
Fig. 5. "Amorphous" extracellular concretion with concentric layers (*arrowheads*) around nucleation centers (*n*). *es* Extracellular space. × 15000. *Bar:* 1 μm. *Inset:* Electron diffraction pattern showing broad diffuse rings indicating the amorphous nature of the deposits.

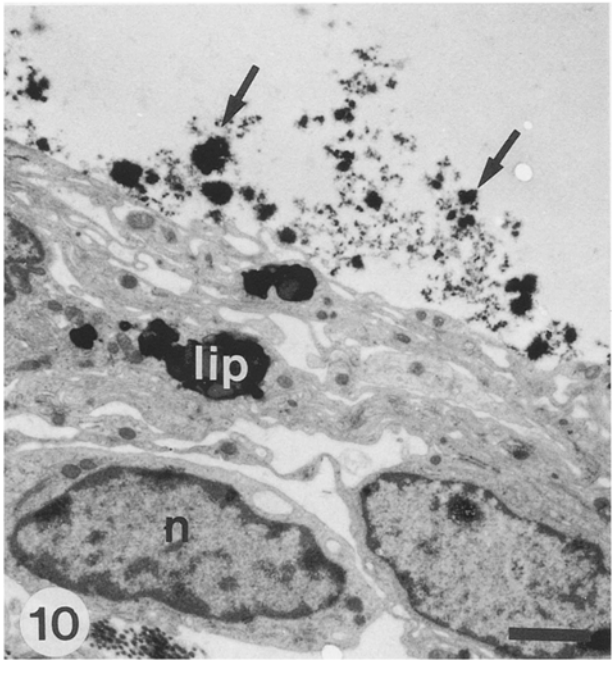
Figs. 6–11. Genesis of the concretions (28-month-old rat)

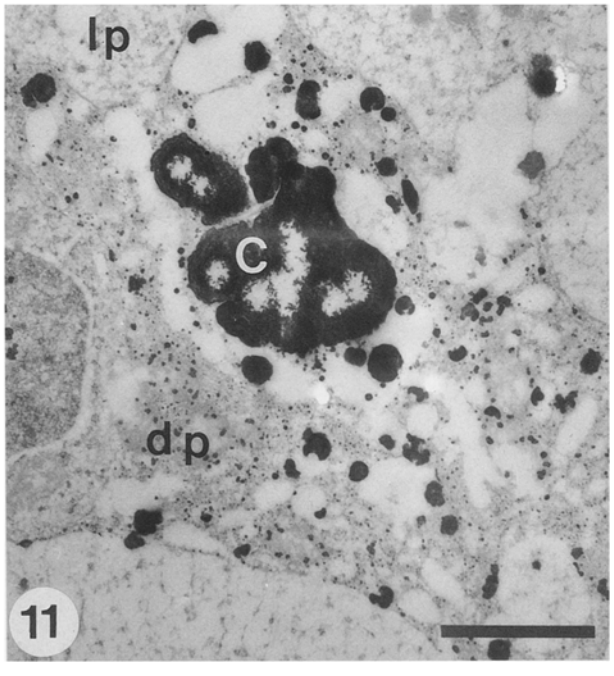
Fig. 6. Pinealocyte with typical vacuoles filled with electron dense material (*arrowhead*). *mi* Mitochondria. Fixation: glutaraldehyde and osmium tetroxide. ×46000. *Bar:* 0.5 μm

Fig. 7. Location of cations by means of the potassium pyroantimonate procedure. Intercellular spaces are outlined by dense precipitates (*arrowheads*). Positive reactions are located in swollen degenerating mitochondria (*mi*); *lip* lipopigments. Note the fine deposits of precipitates on lipid droplets (*li*) ×31000; *bar:* 0.5 μm









Figs. 8–9. Location of cations by means of the potassium pyroantimonate procedure. Note precipitates in the nucleus of dark pinealocytes (*n*), in vesicles of the endoplasmic reticulum (*arrowhead*, **8**) and associated with collagen fibers (*arrowhead*, **9**). **8** ×11000. *Bar:* 1 μm. **9** ×19000. *Bar:* 1 μm

Fig. 10. Location of extracellular electron-dense calcium-rich deposits (*arrows*). Fixation: glutaraldehyde and osmium tetroxide. *lip* Lipopigment; *n* nucleus. ×6000. *Bar:* 2 μm

