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A COMPARATIVE LIGHT AND ELECTRON MICROSCOPIC STUDY OF THE PINEAL COMPLEX IN THREE SPECIES OF SNAKES OF PAKISTAN

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In The Glorious Name of Allah , Most Beneficient, Most Merciful

DEDICATED TO MY HUSBAND

ZAFAR IQBAL

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ABSTRACT

A light and electron microscopic study was carried out on the pineal complex of 3 species of snakes, namely common cobra (*Naja naja*, Family: Elapidae), common krait (*Bungarus caeruleus*, Family: Elapidae) and saw-scaled viper (*Echis carinatus*, Family: Viperidae) belonging to Class Reptilia, Order Squamata and Suborder Ophidia (Serpentes).

The pineal complex in the three species comprises only the pineal organ proper (epiphysis cerebri). The organ in these species is remarkably similar to the pineal of mammals. In none of these species there is evidence of a central lumen and open communication with the third ventricle of the brain and hence has solid organization. The stalk, in all three species, is connected to the posterior commissural-subcommissural area of the brain by a meningeal bridge. The saw-scaled viper is unusual in showing ectopic pineal tissue.

The pineal organ in the cobra and the krait is generally similar in its organization. Both the light microscopic and the electron microscopic details reveal three main cell types in the pineal parenchyma which forms cords and shows follicles only rarely. Electron microscopically these cells are distinguishable as electron lucent, electron dense and of intermediate electron density in both species. The electron lucent and the intermediate cell types are designated as pinealocytes. The electron dense cells have been argued to represent supporting elements of the pineal but the alternative possibility of a single cell type in the cobra has also been discussed. These three cell types show species-dependent differences in the cobra and the krait. There is compelling evidence to suggest that the electron lucent and the intermediate pinealocytes represent the same cell type in different physiological states. Dense core vesicles (100-250 nm), suggesting a secretory potential, are abundant in these cells in the cobra, especially in their processes which terminate on the perivascular space. Although these vesicles are very scanty in the pinealocytes of the krait, the pineal presents a secretory predisposition in this species too. The presence of synaptic ribbon-like structures in the cobra pinealocytes and rare cells resembling modified photoreceptors in the krait pineal is indicative of their origin from the primitive photoreceptor-cell line. The supporting cells in the cobra pineal show a range of variation in both nuclear and cytoplasmic features. Their processes contain numerous vacuoles and show an intimate relationship with the pinealocytes. Some of these cells in the

cobra but not in the krait appear to undergo pycnotic changes depicting cellular degeneration. The possibility that the pinealocytes themselves undergo degeneration with the dark cells being a transient phase of this degeneration process rather than representing supportive elements has been entertained but considered to depict merely loss of the supporting cells. The supporting cells in the cobra do not seem to isolate the pinealocytes from the perivascular space. These cells in the krait show very different morphology, rarely resembling those in the cobra and hardly show any evidence of degeneration. Also, evidence is provided to show that their processes isolate the pinealocytes from the perivascular space. Second order neurons are absent in the pineal of both species. Unmyelinated and myelinated nerve fibers are abundant in the perivascular space in both species, especially the krait. The former also occur in the pineal parenchyma between the pinealocytes. The pineal in both species is very vascular, the endothelium of the capillaries being fenestrated.

The pineal of the saw-scaled viper is relatively smaller in size. It is very unique in showing several noteworthy features. It has a single category of parenchymal cells, the pinealocytes. Cells resemblig supporting elements are entirely absent. The pinealocytes are unusual in showing a massive microfibrillar body occupying nearly as much space in the cytoplasm as the nucleus. This body is a swirling mass of microfilaments not reported previously in the pineal of other vertebrates except the water snake, *Natrix natrix* where microfilaments abound but with a different dispensation. The pinealocytes in the viper also show an outstanding propensity of mitochondria, clusters of which give the semblance of an ellipsoid characteristically seen in the pinealocytes of anamniotes and lizards. The mitochondria contain conspicuous arrays of dense granules of 40-160 nm size in their matrix; a feature not reported earlier in the pineal of any other species. The endoplasmic reticulum is mainly of the smooth type. Lysosomes are also very abundant in most pinealocytes. Dense core vesicles (Vesicle = 100-200 nm, Core = 80-160 nm) abound in both the perikarya and the processes of the pinealocytes and provide strong evidence of their secretory nature. The pinealocytes show some differences in subcellular features. Those which appear to be in an active secretory phase contain much dilated endoplasmic reticulum and partially to completely empty vesicles and fewer lysosomes. Unlike the cobra and the krait, the pinealocyte also release their secretory granules in the residual lumenal spaces and canaliculi which are more common in the viper than in the elapid snakes. It

is argued that the canaliculi may communicate with the perivascular space, though no evidence for this could be gleaned presently. There is no evidence of presence of second order neurons. Also, nerve fibers could not be encountered as readily as in the elapid snakes. Evidence for their presence is provided by rare incidence of an axon which could be detected in the parenchyma next to a pinealocyte. The stromal compartment is rich in blood sinusoids. The endothelium of these vessels is fenestrated.

The morphological details of the pineal in the three species are compared with the known information for the ophidian group, other Sauropsida as well as the mammals. The need for additional studies on the more primitive snakes than the species studied presently is stressed. Special attention is drawn toward studies based on age and season to develop a better idea regarding cellular details, cell biological endowments and the role of the pineal in regulating physiology and behaviour of snakes.

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INTRODUCTION

The pineal complex is well known as mediator of a variety of exogenous and endogenous environmental cues in all vertebrate groups (Wurtman *et al.*, 1968; Reiter, 1973, 1991; Collin and Oksche, 1981; Vollrath, 1981; Oksche, 1983; Korf and Oksche, 1986; Korf, 1994; Reiter, 1994; Cassone, 1995; Edmonds *et al.*, 1995; Lerchl *et al.*, 1995). The complex arises as a dual evagination from the diencephalic roof of the brain of which it is an integral part. Its phylogenetic history is both interesting and complex (Wurtman *et al.*, 1968; Quay, 1974; Collin and Oksche, 1981; Vollrath, 1981). In cyclostomes, anurans and lizards, it is represented by an anterior, median and superficial parapineal (frontal organ or third eye) and a posterior, intracranial component, the pineal proper (epiphysis cerebri). In all other vertebrates, where any evidence of pineal elements has been detectable in the adult stage, only the pineal organ is well developed. The frontal organ ("stirnorgan" in anurans) and the third eye (parietal eye in lampreys, sphenodon and lizards) display a typically eye-like structure with cornea, lens, retina and associated neural elements (Eakin, 1973; Dodt, 1973). The retina contains typical photoreceptor cells which directly respond to environmental light and project photic information toward the brain by a parietal nerve. The pineal organ, in all those vertebrates where its existence has been demonstrated, is characterized by a glandular organization primitively bearing a photoreceptive as well as secretory disposition (Oksche, 1965; Wurtman *et al.*, 1968; Quay, 1974; Oksche, 1971, 1983) but assuming an entirely neuroendocrine capacity in mammals (Quay, 1974; Collin and Oksche, 1985; Vollrath, 1981; Korf and Oksche, 1986; Korf, 1994). In ectothermic vertebrates (lampreys, fishes, amphibians), it possesses photoreceptor cells which are modified first-order neurons. These cells are synaptically associated with second-order neurons, the axons of which constitute a pinealofugal nerve tract projecting over various centers in the diencephalic and mesencephalic areas of the brain (Paul *et al.*, 1971; Hafeez and Zerihun, 1974; Ekstrom, 1984; Ekstrom and van Veen, 1983, 1984; Ekstrom and Korf, 1985). The organ varies in its organization from a saccular or tubular structure to a compact nearly solid organ (Vollrath, 1981). The central lumen in either case opens into the third ventricle of the brain. Among the endothermic vertebrates (birds, mammals), the mammals possess a pineal which is an entirely solid organ having lost the

central lumen (Quay, 1974; Vollrath, 1979, 1981; Collin and Oksche, 1981). The chief cells of the pineal, the pinealocytes, lack the apical photoreceptive pole and the basal pedicle terminates in the perivascular space. These cells thus depict a secretory disposition. Concomittant with such structural change, direct neural connections between the pineal and the brain have undergone reduction (Collin and Oksche, 1981). Until quite recently, the innervation of the organ was thought to be exclusively autonomic (Kappers, 1960, 1965; Collin and Oksche, 1981) but there is now evidence that direct pinealopetal and pinealofugal pathways between the pineal and the brain also exist in some mammals (Korf and Moller, 1984, 1985; Korf, 1990; Moller, 1992, Korf, 1995).

The shift in the structural make up of the pineal in mammals is foreshadowed by the structural multiplicity of the pineal in the reptilia (particularly the lizards) (Quay, 1979), the group from which birds and mammals have evolved. In the lizards, there is a diversity of structure ranging between well developed photoreceptor cells and rudimentary photoreceptors (Collin, 1969, 1971; Petit, 1969; Collin and Oksche, 1981) possessing modified apical and basal poles. This has suggested a phylogenetic transformation of the typical photoreceptor cells of the anamniote pineal into the mammalian pinealocyte by rudimentation and ultimate loss of the photoreceptive pole as well as the synaptic associations of the basal pedicle (Collin and Oksche, 1981; Vollrath, 1981). The Aves also provide evidence of rudimentation of photoreceptor cells and changes in the organization of the pineal, though not quite to the same extent as in the mammals (Oksche and Kirschstein, 1969; Oksche *et al.*, 1969, 1972; Ueck, 1970; Calvo and Boya, 1979; Boya and Calvo, 1979).