Fig. 11. Dense deposits of pyroantimonate precipitates in dark pinealocytes (*dp*) and an early stage of the genesis of the concretion (*C*). Note the abundant precipitates in dark pinealocytes (*dp*) compared with the neighboring light pinealocyte (*lp*). ×16000. *Bar:* 1 μm

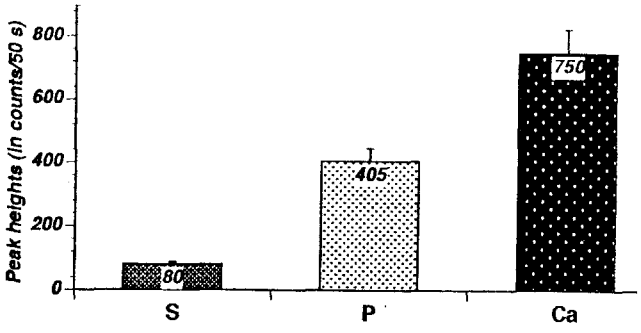


Fig. 12. Peak heights of sulfur, phosphorus, and calcium in concretions as determined by X-ray microanalysis (in counts/50 s; n=5)

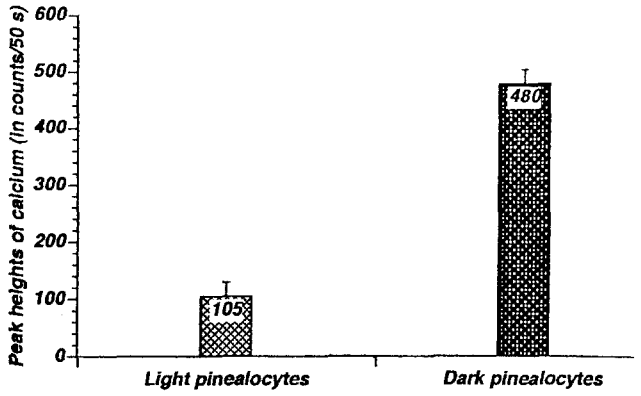


Fig. 13. Peak heights of calcium in the cytoplasm of light and dark pinealocytes as determined by X-ray microanalysis (in counts/50 s; n=8)

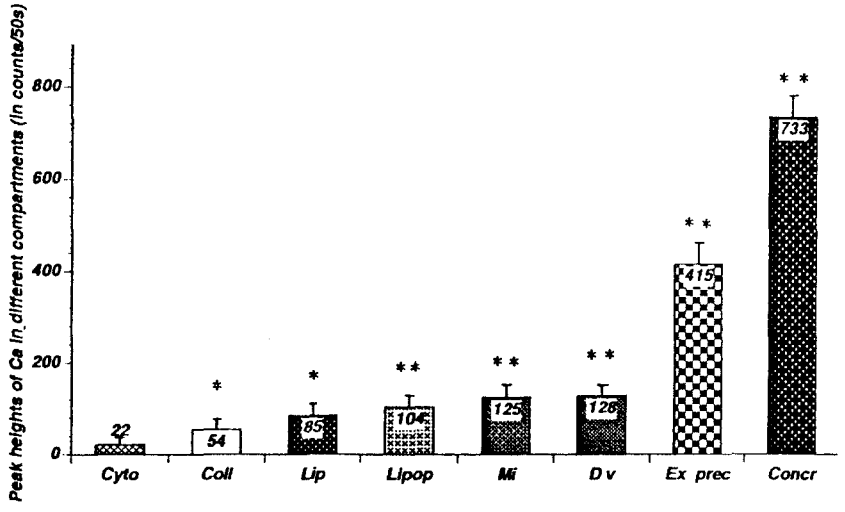


Fig. 14. Peak heights of calcium in different cellular and extracellular areas of the pineal gland as determined by X-ray microanalysis (in counts/50 s; n=8). *Cyto* Cytoplasm; *Coll* collagen; *Lip* lipids; *Lipop* lipopigments; *Mi* mitochondria; *Dv* dense vacuoles; *Ex prec* extracellular deposits; *Concr* concretions. Calcium emissions are significant (* P<0.01) or highly significant (** P<0.001) in studied areas when compared with cytoplasm (according to Student's t test)

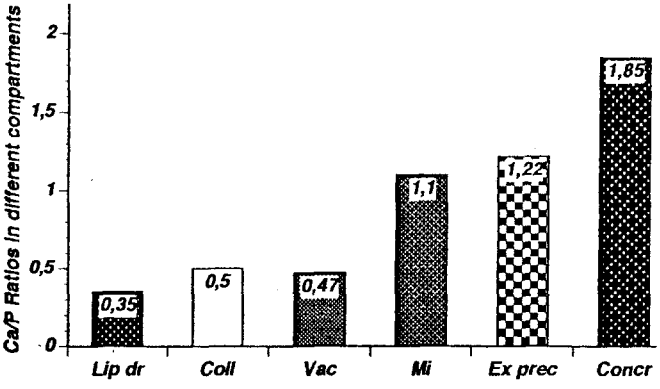


Fig. 15. Calcium/phosphorus (Ca/P) ratios calculated in different "compartments" of pinealocytes and concretions after determination by X-ray microanalysis. *Lip dr* Lipid droplets; *Coll* collagen; *Vac* vacuoles; *Mi* mitochondria; *Ex prec* extracellular precipitates; *Concr* concretions

Vesicles, vacuoles, mitochondria, lipid droplets appeared to contain more calcium than the cytoplasm (Fig. 14). Extracellular deposits (Fig. 10) showed strong calcium emissions (Fig. 14).

Calcium/phosphorus ratios were determined by X-ray microanalysis in the same cellular compartments. They showed marked changes in the calcium/phosphate phase, with the highest value being obtained on concretions (Fig. 15); this value was close to 1.66, which is the Ca/P ratio of stoichiometric hydroxyapatite.

Discussion

Our results in aged rats are concordant with previous data published on calcium localization, and on the structure and distribution of calcified concretions (corpora arenacea, acervuli) in the pineal gland of different mammalian species, including man (Japha et al. 1976; Welsh 1984; Krstić 1985, 1986). However, the main questions of pineal calcification in old rats are why, how and where do primary loci transform into concretions.

Notable amounts of Ca pyroantimonate deposits have been found in the cytoplasm and nuclei of electron-dense cells in the pineal organ of 4 to 6-month-old rats (Vigh et al. 1989). The intracellular accumulation of Ca-pyroantimonate precipitates around calcified concretions has been described in the Mongolian gerbil by Krstić (1985) who has proposed that the high Ca^{2+} concentration in gerbil pinealocytes creates favorable conditions for the formation of acervuli. These data correspond

with the elevated calcium concentration found in the pineal gland of aged rats containing concretions compared with the low calcium values in young rats (Humbert and Pévet 1991).

Origin of the concretions

Several hypotheses regarding the origin of the concretions have been put forward. In the light of the above results, viz., the elevated amount of calcium in dark pinealocytes, which degenerate gradually (Humbert and Pévet 1992), we have suggested that concretions originate from these cells. The accumulation of electron-dense material in vesicles, vacuoles, mitochondria, and lipid droplets suggests that these organelles are involved in the genesis of the concretions. A local increase in the concentration of calcium and phosphorus could facilitate the nucleation of calcium phosphates and could therefore represent possible sites of genesis for the concretions. These dense deposits or loci could transform into concretions by further addition of material.

Although the intracellular mode of formation is the classical route of mineralization (Simkiss 1976), the extracellular route appears to be a possible alternative. Our present results suggest that floculent extracellular calcium, viz., rich material resulting from degenerated cells or representing the proteinaceous secretory product described by Lukaszyk and Reiter (1975), could also initiate formation of the concretion.

Role of mitochondria and endoplasmic reticulum

Mitochondria and endoplasmic reticulum have been considered as the cellular sites of calcium regulation (Somlyo 1984). However, these organelles have also been demonstrated as being capable of becoming mineralized (Lehninger 1970; Glimcher 1959, 1981; Wuthier 1982). Evidence for the intracytoplasmic and intramitochondrial accumulation of calcium in swollen pinealocytes of the gerbil has previously been reported by Krstić (1985). Large amounts of calcium and phosphorus are present in mitochondria and bone during mineralization (Landis et al. 1977). Some authors consider this to be the first phase of the formation of hydroxyapatite (Boskey and Posner 1976; Posner 1969; Termine and Posner 1967; Wuthier 1982). The increase of intramitochondrial calcium increases mitochondrial swelling, leading to the loss of mitochondrial function.

In the present work, we have observed that vesicles and vacuoles of endoplasmic origin have the same sequestering capabilities, since calcium and phosphorus have been demonstrated in these organelles by cytochemistry and X-ray microanalysis; they may play an important role in the genesis of concretions. These results, which are in agreement with observations obtained in the gerbil pineal gland, indicate that concretions develop within vacuoles of pinealocytes (Reiter et al. 1976; Welsh and Reiter 1978; Welsh 1984, 1985; Krstić 1985, 1986).