The interesting aspect of the pineal story is that in all vertebrates, from the cyclostomes to the mammals, where the pineal has been studied and shown to exist, the organ maintains an active indoleamine metabolism (*Lamprey*: Joss, 1973, 1977; Meiniel, 1980; Meiniel and Hartwig, 1980; Tamotsu *et al.*, 1990; *fishes*: Hafeez and Quay, 1969, 1970; Owman and Rudeberg, 1970; Fenwick, 1970; Oguri *et al.*, 1968; Smith and Weber, 1974; Hafeez and Zerihun, 1976; Falcon, 1978; Gern, 1978, McNulty, 1984; *Amphibia*: Quay, 1965; Van de Veerdonk, 1967; Baker, 1965; *Reptilia*: Quay, 1965; Collin, 1972; Collin and Meiniel, 1972; Petit, 1969; Vivien-Roels, 1970; Vivien-Roels *et al.*, 1981; *Aves*: Quay, 1966; Lauber *et al.*, 1968; Ralph and Hedlund, 1967; *Mammalia*: Quay, 1963, 1964, 1974). One of the

indoleamine metabolites, melatonin, is synthesized and secreted rhythmically in the pineal of all vertebrate groups (Quay, 1974; Reiter, 1980, 1991). The rhythm of synthesis and release is circadian (endogenous) but entrained by environmental light (Reiter, 1991; Korf, 1994). Thus, the functions of the pineal are profoundly linked to day-length and the organ acts as part of a photoneuroendocrine system and an integral component of the biological clock (Oksche, 1983; Korf and Oksche, 1986; Klein, 1985; Reiter, 1991; Korf, 1994). In the poikilotherms (fishes, amphibians and many reptiles), it is directly photosensitive and transforms light stimuli into hormonal as well as neural output. In the mammals, it is indirectly photoresponsive receiving photic information from the lateral eyes via retinohypothalamic tract, the nucleus suprachiasmaticus (the endogenous oscillator in mammals), intermediolateral nucleus in the spinal cord, superior cervical ganglion and finally the sympathetic innervation (Moore, 1978; Korf, 1995). The photic information is then translated into hormonal output with melatonin acting as the major known hormone. Since synthesis and release of melatonin show pulsatile increase during darkness, it is aptly called the "signal for darkness" (Reiter, 1991; Korf, 1994).

Information on the structure, biochemistry and functional relationships of the pineal systems has grown rapidly especially during the last decade and a half (Dodt, 1973; Quay, 1974; Oksche, 1983; Collin and Oksche, 1981; Reiter, 1989; Vollrath, 1981; Korf and Oksche, 1986; Korf, 1994). All major vertebrate subgroups have attracted attention of researchers in the field. Surprisingly, one subgroup of the vertebrates, the Serpentes (snakes) has received scant focus. Several electron microscopic studies have been done (Vivien, 1964, 1965; Milcou *et al.*, 1968; Petit, 1971) on only one species of snakes (*Tropidonotus natrix* now renamed as *Natrix natrix*) during the recent period of pineal research. A few other publications concern development of pineal complex (Petit, 1969, *Natrix natrix* ; Haldar and Pandey, 1987, *Natrix piscator*), sympathetic innervation (*Natrix natrix*, Quay *et al.*, 1968), immunocytochemistry (*Thamnophis sirtalis, T. radix*, Kalsow *et al.*, 1991) and thermoregulatory role of pineal in reproductive behaviour (*Thamnophis sirtalis*, Nelson *et al.*, 1987; Crews *et al.*, 1988). Much of the work on developmental and structural aspects of the pineal of snakes dates back to late 19th century and early 20th century. This earlier work on several species of colubrid snakes and a few species of Boidae and Viperidae has been reviewed by Studnicka (1905) and Tilney and Warren (1919). These studies have collectively suggested that the ophidians (snakes) possess

only the pineal organ which is solid as in the mammals and possesses a single parenchymal cell type, the pinealocytes (review: Collin and Oksche, 1981; Korf, 1994). Considering the diversity of the Serpentes group (about 10 families and around 3500 species, Stidworthy, 1974), it becomes obvious that very little is known about this organ in snakes. There is dire need to examine possible variety in structure of the pineal in the snakes to fully understand its phylogenetic and functional status. The present study was, thus, carried out keeping in view the above vacuum. Three species of poisonous snakes, *Naja naja* (common cobra, Family : Elapidae), *Bungarus caeruleus* (common Krait, Family : Elapidae) and *Echis carinatus* (saw-scaled viper, Family : Viperidae) were selected for this study. Both families contain phylogenetically advanced snakes. Although the pineal of two viperid species belonging to a single Genus of Viperidae has been studied light microscopically in the past (*Vipera berus*, Hanitsch, 1888; Studnicka, 1905; *Vipera ursinii, Leydig,* 1897), none of the elapid snakes has been subjected to light or electron microscopic examination of this organ.

MATERIALS AND METHODS

Three species of common poisonous snakes of Pakistan, common cobra (*Naja naja*, Fig. 1), common krait (*Bungarus caeruleus*, Fig. 2) and saw-scaled viper (*Echis carinatus*, Fig. 3) were collected from the province of Sind through local dealers. The snakes were defanged and brought live to the Department of Biology at Quaid-i-Azam University, Islamabad, within three days of collection. The cobra and the krait are oviparous snakes belonging to the Family Elapidae of the Suborder Ophidia, Order Squamata and Class Reptilia (Smith, 1943; Stidworthy, 1974). The saw-scaled viper, on the other hand, is a viviparous species and belongs to the Family Viperidae. Both the cobra and the Krait inhabit not only wooded (grassy or cultivated) and deserted buildings but also near places of human habitation (gardens and human dwellings). The krait prefers open grassy country, gardens, semidesert areas and places close to human dwellings. The cobra and the krait are nocturnal to seminocturnal (Khan, 1990; Ali and Begum, 1990). Although both species are dangerously poisonous, the krait is the most dreaded snake. The elapids lay eggs in the months of April to May. The saw-scaled viper is a desert or semi-desert species, occurs in sandy and alluvial soil with vegetation (Khan, 1990) and is very common throughout Sind. Most human fatalities in its areas of distribution are due to this snake because it is difficult to spot in the sandy background and is thus liable to be easily stepped upon by the unwary. It is also nocturnal but becomes diurnally active on hot days and likes to bask in the brightest of the sun with its head sticking out of the sand. It gives birth to 4-10 young ones in warmer months of the year. All three species hibernate in winter.

The specimens of the cobra and the viper were collected in two separate lots in the months of October and March. The specimens of the krait were collected in June. On the day of sacrifice, the snakes were individually anesthetized with diethyl ether. The specimens of the cobra (*Naja naja*) included 6 females (223-386 g, 117-140 cm snout to vent length) and 4 males (280-500 g, 115-130 cm). Of the 4 Kraits (*Bungarus caeruleus*), 3 were females (170-180 g, 95-112 cm) and 1 male (204 g, 101 cm). A total of 10 vipers (*Echis carinatus*) were used which included 4 females (142-180 g, 62.5-67.5 cm) and 6 males (147-196 g, 62.0-82.5 cm). All snakes were adults.

The animals were decapitated immediately following anesthesia. The dorsal skin of head was removed and the skull was opened with a bone cutter. The exposed dural surface was immediately irrigated with fixative (aqueous Bouins for light microscopy, 2% buffered Glutaraldehyde for electron microscopy). The brains with intact pineal were removed under a dissecting microscope. The pineal organ in the cobra and the krait could be readily located under the dural membrane as a nodular body resting over the confluence of the cerebral hemispheres and infront of the optic lobes. The anterior aspect of the organ in the cobra is usually heavily pigmented. The pineal in the viper is barely visible superficially with the naked eye between the optic lobes and the cerebral hemispheres as a relatively smaller nodule than in the elapids.

For light microscopy, the entire brains were immersed in aqueous Bouins fixative, rinsed briefly 24-48 hours later in running water, dehydrated in graded ethanol series, cleared and finally embedded in paraffin wax. Tissue blocks were sectioned at a thickness of 6-8 u in sagittal and transverse planes. The sections were affixed to precleaned albuminized glass slides, stained with Harris's hematoxylin and eosin (H and E), examined and photographed under a Nikon Optiphot Research microscope.

For electron microscopy, the pineal organs were excised with a pair of microscissors under a dissecting microscope and immersed in chilled 2% phosphate buffered Glutaraldehyde (pH 7.2) for 2-4 hours. The stalk of the pineal was usually difficult to locate in the excised portion. The pineals were postfixed in 1% cold OSO4 for 1-2 hr, rinsed thrice in phosphate buffer and dehydrated in graded ethanol series, propylene oxide and embedded in Epon resin or Durcupan. Semithin and ultrathin sections were made with glass Knives on a LKB Ultrotome V. The semithin sections were stained with 1% toluidine blue. The ultrathin sections were placed on copper grids (150-300 mesh), contrasted in Reynold's uranyl acetate-lead citrate sequence and photographed with Joel SX-100 Electron Microscope.

RESULTS

Pineal of Cobra (*Naja naja*)

Light microscopic structure

The distal part of the organ forms a vesicle (Fig. 4) which rests behind the hemispheric vault and, thus, can be seen superficially marked by a heavily pigmented area of the dura mater. The posterior part of the vesicle rests partly over the optic lobes. The vesicle narrows sharply at its anteroventral end gradually forming a short stalk. The vesicle is solid, oval to pear-shaped in sagittal plane (Fig. 4). In cross sectional view too, it has an oval appearance with the greatest diameter being nearly comparable in the two planes. The broader anterior end of the vesicle abuts against the cerebral eminences. The arrangement of the dorsal dura and the pia-arachnoid is such that the entire vesicle appears to be cradled in a meningeal encasement (Fig.4). A definite but narrow, dorsoventrally oriented (vertical) lumenal cleft is present in a rare specimen in the extreme anterior part of the vesicle (Fig. 4) but it is generally absent. It is immediately ventral to this posterior area that the vesicle abruptly narrows as a slender stalk wrapped in meningeal connective tissue and which is traceable in serial sections to approach the posterior commissural area. Thus confluence of the stalk epithelium with the habenular and the subcommissural epithelium does not occur. Instead, the stalk appears to be attached to the posterior commissure and the subcommissural area by fibrous meningeal tissue. The pineal organ, therefore, seems to have lost open connection with the third ventricle of the brain. The pineal is surrounded by a connective tissue capsule of its own, strands and trabeculae from which profusely permeate the vesicle. The trabeculae and connective tissue septa are rich in fibroblasts, sinusoids and capillaries. Patches of melanin pigment granules are conspicuously seen along the connective tissue elements as well as in the parenchymal tissue, generally in the anterodorsal part of the vesicle but are present in other regions as well. The parenchymal tissue of the pineal is delimited from the stromal tissue usually in the form of cords of variable size but rosettes and follicles are also distinguishable here and there (Fig. 5). Excepting the proximal lumenal cleft in a single specimen, the entire vesicle is compact and solid in all other specimens with evidence of canalicular residues only here and there and in the follicles. The demarcation between the stromal and the parenchymal cords and the compactness of the pineal

epithelium are discernible to greater advantage in semithin sections stained with toluidine blue (Fig. 6).

The parenchymal cells reveal striking tinctorial differences in semithin sections. Such a distinction is not readily evident in H and E stained pineal tissue. At one extreme are intensely dark cells and at the other are clear cells (Fig. 6). These two types are intermixed with cells of intermediate tinctorial density. The latter are by far the most abundant type. In places, the clear and the intermediate types intergrade imperceptibly and, as judged from ultrastructural details also (see below), constitute pinealocytes which show striking differences from the dark cells. The relative abundance and distribution of these dark, intermediate and clear cells is somewhat variable. In some places, a mix of the three types exists. In others, either mostly light cells or entirely intermediate type of cells occur (Fig. 6). The dark cells are associated with both of these cell types. The dark cells are generally small in size and vary in shape from stellate to elongate (Fig. 6). They often lie close to the surrounding connective tissue strands. Their cytoplasmic processes extend freely into the cords and even entire cells are interspersed among the clear and the intermediate cells. In the latter position, they often look like satellites (Fig. 6). The dark cells have angular processes which meander within the cords far and wide in various directions occupying the intercellular spaces. The nuclei of these cells are highly irregular and show a variety of shapes and size.

The intermediate cells vary in the degree of chromophilia (Fig. 6). Their nuclear configuration ranges from round, oval, oblong to elongate and kidney shapes. A nucleolus is prominent and is generally eccentric in position. The cytoplasm is abundant and forms interdigitating processes. The general impression, based on the intergradation of the intermediate and the clear cells is that the former undergo hypertrophy, become increasingly less chromatic and frequently develop vacuoles and "transform" into clear cells.

The clear or light cells contain a relatively larger nucleus (Fig. 6). It varies from round, oval to oblong shapes and often shows a crease or indentations. The nuclei stain more lightly than those of intermediate cells and reveal a finer texture. The nucleolus is eccentric or marginal in position (Fig. 6). Since these cells stain lightly, they clearly stand out as elliptical, oval or round cells but bizarre shapes are also seen. Their cytoplasmic compartment is generally very spacious, especially in those cells which appear tinctorially most clear. The cytoplasm often

shows vacuoles and, in some cells, is expanded in such a way that their shapes become irregular. The overall impression that these cells are hypertrophied is unavoidable. The vesiculation and vacuolation in the intermediate and the clear cells together with canalicular spaces imparts a fenestrated appearance to the pineal in semithin sections (Fig. 6).

Electron microscopic structure :

Under the electron microscope as well, the pineal reveals three categories of epithelial cells on the basis of their electron density (Figs. 7,8,9). The surrounding leptomeningeal capsule is rich in sinusoidal vessels, fibroblasts and collagen fibers (Fig. 7). The connective tissue strands invade the pineal from all sides breaking the parenchyma into lobules. Nerve fibers of the unmyelinated type abound in the connective tissue strands (see below).

The cells in the cords consist of electron dense, electron lucent and the intermediate types (Figs. 7,8,9). The electron dense cells show a range of variation both in terms of morphology and electron density. Similarly, the intermediate cells, which are the most abundant, (Figs. 8,9,10,11,12) intergrade with the electron lucent cells suggesting that these may be variants of the same cell type. The electron microscopic images of the pineal vesicle of the cobra are such that no single criterion, electron density or given structural feature alone is sufficient to separate the parenchymal cells into distinct types. It is only use of several criteria which collectively help in this respect.

The subcellular details and intercellular relationships of the intermediate types indicate that these are the chief pineal cells, the pinealocytes. These cells have a generally round to oval nucleus but other configurations are also seen. Often lobed, indented and irregular nuclear shapes are encountered (Figs. 7,8). The heterochromatin not only forms a prominent marginal rim but also forms scattered and widely spaced clumps of variable size in a uniformly granular nuclear matrix (Figs. 7,9,10,11). A usually eccentric nucleolus is very prominent, sometimes flanked by a dense clump of heterochromatin (nucleolar satellite, Figs. 8,11,14). The membranes of the nuclear envelope show a narrow but clear space (Figs. 10,11). The cytoplasm is abundant and extended into processes of variable dimensions (Figs. 8,9,11,13). The plasma membrane of adjacent cell processes interdigitates intimately. The cytoplasmic processes of the electron dense cells not only cap these cells but also thread their way between

them (Figs. 7,8,9). Rough endoplasmic reticulum (RER) is pominent and often forms parallel stacks (Figs. 10,11). It is frequently dilated and forms vesicles and cisternae. The latter constitute a very common feature of these cells. Free ribosomes are abundant throughout and form polysomal aggregates in many places (Figs. 9,10,11). Mitochondria with discontinuous cristae are scattered in the perikarya and the processes.Their number in individual cells is low to moderate. Often they form clusters in the vicinity of the nucleus (Fig. 10). Golgi saccules are present in a juxtanuclear position (Fig. 12). Although clear vesicles are sometimes noticeable in the Golgi zone, dense core vesicles are hardly detected in association with the saccules. Clear and dense core vesicles (100-250 nm, core = 80-150 nm) are generally sparse in the cell body but are abundant in the processes of the intermediate cells (Figs. 9,11,12,13,14) bordering the perivascular space and bespeak of secretory potential of these cells. Clusters of such granules are present in the basal cytoplasmic processes terminating on the perivascular space, many of which are nearly or partially empty (Fig. 12). Lysosomes are common (Figs. 10,11,12). Membrane whorls inside vacuoles are also encountered (Fig. 11). Some of the vacuolar profiles resemble swollen mitochondria. Microtubules and microfilaments are barely detectable in these cells. A usual feature of the cobra pineal is presence of abundant melanosomes. These are not restricted to any particular category of cells. They exist in the intermediate cells as well as in the dark cells (Figs. 14,18). Figure 16 shows several cells of the intermediate type resting on the perivascular space. Although all of these appear similar in shape and nuclear characteristics, some show a distinctly vesiculated cytoplasm, are entirely devoid of melanosomes and contain dense core vesicles and granules. Other cells which contain melanosomes lack such conspicuous vesiculation and dense core vesicles. Vacuoles with a flocculent substance (lipid ?) also exist in the processes of these cells (Fig. 14). In spite of these slight differences in melanosome-bearing cells and those devoid of these, the two have the general disposition of the intermediate cell type.

In contrast to the intermediate cells, the electron lucent (or clear) cells display some unique features (Figs. 9,10,15,16) in spite of the fact that in places the two types intergrade imperceptibly (Figs. 9,12) and represent pinealocytes in a different physiological state. Generally, the lucent cells are larger in size and look hypertrophied (Figs. 15,16). Their shape varies, often imparting to them a bizarre appearance (Fig. 15). Their nucleus is characterized

by generally less heterochromatin than is the case in the intermediate cells but this is variable Rough endoplasmic reticulum as well as free ribosomes are scantier in them (Figs 9,10,12,15,16) than in the intermediate cells. Mitochondria are of the same size and structural features as in the intermediate cells. Cisternae and vacuoles are a common feature of the lucent cells. Golgi saccules occur in the vicinity of the nucleus (Figs. 9,16). Dense core vesicles are sparse in the perikaryon but lysosomes are of frequent occurrence (Figs. 9,15,16). The processes of the lucent cells adjacent to the perivascular space are also rich in dense core vesicles (100-250 nm, core = 80-150 nm) indicating the secretory nature of the cells (Figs. 9,13,15). Microtubules and microfilaments are not visible. In some cells a cilium and associated basal body are also encountered (Fig. 12). A very striking feature of the lucent cells is their relationship with the electron dark cells. The latter embrace them very intimately and often envelope the lucent cells completely (Figs. 15,16). Many lucent cell processes rich in dense core granules (Figs. 9,15) are seen surrounded by the processes of the dense cells.