Another explanation of the mineralization process involves the matrix vesicles. For many authors, matrix vesicles are considered to play an essential role in primary nucleation (Wuthier 1982). Matrix vesicles of chicken epiphyseal plate and cartilage contain calcium and phosphorus. Initial mineralization phases have been found free or located in small vesicles in the midgut of Collembola (Humbert 1978), in the cytoplasm of fibroblasts in culture (Yajima et al. 1984), in necrotic thyroid tumor cells (Johannessen and Sobrinho-Simoes 1980), and in vacuoles and mitochondria of fibroblasts during experimental cutaneous calcinosis (Boivin 1975; Walzer et al. 1980). Similar vesicles have been described in this study in the cytoplasm of pinealocytes, but whether they correspond to the equivalent of matrix vesicles has yet to be determined.

Role of lipids

Earlier observations have indicated that lipids and more specifically phospholipids present in extracellular membrane vesicles (matrix vesicles) are involved in the initial formation of cartilage, bone, and dentine (Shapiro 1973; Irving and Wuthier 1968; Wuthier 1968).

Data presented by Goldberg and coworkers (1988) provide evidence that lipids associated with other groups of glycosaminoglycans and structural proteins in the dental basal membrane of developing rat incisors play a role during dentinogenesis. Other types of calcifications are associated with lipid-rich organic lamellae (Ennever et al. 1979) and necrotic cell membrane fragments (Kim 1976).

This study has shown that lipids are capable of being located with calcium (associated with phosphorus). Whether they play a role in trapping an excess of calcium present in the cell, or whether they play an active role in the genesis and growth of concretions remains to be investigated.

Role of collagen

In aged rats, collagen fibers are particularly numerous in extracellular and perivascular spaces. We have reported that collagen fibrils are able to accumulate calcium and phosphorus (in preparation). They could thus act as sites of nucleation leading to the precipitation of hydroxyapatite crystals and therefore may be important initiators of mineralization (Glimcher 1959, 1981; Termine and Posner 1967; Landis et al. 1977). These fibrils may also play a role in the growth of extracellular concretions (in preparation).

Amorphous and crystalline concretions

The presence of two types of concretions (amorphous and crystalline) raises the following questions: Are there two different types of concretions present, or is there one type, existing in two different forms, the amorphous

form being able to transform into the crystalline form, depending on physicochemical conditions? Amorphous calcium phosphates are readily transformed to the apatite crystalline phase during exposure to histological fluids (Eanes 1972; Landis et al. 1977). According to Houben (1971), the amorphous fraction has indeed a sub-microcrystaline structure. It appears to be constituted by such small hydroxyapatite crystals that they cannot be identified by the available analytical methods, because they are beyond the limit of resolution. This could be the case in the pineal gland in which high magnifications of amorphous concretions show small crystallites. The transformation of calcium-carbonate phases (calcite-vaterite) has been reported in a previous study of intestinal biocrystals of seawater eel (Humbert et al. 1989). Amorphous calcium phosphates have been demonstrated to be the main component of a human pineal tumor (Møller et al. 1979), whereas normal human calcification consists of crystalline hydroxyapatite (Mabie and Wallace 1974). In the light of our results, we suggest that pineal concretions probably exist in two different forms, but we do not exclude the possibility of a transformation of phases during tissue processing.

Chemical composition

Our results show that concretions are mainly composed of calcium and phosphorus, suggesting the presence of hydroxyapatite (in preparation); sulfur, iron, silicon, chlorine, potassium, copper, and zinc have been detected with freeze-drying techniques (Humbert and Pévet 1991). Sulfur can be attributed to the presence of sulfated mucopolysaccharides within the matrix, which is composed of carbohydrates and which has a proteic component (Lukaszyk and Reiter 1975; Michotte et al. 1977; Humbert and Pévet 1991).

Conclusion

The above results and the data reported in the literature suggest that pineal concretions in aged rats (1) are not indicators of a secretory activity similar to that supposed to occur in the pineal gland of the gerbil (Lukaszyk and Reiter 1975; Champney et al. 1985; Krstić 1986), (2) are not indicators of an active storage site for calcium and organic material (Krstić 1985), (3) seem to be indicators of mineralization, resulting from the death or degeneration of pinealocytes. An increase of pineal calcification (related to the aging process) could thus lead to a decrease of pineal activity, or vice versa, a decrease of pineal activity could induce pineal calcification. These questions remain to be investigated.

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