The electron dense cells stand out on the basis of their distinctly different nuclear and cytoplasmic details and have a predisposition which suggests that they are supportive elements of the pineal. Their nuclei are small and of a highly irregular configuration (Figs. 7,9,15,16,17,18,19). The distribution of heterochromatin imparts a highly speckled and variegated appearance to the entire nucleus. The heterochromatin also forms a thick, nearly continuous marginal condensation. A nucleolus is present eccentrically, even marginally (Figs. 15,17,19) and is often associated with a dense clump of heterochromatin resembling nucleolar satellite. The inner and outer membranes of the nuclear envelope show a wider space (Figs. 7,9,17) than in the pinealocytes. The nuclear shape is elongate, squarish, polygonal and even pronged with lobes and indentations. The cytoplasm is stretched into processes which reach far and wide as spidery extensions (Figs. 7,8,9,15,17). The dark cells are so intimately intertwined that in many places it is generally not possible to delineate the boundaries of individual cells The cytoplasm of these cells contains Golgi saccules and always displays a preponderance of rod-shaped or worm-shaped mitochondria (Figs. 7,9) which appear swollen in some cells and are difficult to distinguish from vesicles or vacuoles with a flocculent content. The endoplasmic reticulum forms dilated channels sometimes in parallel rows (Figs. 7,9). Vacuoles containing granular or flocculent material (Figs. 8,12,16) are very common and frequently show extrusion

of their content into the pinealocytes (Figs. 15,16). Dense granules or vesicles are rare (Fig 15). Melanosomes sometimes fill their cytoplasmic processes (Fig. 18).The electron dense cells generally occupy a peripheral position in the parenchymal cords and appear quite distinct from the intermediate and the lucent types. Their relationship with the intermediate and the lucent cells is intimate and together with their unique ultrastructural features betrays a supportive status. The electron dense cells themselves show variations in both electron density and their subcellular features. Those cells which are relatively less electron dense (Figs. 17,19) possess a larger and robust nucleus with comparatively fewer clumps of heterochromatin. The cytoplasm contains mitochondria, Golgi saccules, cisternae of the endoplasmic reticulum with little evidence of dense granules. In the darkest of these cells, the nucleus assumes a dark, prunish, lobed and even pronged appearance (Figs. 9,15,16). The heterochromatin is condensed in the form of discrete patches along the nuclear margin as well as within the nuclear matrix. Enlarged cisternae of the RER and vacuoles with a flocculent deposit (lipid ?) abound in such cells (Fig. 18). Some of these cells also contain heavy deposit of melanosomes (Fig. 18). The changing nuclear and cytoplasmic morphology of these electron dense cells indicates a changing metabolic state. The darkest of the cells even give the impression of pycnotic change and cell degeneration (Figs. 9,18), especially those which contain melanosomes and vacuoles with flocculent content. Profiles containing granular condensate are sometimes encountered in the parenchyma which seem to be cell remnants (Figs. 14,18) and suggest cellular degeneration. The noticeable variations in nuclear features accompanied by cytoplasmic alterations noted above, at times, compel the impression that the dark cells are a transient stage in an ongoing degenerative loss of pinealocytes themselves. This would force the view that only a single category of cells, the pinealocytes, characterize the pineal in the cobra. It is, however, noteworthy that mitotic cells are never seen which would otherwise indicate cell replacement.

Second order neurons are entirely lacking in the parenchyma. On the other hand, abundant myelinated and unmyelinated nerve fibers are present in the stromal compartment (Figs. 13,18,20) where sinusoids and capillaries with fenestrated endothelium exist. These are characterized by microtubules (18-20 nm diam), mitochondria and often dense core vesicles (80-160 nm). Nerve axons are also discernible in the pineal parenchyma (Fig. 20). Typical

synaptic structures (ribbons, rods or spherules with clear vesicles) are also not noticeable. However, a pair of rod-shaped thickenings (Fig. 12) resembling synaptic rods could be detected in one of the intermediate cells near the cell margin. A few vesicles (clear and dense) exist in their vicinity.

Pineal of Krait *(Bungarus caeruleus)*

Light microscopic structure:

The pineal of this snake shows a general similarity in shape and organization to that of the cobra (Fig. 21). It is entirely solid with a distal vesicle which appears pyriform in sagittal plane. Compared with the cobra pineal, the vesicle in the krait is more dorso-ventrally compressed and laterally expanded as is also evident in cross sections. Its anterior end is broader and abuts on the cerebral eminences. The posterior end is tapered to a blunt point (Fig. 21). The major part of the vesicle rests on the anterior part of the midbrain and is visible superficially. The dorsal dural layer and the deeper pia-arachnoid tissue form an encasement which enloses the vesicle entirely. A thin connective tissue capsule forms the immediate investment of the pineal. Strands and trabeculae of the connective tissue together with blood vessels invade the pineal forming its stromal part (Fig. 22). The stroma and parenchyma are seen well demarcated in semithin sections (Fig. 23). The vesicle and the short stalk are entirely solid with no evidence of a central lumen. The parenchyma of the vesicle is compact throughout. It generally forms cords but few follicles enclosing lumenal residues are also noticeable in H and E as well as semithin preparations (Figs. 22,24). The stalk comprises continuation of the anteroventral aspect of the vesicle (Fig. 21). It is relatively broad at its origin but becomes very slender as it joins the posterior commissural region of the brain by a meningeal bridge, there being no evidence of a connection of the pineal epithelium with the subcommissural organ or communicaton with the third ventricle.

Three major cell types are noticeable in the semithin preparations, namely, deeply chromatic, clear and intermediate cells (Figs. 23,24). As shown for the cobra, the former are supportive cells and the latter two the pinealocytes. The deeply chromatic cells are much fewer than in the pineal of the cobra. They are comparatively smaller than the other cells with dark nuclei of irregular shapes. They generally occupy a peripheral position in the cords. The clear

cells are by far the largest in size with pale nuclei and clear cytoplasm. The intermediate cells are the most abundant. In some specimens, nearly the entire parenchyma contains intermediate cells. The hyperchromatic and the clear cells are fewer. Both the clear and the intermediate cell nuclei possess an eccentric nucleolus. They also contain granules and vacuoles (Fig. 23). The hyperchromatic cells and their processes often cap the other cell types. Myelinated nerve fibers are readily distinguishable invading the pineal along the connective tissue trabeculae and blood vessels (Fig. 23). The origin of these fibers could not be determined since these were seen in semithin sections of excised pineals stained with toluidine blue.

Electron microscopic structure:

As in the cobra pineal, at least three categories of parenchymal cells are distinguishable under the electron microscope in the krait as well (Figs. 25,26,27,28,29,30) and represent the pinealocytes and the supportig cells. These are electron lucent (clear), electron dense (dark) and intermediate cell types. The distribution of these cells in the pineal is variable. Many cords contain mainly the pinealocytes of intermediate electron density (Fig. 26) surrounded by the processes of the electron dense supporting cells. In other cords all three types exist (Fig. 25). Strands and trabeculae of connective tissue are rich in fibroblasts, collagen fibers and capillaries. One of the striking features of the krait pineal is the profusion of nerve fibers in the perivascular space (Figs. 26,33,35).

The electron lucent pinealocytes are the largest in size and usually round in shape. Nowhere do they present such bizzare shapes as the lucent pinealocytes in the cobra. Their nuclei are also round with a marginal rim of heterochromatin and widely scattered clumps of it in the nuclear matrix (Fig. 25). The nucleolus is eccentric. The inner and outer membranes of the nuclear envelope are separated by a narrow space. The cytoplasm is abundant and contains free ribosomes and evenly distributed rough endoplasmic reticulum which is scanty (Figs. 27,28). Mitochondria with thick transverse cristae are present throughout. They form small clusters in the vicinity of the nucleus and also elsewhere in the more remote parts of the cells (Figs. 25,27,28). Golgi saccules are also discernible in juxtanuclear position but with some difficulty. Lysosomes of variable size are frequent but dense-core or clear vesicles are rare in the

perikaryon. A few dense core vesicles (100-150 nm, core = 80-120 nm) are noticeable in the cytoplasm (Fig. 29). Microtubules and microfilaments are barely visible.

The most abundant cells in the parenchyma are the pinealocytes of intermediate electron density which seem to intergrade imperceptibly with the lucent cells. Their nuclei are generally similar in shape, electron density and distribution of heterochromatin to those of the lucent cells. However, squarish, oblong and elongate nuclear shapes are also common (Figs. 25,26,29). A single nucleolus occupies an eccentric position. A narrow space separates the inner and outer nuclear membranes. The cytoplasmic compartment of these cells is spacious which characteristically forms processes stretching between the adjacent cells. A very notable feature of these cells is presence of large vesicles and vacuoles in the cytoplasm (Figs. 25,26). Rough endoplasmic reticulum is conspicuous (Figs. 29,30) and free ribosomes and polysomes are more abundant in these cells than in the lucent pinealocytes. The greater electron density of the intermediate cells is largely due to the preponderance of these organelles. Rod-shaped mitochondria with thick transverse cristae resemble those in the lucent cells. They form clusters both in the vicinity of the nucleus as well as in the cell processes (Figs. 25,28,29,30). A Golgi complex comprising few saccules is more readily discernible in these cells than in the clear pinealocytes and occupies a juxtanuclear position (Fig. 30). Dense core vesicles (100-150 nm, core = 80-120 nm) are present and provide evidence of secretory activity but are not very abundant at least in the perikarya (Figs. 29,30,31). Lysosomes are of common occurrence (Figs. 26,29,30). Microtubules and microfilaments are barely visible. The intermediate cells are tightly packed, the plasma membrane of adjacent cells interdigitating intimately. Junctional densities are frequently seen between these cells (Figs. 29,30,31). Canalicular spaces apparently belonging to follicles are encountered in some places and represent highly attenuated portions of the lumen (Figs. 29,31).

Figure 31 shows a group of several cells whose apical ends protrude into a small diminutive lumenal space. A few microvilli project from the apical plasma membrane into this space. A cilium is seen arising from one of the cells and protrudes into this cavity. The apical cytoplasm of one of the cells narrows to a conspicuous neck beyond which it projects as a bulbous protrusion into the extracellular space. It bears microvilli and contains a prominent multivesicular body. The neck shows junctional specializations of the zonula adherentes type.

Such junctional complexes also exist between the adjacent cell processes of intermediate cells located in the immediate vicinity of the lumenal space. Mitochondria and lysosomes are abundant in the cytoplasm. Dense core vesicles are rare. These cells with inner and outer segment-like apical configuration resemble rudimentary photoreceptors of lizards. In other subcellular features they resemble the pinealocytes of intermediate electron density.

The electron dense cells are fewer in number than the pinealocytes (Figs. 25,27,32,33,34) and structurally quite distinct. Although these cells may occur anywhere in the cords and the follicles, they generally occupy a peripheral position. Basally they are separated from the perivascular space by a basal lamina (Figs. 27,32). Their contrasting structural identity and disposition indicates that these are supportive elements. Their nuclei are small in size and of highly variable configuration. Clumps of heterochromatin form interlacing dense patches throughout the nuclear matrix. The nuclei thus appear variegated. Nucleoli are not usually noticeable. The cytoplasm of the cells forms extensive processes which do not reveal microfilaments or microtubules but embrace and even envelope the lucent and the intermediate cells (Figs. 25,27,32,33,34). Their processes also thread their way between the pinealocytes. Near the perivascular space, their processes are seen to isolate the lucent and the intermediate pinealocytes from the basal lamina (Figs. 27,32). Mitochondria in these cells display the same features as in the pinealocytes (Fig. 32). Lysosomes are of frequent occurrence but vesicles and dense granules are not visible. Rough endoplasmic reticulum is present. In many processes, it is arranged in parallel stacks (Figs. 41,42). A Golgi complex is not readily discernible. Only rarely some of the supporting cells show cytoplasmic features similar to those seen in the supporting cells in the cobra and are characterized by vesicles which appear to be swollen and noodle-shaped mitochondria (Fig. 33,34). Vacuoles and cisternae are common in these cells. The proportion of such cells is rather low. Whether these variants of the supporting cells reflect a stage depicting degenerative change is difficult to judge. There is hardly any evidence of such advanced pycnosis in these cells as noted for the cobra.

There is a profusion of nerve fibers in the pineal of the krait. Both myelinated and unmyelinated fibers occur in the stromal tissue (Figs. 33,35,36). The origin of these fibers could not be ascertained. The myelinated fibers are very abundant and run in bundles. Unmyelinated fibers are also seen running along the myelinated fibers in the stromal

compartment (Fig. 36). The fibers are not restricted to the stromal compartment but also break through the basal lamina and enter the parenchymal tissue. Their longitudinal and cross sectioned profiles characterized by microtubules and occasional mitochondria are frequently detected between the intermediate as well as the clear cells (Figs. 37,38).Second order neurons are not detectable. Synaptic ribbons have also not been encountered.

Sinusoids and capillaries permeate the stroma abundantly.The endothelium of the capillaries is fenestrated and canaliculae communicate with the perivascular space (Fig. 38).

Pineal of saw-scaled viper (*Echis carinatus*)

Light microscopic structure:

The pineal of this viper shows striking differences when compared to that of the two elapid snakes described above. The organ consists of a conspicuous vesicular part. As in the krait, it is backwardly directed with its distal portion running over the midbrain (Figs. 39,40). The middle part of the vesicle is robust almost pear shaped in dorsoventral orientation. It narrows to a point (Fig. 40) as it courses caudally over the anterior third of the midbrain. Proximally (ventrally), the vesicle gradually narrows to a stalk which runs wrapped in pia-arachnoid tissue toward the subcommissural-postcommissural region (Fig. 41). The entire vesicle is a solid organ with no evidence of a central lumen (Figs. 39,41). Lacunae or canalicular spaces are present in different parts of the vesicle (Fig. 42). The stalk also does not readily reveal a central canal. The stalk seems to end blindly with only meningeal connective tissue forming a bridge with the posterior commissural area of the brain. The pineal, thus, seems to be isolated from the third ventricle. An interesting feature in this species is presence of small ectopic masses or clusters of the pineal tissue which lie outside the capsule of the pineal vesicle (Figs. 39,42). The median sagittal vein runs dorsal to the vesicle. The entire pineal, surrounded by a connective tissue capsule of its own, is encased by the dural layer dorsally and the pia-arachnoid tissue ventrally (Fig. 39). Connective tissue strands from the capsule invade the pineal and break up its parenchyma into lobular compartments of variable size. The pineal appears very vascular as is evident from the abundance of sinusoids and capillaries.

The epithelium of the pineal is organized in parenchymal cords but follicles are also seen here and there (Fig. 42). In H and E preparations, the arrangement of the parenchymal cells

appears somewhat loose due to some shrinkage of the tissue but in semithin sections (Fig. 43) and particularly in ultrathin sections (see below) it is very compact. An outstanding feature of the pineal of the viper is the extreme uniformity in cellular composition of its parenchyma. The entire parenchyma consists of cells which seem to be of a single type. All cells contain a large round to oval nucleus with a central or eccentric nucleolus (Figs. 42,43). Only rarely do nuclear shapes vary. Some cells display hyperchromatic, spindle shaped or shrunken nuclei and appear to be pycnotic (Fig. 42). That all of the parenchymal cells belong to a single type is further supported by presence of a round and pale staining body which occupies much of the cell cytoplasm in nearly the entire parenchymal cell population (Figs. 42,43). Owing to this the nucleus is pushed to the periphery of the cell. In some cells it is as large as the nucleus itself. In semithin sections it stands out as a dark osmiophilic structure adjacent to the pale-staining nucleus. Since ependymal, glia-like or other interstitial types of cells are not detectable in the pineal of the viper, it is deemed to consist of pinealocytes only.

Electron microscopic structure:

The surrounding connective tissue capsule (Fig. 44) and the strands which invade the pineal are rich in collagen fibers and carry with them sinusoidal blood vessels. The collagen fibers form a criss-cross latticework around the lobules of the pineal tissue. The single type of parenchymal cells reveals consistently similar structural details and electron density. Nearly all cells in the pineal series examined contain round to oval nuclei peripherally located inside a spacious cytoplasmic domain. The cells are arranged in cords and sometimes in rosettes and vary from nearly round to polygonal shapes with cytoplasmic processes emanating from various sides particularly from their angular aspects. Canaliculi are often seen in the interstices (Fig. 44). The plasma membranes of adjacent cells are very closely apposed (Figs. 44,45,46,47) indicating a very compact arrangement of the cells. Desmosomes and zonula adherentes-like membrane thickenings are frequently seen (Fig. 48). None of the cells presents contrasting electron density in contradistinction to the situation in the cobra and the krait. The nuclei appear euchromatic with an even disposition of chromatin. A conspicuous nucleolus occupies an eccentric to nearly central position. Only rarely are cells encountered whose nuclei deviate from round or oval shapes and possess lozenge-shaped, elliptical and indented nuclei (Fig. 46).

That these belong to the same category of cells is evidenced by the general similarity of their ultrastructural details. An unusual and outstanding feature of all of the parenchymal cells in the viper is presence of a large microfilament body located next to the nucleus in individual cells (Figs. 44,45). It is this mass of microfilaments which is noticeable as the pale-staining round body in H and E and osmiophilic body in semithin preparations. This patent feature of all of the parenchymal cells together with numerous dense granules and the characteristic nuclear configuration shows that these cells constitute the chief cells, the pinealocytes. Satellite cells comparable to the electron-dense supporting cells characteristically seen in the pineal of the cobra and the krait, "glial" or "interstitial" cells characteristic of the pineal of other vertebrates are conspicuously absent.

The nucleus of the pinealocytes occupies a peripheral position in the cell. The inner and outer nuclear membranes are separated by a clear space which appears dilated in places (Figs. 45,46). Much of the perikaryon of the pinealocytes is occupied by the microfilament body which can be as large as the nucleus itself. The body is a swirling ball of tightly packed microfilaments, one aspect of which presses against the nucleus and pushes the latter to the periphery of the cell (Figs. 44,45). Microtubules are hardly visible in this mass or in the surrounding cytoplasm. Along the margin of this body, individual microfilaments or their bundles swirl outward and spread to more remote parts of the cell. The density of the microfilaments decreases sharply towards the cell periphery. The cytoplasmic space around the nucleus and the microfilament body is occupied by scanty, discontinuous membranes of endoplasmic reticulum, free ribosomes, mitochondria, lysosomes, small stacks of Golgi saccules, clear vesicles and dense granules (Figs. 44,45,46,48). Dilated cisternae of the E.R., vesicles and vacuoles are very prominent in most cells. The E.R. appears to be mainly of the smooth type. Membranes and granules are present in some of the vacuoles (Figs. 46,48). The abundance of rod-shaped or worm-shaped mitochondria and lysosomes is a very impressive feature of the pinealocytes in the viper (Figs. 44,45,46,47,48). The mitochondria contain lamellar cristae oriented in oblique or longitudinal direction. They are particularly very abundant in the broad processes of the pinealocytes where their density is comparable to the ellipsoid (Figs. 46,47,48) so very characteristic of the inner segments of the pineal photoreceptors in anamniotes and lizards. A unique feature of the mitochondria and hence that

of the pineal of the saw-scaled viper is presence of dense granules in their matrix. These granules are 40-160 nm in diameter and are often arranged in rows consisting of 3-4 granules (Figs. 46,47,48). Similar granules exist free in the cytoplasm as well. It is difficult to discern whether their content is taken up from the surrounding cytoplasm and sequestered as granules by the mitochondria or synthesized intramitochondrially. Clusters of similar sized granules are seen free in the immediate vicinity of the mitochondria. Compared to the pinealocytes in the cobra and the krait, the propensity of lysosomes in the pinealocytes of the viper is strikingly very great (Figs. 44,45,46,47,48). These are freely interspersed with the mitochondria. Their preponderance imparts somewhat greater electron density to these pinealocytes compared to those where they are absent or scanty (see below). The lysosomes vary in size between 120-1200 nm. In some rare large cells they form very dense clusters exclusively of extremely large lysosomes (Fig. 47). Such cells are exceptional in the parenchyma and may be macrophages which have invaded the pineal. They lack the other ultrastructural features (dense core granules, cisternae of the E.R. and mitochondrial clusters) which otherwise characterize the pinealocytes. Many of the lysosomes in these cells are tailed or of dumb-bell shape indicating a pinching-off process. The lysosomes in the pinealocytes and their processes are often intimately associated with the dense granules which are also seen endocytosed by the lysosomes (Figs. 47,48). Unlike the very abundant lysosomes and mitochondria in the pinealocytes, Golgi saccules are not encountered as extensively. Whenever seen, they are represented by small stacks in a juxtanuclear position as well as in remote parts and processes of the pinealocytes (Figs. 48,50,53,54). Although clear vesicles occur in the immediate vicinity of the saccules, surprisingly dense-core vesicles and granules are only rarely seen in the Golgi fields. This is somewhat surprising considering that these vesicles (100-250 nm, core = 80-160 nm diameter) are abundant elsewhere in the pinealocytes (Figs. 45,47,48,50,51,52,53,54). The abundance of these granules is markedly very high in the processes of the pinealocytes, although clusters of these occur in the perikarya as well. Their intimate association with the lysosomes and among the mitoctondrial clusters has already been noted above. Very often they form linear arrays of unmiformly spaced vesicles pressed against the inner face of the plasma membrane of the cytoplasmic processes of the pinealocytes (Figs. 47,48). In their distribution, the vesicles do not follow any particular polarity format. They occur in parts of the cells adjacent to the

perivascular space as well as in areas of cell processes around canaliculi and lacunae representing the residual lumenal system (Figs. 51,52,53,54). The presence of the dense core vesicles and empty vesicles in the pinealocytes speaks in favour of their secretory status. In terms of their origin and packaging, it appears that the endoplasmic reticulum plays a major role as can be discerned in Figs. 48,49. Here numerous membrane-bound granules of 100-200 nm coexist with small clear vesicles and dense granules of variable size. Many of these granules form clusters in the immediate vicinity of the membrane-bound granules. Although the granular clusters resemble glycogen, they seem to be undergoing a process of coalescence and packaging in vesicles which ultimately achieve the size of the dense-core vesicles.

While all pinealocytes share the common features of large cell size, round to oval nuclei and presence of a microfilament body, they reveal differences in respect to some other ultrastructural features. Some clusters of interdigitating cells and their processes are encountered which are relatively less electron dense (Figs. 49,50). The nuclei of these cells contain conspicuous patches of heterochromatin which are particularly prominent along the nuclear margin. Lysosomes are not as abundant as in the other pinealocytes. Their paucity, in fact, is very striking. These cells contain abundant cisternae of the endoplasmic reticulum. The cells and their processes appear highly vesiculated and vacuolated (Figs. 49,50). The vesicles and vacuoles not only surround the massive microfilament body in the individual cells but also exist within it (Fig. 50). Dense core vesicles are very abundant. Many of these are entirely or partly empty. These also abound both in the microfilament body and around it. Dense granules occur in the cisternae as well as in the vacuoles and their sequestration in them is evidenced by endocytotic profiles (Fig. 49). They are present in the vicinity of the Golgi saccules and in the mitochondria (Fig. 50). The cells appear to be in an active secretory phase. The distinction between the two variants of the pinealocytes is particularly discernible in Fig. 51 where processes of several cells are seen in the vicinity of the perivascular space.

The secretory nature of the pinealocytes is not only very evident from association of their granule-rich processes with the perivascular space (Fig. 51) but also by their intimacy with the intrapineal canaliculi and the lacunar spaces (Figs. 52,53,54). In the latter case, the processes surround the spaces and contain abundant dense core vesicles. The clear spaces also contain numerous membrane-free granules. The cell processes seen in Fig. 52 are devoid of microvilli

but those shown in Fig. 53 bear microvilli, and free granules are abundant in the lacunar space which also is wider than that shown in Fig. 52. Numerous dense core vesicles occur pressed against the plasma membrane of the processes and there is also evidence of release of the granules by exocytosis (Figs. 52,53,54). Allthough some of the granule-like profiles are cross sections of the microvilli, most represent granules.

In contrast to the pineal of the cobra and the krait, neural elements could not be readily discerned in the pineal of the viper. Nerve cells (second order neurons) are absent. While the pineal stroma is rich in sinusoidal capillaries with fenestrated endothelium (Fig. 55) and collagen fibers, nerve fibers are difficult to see. The presence of one rarely detectable axon in the pineal parenchyma (Fig. 49) indicates that nerve fibers exist and that they also break through the perivascular space and make contact with the pinealocytes. Besides microtubules and neurofilaments, these axons contain dense-core vesicles as well (40-120 nm, Fig. 49). Whereas second order neurons are not discernible in the pineal, the processes of the pinealocytes contain synaptic ribbons crowned with dense-core vesicles. These ribbons lie next to the plasma membrane of the processes which face intercellular canaliculi (Fig. 56) or lie adjacent to the perivascular space (Fig. 56).

DISCUSSION

The parapineal (parietal eye, third eye) and the pineal proper (epiphysis cerebri) constituting the two components of the pineal complex present one of the most fascinating examples of structural, functional and evolutionary development (Dodt, 1973; Collin and Oksche, 1981; Vollrath, 1981). A detailed treatment of this subject has been documented in several treatises (Studnicka, 1905; Tilney and Warren, 1919, Bargmann, 1943; Kappers and Schade, 1965; Wurtman *et al.*, 1968; Oksche, 1971; Dodt, 1973; Eakin, 1973; Quay, 1974, 1979; Collin and Oksche, 1981, Vollrath, 1981; Oksche, 1986, 1989; Korf, 1994, 1995). The major trends in evolution of this complex are a gradual reduction (fishes) and ultimate loss of the parapineal (parietal organ) having reached its zenith in the lampreys, anurans, sphenodon and lacertilians, and a simultaneous shift in the pineal from a directly photosensitive-neuroendocrine organ in anamniotes to a purely neuroendocrine organ in the mammals which is only indirectly photoresponsive. The evolutionary direction taken by the pineal has involved changes in its morphological substrate, the most principal of which is transformation of the primitive photoreceptor cell of anamniote pineal into a secretory pinealocyte in mammals (Quay, 1974; Collin and Oksche, 1981; Oksche, 1983; Korf and Oksche, 1986; Korf, 1994). The reptilian group, in this scheme, has yielded valuable information pertaining to this transition and provided major support to the idea that the mammalian pinealocyte is a derivative of the photoreceptor cell-line. Concomittant with this change there has also been loss of the primitive central lumen of the pineal at the mammalian level depicting transformation of tubular or saccular pineals of lower vertebrates into solid pineals in mammals. The lacertilians, chelonians and most avian pineals have retained this lumen but with evidence for its diminution in certain avian groups (Galliformes : Renzoni and Quay, 1963, 1967; Collin and Oksche, 1981; Vollrath, 1981). The ophidians among the reptiles are intriguingly significant in showing a departure from the rest of the reptiles in revealing remarkable closeness of the pineal to the situation in mammals in many respects and, at the same time, displaying some unique morphological features of their own (Studnicka, 1905; Trost, 1952, 1953; Vivien, 1964, 1965; Milcou *et al.*, 1968; Petit, 1968, 1971).

In spite of voluminous information gathered during the last two to three decades on the pineal systems in different vertebrate groups, surprisingly the ophidian pineal has attracted the least attention of researchers in the field. Studnicka published his valuable review of the light microscopic studies on the pineal of snakes in 1905. Since then only a single species, *Tropidonotus natrix* (now revised as *Natrix natrix*) has been subjected to light and electron microscopic examination by several workers (Trost, 1952, 1953; Vivien, 1964, 1965; Milcou *et al.*, 1968; Petit, 1968, 1971). Thus, the work reported here on three species of snakes (*Naja naja, Bungarus caeruleus*, and *Echis carinatus*) fills a noticeable vacuum in the field of pineal research. Quay (1979) has provided a comprehensive review of the past work on the snakes. Work done in the late 19th century pertains to species blonging to only three Families, Boidae (Pythons, 2 species), Colubridae (5 species) and Viperidae (2 species of a single Genus). The present study for the first time provides information on the pineal of members of the Family Elapidae (Cobra, Krait) and adds original details for an as yet untouched Genus from the family Viperidae (*Echis carinatus*). This is also the first major study on poisonous snakes. According to the widely accepted classification of snakes, the families Elapidae and Viperdae represent higher snakes (See Smith, 1943; Stidworthy, 1974). In a recent systematic revision of Ophidia, the family Colubridae has been raised to a higher rank than the Elapidae (McDowell, 1987) but this proposition awaits wider acclaim.

The earlier work on snakes reviewed by Studnicka (1905) deals with young as well as adult animals examined at the light microscopic level. More recent developmental studies have been presented by Petit (1968, *Natrix natrix*) and Haldar and Pandey (1987, *Natrix piscator*). These researches on early development have shown that the pineal anlage includes both a parietal and a pineal rudiment. However, by the time adult stage is reached the two merge and form a single intracranial pineal vesicle connected to the brain by a short stalk. The adults of these snakes, thus lack a parietal organ. The same holds true for the adult cobra, krait and saw-scaled viper as demonstrated in the present study. Also, the general topographic relationships of the pineal in these species are similar to those known for the other vertebrates in general. The organ comprises a distal vesicular part and a proximal stalk. Compared to the other reptiles, however, the vesicle appears to be considerably enlarged especially in the two elapids (cobra, krait), being very prominent superficially. On the other hand, its shape is different and

also the size is comparatively smaller in the viper than in the elapids. This relative preeminence of the vesicular part of the pineal regardless of the species is accentuatd owing to a concomittant attenuation of the stalk. The most noteworthy aspect of the pineal in these snakes is the extremely compact organization of the vesicle with profound diminution of the lumen in the adult stage. A lumen is not clearly visible even in the pineal stalk in any one of the three species studied. A tiny remnant of it in the extreme terminal part of the stalk, however, suggests its presence in early development of the pineal. The entire pineal is thus solid containing compact cords and some follicles which retain portions of the lumen presumably pinched off during the course of early development. The vesicle in the saw-scaled viper is also solid but contains lumenal spaces reduced to small lacunae and canaliculi of variable size spread in its various parts. Whether these lacunae intercommunicate could not be fully resolved in the present study. Notwithstanding the above species-specific or even individual variations, the remarkable resemblance of vesicular compactness and organization in these snakes to the mammalian pineal is a most striking fact. Extreme diminution of the pineal lumen has been noted in other snake species studied to date (Studnicka, 1905) and also emphasized by Petit in her more recent study on *Natrix natrix* (1968, 1971) where it is reduced to merely small spaces here and there. Haldar and Pandey (1987) have also described a similar situation in their study of development of the pineal in *Natrix piscator*. In these species too the stalk is connected to the posterior commissural area by fibrous tissue. In respect to both the degree of compaction, attenuation of the lumen and reduction in size of the stalk, the pineals of these snakes stands apart from those which are characteristic of anamniotes as well as other sauropsida (non ophidian reptiles, birds), and shows greater affinity to that in the mammals -- a fact already appreciated by earlier workers (reviews: Tilney and Warren, 1919; Quay, 1979; Collin and Oksche, 1981; Vollrath, 1981; Korf, 1994). A tendency for formation of compact pineals has been evident as early as the fish level (Oguri, 1969; Hafeez, 1971) reaching a near mammalian situation in Galliformes birds (Quay and Renzoni, 1963, 1976; Ralph, 1970; Collin and Oksche, 1981). The follicular pineals of Passeriformes birds represent an intermediate situation. However, the degree of compactness achieved by the ophidian pineals remains unsurpassed at lower taxonomic levels and even in the birds. The pineal of snakes contains a

mix of cords and follicles; the latter always being much fewer as is evident in the cobra, the krait and the saw-scaled viper (See also Milcou *et al.*, 1968; Petit, 1971).

The cobra and the krait pineal organs reveal an apparent diversity in parenchymal cells particularly when nuclear shape, size and stainability of the cells are considered. The pineal of the viper, in contrast, shows remarkable uniformity in shape and size of the cells, thus, suggesting a single parenchymal cell type. The current view in this context is that the pineal parenchyma in the snakes studied so far contains only pinealocytes (Studnicka, 1905; Tilney and Warren, 1919; Vivien, 1964; Milcou *et al.*, 1968; Petit, 1971; Quay, 1979; Collin and Oksche, 1981; Vollrath, 1981; Korf, 1994). Although earlier discourses on the subject (Studnicka, 1905) have failed to discuss the supportive components of the pineal parenchyma in detail, Petit (1971) has reported absence of "glial" or other supportive types in *Natrix natrix* which are otherwise characteristically present in the pineals of all other vertebrates. Thus in *Natrix natrix*, the parenchymal cords have been argued to contain a single cell type, the pinealocytes (Petit, 1971). In spite of showing tinctorial differences in semithin sections and differential electron density at the ultrastractural level, Petit has argued that the differences in their electron density reflect variation in the physiological state of a single cell type, the pinealocytes. The pineal of the cobra and the krait also reveals this very unique feature, that is electron lucent, electron dense and intermediate cell types. However, dense core vesicles and granules as well as a secretory polarity depicted by perivascularly directed terminals is evident to any appreciable extent in these species in the intermediate and the lucent cell-variants only. These cells which also possess the other usual cell organelles thus represent pinealocytes. The electron dense (dark) cells, on the other hand, have a very different morphology and in view of their relationship to the pinealocytes seem to be supportive components of the pineal parenchyma. Subtypes of pinealocytes *sensu stricto* represented by electron dark and electron light cells are a characteristic feature of the pineal in many mammalian species (Pevet, 1974, 1976, 1977; Reiter, 1978; Calvo *et al.*, 1988). Such a distinction into subtypes is rare in the pineal organs of anamniotes, lizards, chelonians and birds.

Although the pinealocytes in the cobra and the krait show remarkable similarily to the mammalian pinealocytes in their unipolar to multipolar structure and vascular polarity, they reveal a paucity of both microtubules and microfilaments. Microtubules are generally, though

not always, known to be abundant in the photoreceptors of anamniotes, Sauropsida (McNulty, 1984; Collin and Oksche, 1981) and mammals (Wolfe, 1965; Anderson, 1965; Arstila, 1967). Microfilaments are a characteristic feature of the pineal glia cells (Wolfe, 1965; Anderson, 1965; Arstila, 1967; Wartenberg, 1968). The secretory cells in the pineal of *Natrix natrix*, designated by Vivien (1964) and Petit (1971) as pinealocytes, are similar to the pinealocytes in the cobra and the krait in showing variations in electron density but contain a rich supply of microfilaments. In spite of this enigmatic preponderance of microfilaments which are otherwise more characteristic of glia, Petit has designated them as pinealocytes on the strength of their secretory predisposition and other features. Glial cells are thus not claimed to exist in *Natrix natrix*. Both the pinealocytes and the glial cells are derivatives of neuroectoderm with ependymal cells being the remote precursors of both. Detailed developmental studies would go a long away in shedding light on this aspect of snake pineals. In these two species much of the ultrastructural evidence also suggests that the intermediate and the lucent cells represent two different physiological states of the same cell type (See also Petit, 1971, *Natrix natrix*; Pevet, 1977; Pevet and Kuyper, 1978: some mammals). This interpretation is supported by the fact that the two variants intergrade into each other imperceptibly in many parts of the pineal parenchyma. Thus, it is sometimes difficult to designate given cells as lucent or of intermediate type. Furthermore, the relative proportion of the intermediate and the lucent cells in the krait shows variations from specimen to specimen aside from showing lack of a sharp distinction between the two in many parts of the epithelium. The intermediate and the lucent cells, both contain secretory granules, have vascular polarity and a similar intrapineal regional distribution as well as features of the Golgi saccules and mitochondria. They differ from each other in size, electron density, and to some extent in abundance and organization of the rough endoplasmic reticulum. Experimental studies involving manipulation of environmental factors (such as photoperiod or even temperature) or collection of animals at various ages and seasons may be needed to further resolve whether the lucent and the intermediate cells are pinealocytes with fundamentally different functions as has been suggested for the light and dark pinealocytes in gerbil, macaque, mouse, rabbit and rat by Arstila (1967). It is also noteworthy that in both the cobra and the krait, neither synaptic ribbons nor axial ciliary filaments are as frequently encountered as are characteristic of the rudimentary photoreceptor cells of other reptiles

(review: Quay, 1979), birds (Ueck, 1973) and to some extent in the pinealocytes of mammals (Pevet *et al.*, 1977a,b; Chang *et al.*, 1978; Calvo *et al.*, 1988, 1990). Profiles with a 9 x 2 + 0 axonemal arrangement and cilia are detectable rarely and thickenings resembling synaptic rods in some cells would suggest that the pinealocytes in these snakes are phylogenetically related to the primitive photoreceptor cells. The likehihood that some populations of the pinealocytes in snakes are also related to the line of mammalian-like pinealocytes described in the lamprey pineal (Tamotsu, *et al.*, 1990) should not be entirely ruled out. Concerning the question of synaptic ribbons, it is well known that they are responsive to environmental light and show 24-hour variation in their numbers in the pineal of mammalian species (Vollrath, 1973; Vollrath and Huss, 1973; Karasek, 1976; Soriano *et al.*, 1984; Struwe and Vollrath, 1990). In this connection it may be noted that all snake specimens in this study were sacrificed in day time and infrequency or insufficiency of the synaptic ribbons in their pineal may be related to the time of autopsy. Infrequency or absence of the synaptic ribbons has also been demonstrated for the pineal of the horse (Cozzi, 1986) and the vampire bat (Bhatnagar, 1988). In the present study, ultrastructural details of parenchymal cells lining the lacunae or the residual lumenal spaces could be visualized under the electron microscope in the krait and the viper only. Some of these cells in the krait resemble the highly reduced photoreceptors (rudimentary) common in the pineal of lizards and birds (Collin and Oksche, 1981). The presence of a cilium or an apical projection into the small lumenal space as well as necks with junctional thickenings is reminiscent of modified photoreceptor cells and lends support to the idea that the pinealocytes in the krait belong to the photoreceptor-cell line. Dense granules are present but are not very abundant and suggest a secretory nature of these cells. Ependymal cells lining the residual lumena in *Natrix natrix* have similar features except that in this species they are very rich in melanin pigment and bear processes which Petit (1971) has considered to be apocrine secretion but the possibility that these represent rudiments of outer segments has not been entriely discarded by her. Presence of photoreceptor type cells with outer segment-like protrusions has also been claimed in *Natrix natrix* by Trost (1953). A study of the pineal of the krait and even the cobra in early development is warranted to gain deeper insight in this connection.

The electron dark cells in the pineal of the cobra and the krait show very different ultrastructural features from those of the pinealocytes. Cells with such features have not been

reported in the pineal of any other vertebrate. They do, however, show a superficial resemblance to the electron dark cells in the pineal of the rat (Calvo and Boya, 1984), the interstitial cells in the pineal of the horse (Cozzi, 1986) and the glia in the pineal of the vampire bat (Bhatnagar, 1988). These dark cells in the cobra and krait show wide structural differences. In the cobra, these cells show a wide range of variation in nuclear shape and the pattern of heterochromatin distribution. At one extreme are cells with a relatively large robust, variegated nucleus and at the other are cells with very irregular, even highly indented and multipronged nuclei containing heavy and clumpy condensation of heterochromatin. In addition, cytoplasmic variations are also evident reflecting a changed physiological state. In the most advanced stage of change, the cytoplasm looks highly vesiculated and vacuolated apparently owing to changes in mitochondrial integrity and formation of vacuoles with granular, flocculent and lipid-like content. Parallel arrays of flattened or dilated endoplasmic reticulum as well as cisternae constitute characteristic feature of these cells. Perhaps, the most outstanding feature of these electron dense cells is the presence of extensive, almost spidery, thread-like processes which meander far and wide between the pinealocytes. It is difficult to suggest homology of these cells with any of the typical glial cell varieties or the interstitial supportive cells described in other vertebrate pineals. In respect to their cytoplasmic extensions and intimate satellite-like association with the pinealocytes they resemble glial elements of the mammalian pineal even though microfilaments are difficult to discern. No valid arguments concerning their exact indentity can be extended at this stage. An exogenous origin (microglial) of these cells with invasion of the parenchyma remains an open question but appears to be very remote. Enigmatically, some of these cells look pycnotic and hence verge on a state of degeneration. Regardless of this, the general relationship of the dark cells with the pinealocytes and position in the parenchymal cords is very similar to the supportive elements of the pineal organs in general. Cisternae and vacuoles containing a flocculent or granular material are abundant in these cells in the cobra. The cells show a very intimate association especially with the lucent pinealocytes. The exact significance of their relationship is unclear but mutual exchange of materials seems to be the most possible one. Supportive cells (interstitial, glia) in the anamnia, sauropsida and even in several mammalian species (See Wartenberg, 1968) are known to isolate the photoreceptor and pinealocyte basal processes from the perivascular space and

serve as intermediaries in exchange of materials between them and the blood (Collin and Oksche, 1981). Since most pinealocytes in the cobra seem to have direct access to the perivascular space via their terminals here, a role of these cells in controlling release of pinealocyte secretions into the blood and exchanges with it may be only limited. Whether these cells have a bearing on the hypertrophied appearance of the lucent pinealocytes deserves to be explored. It should be noted that hypertrophied pinealocytes have been demonstrated in the pineal of rodents and humans as well (Bargmann, 1943). The electron microscopic images of the advanced postnatal (45 day) pineal of the rabbit (Garcia-Maurino and Boya, 1992) show a general similarity to the images described for the cobra pineal here, especially the relationship of the dark and the lucent cells there.

In the context of single and multiple cell types, an alternative view which cannot be entirely set aside is that the pineal in the cobra comprises only a single category of parenchymal cells, the pinealocytes. The electron dense cells, given the range of variation, represent a transient stage in an ongoing process of pinealocyte degeneration. The extremely electron dense cells with pycnotic and prunish or multipronged nuclei and highly vacuolated melanosome-rich cytoplasmic processes depict an advanced state in the sequence of events. Such a view tends to negate existence of supportive elements in the pineal of the cobra and would be in line with the currently accepted idea. It also leads to the unavoidable conclusion that the pineal in the adults of this species undergoes extensive degeneration as time progresses, especially since mitotic process reflecting cell replacement is not evident. The weight of evidence presented in the present study, however, speaks more in favour of multiple cell types in the cobra pineal with, at best, the supporting cell category showing degenerative changes.

Interestingly, the structure, proportion and relationships of the electron dark cells in the krait pineal differ much from the situation in the cobra pineal and argue in favour of multiple cell types in the elapid pineals. There is hardly any evidence of pycnosis and cell degeneration. Also, although some pinealocytes in this species may send processes directly to the perivascular space, in most places they seem to be isolated from this space by the electron dense cell processes. Thus, there is compelling evidence here in favour of their identity as supportive elements and that these cells have a major role in controlling exchanges between the pinealocytes and the blood.

In contrast to the pineal in the cobra and the krait, the pineal of the saw-scaled viper shows several unique features of its own. Unlike the pineal in anamnia and sauropsida including most other snakes, its vesicular part tapers to a point posteriorly and runs over the midbrain surface for quite some distance. A posteriorly directed extension of pineal vesicle is evident in the cobra and in the krait but it does not extend too far over the midbrain. The situation in this viper is very different from that in the lizards where the tapering vesicular part of the pineal is directed anteriorly and runs forward over the cerebral hemispheres for a considerable distance. The middle part of the vesicle in the viper is generally very robust. It narrows as it curves ventrad to run toward the subcommissural region of the brain where it ends blindly attached to this area by a meningeal bridge. In being entirely solid, it conforms to the mammalian type of pineals. Canaliculi or lacunae, represent the only remains of a lumen which otherwise is a characteristic feature of the pineal of the anamniotes and other reptiles. In this respect, the pineal of the viper is also comparable to the pineal in the elapid snakes where a lumen is not detectable even in the stalk. Presence of ectopic masses of pineal tissue is another noteworthy feature of this viper. Ectopic pineal tissue has neither been seen in the elapid snakes studied presently nor in *Natrix natrix* (Vivien, 1964; Trost, 1952; Petit, 1971) or demonstrated in any other vertebrate species so far.

One of the most striking features of the pineal of the viper is that its parenchyma consists of a single cell type, the pinealocytes. Although it is possible to detect what appear to be two variants of the pinealocytes, "supporting", "interstitial" or "glia" cells are not recognizable in the pineal of this species. In this respect it contrasts with the pineal of the cobra and the krait where the electron dense cells have been demonstrated in the present study to represent the supporting elements. The situation in the saw-scaled viper conforms to the prevalent view that the ophidian pineal parenchyma contains only pinealocytes (Trost, 1952; Vivien, 1964; Petit, 1971; Collin and Oksche, 1981).

Light microscopically, the pineal parenchyma in the viper reveals cells with remarkably similar morphological appearance, that is, large, round to oval, eccentric nuclei and a globular cytoplasmic body (microfilament body) next to the nucleus. Ultrastructurally, also the nuclei are consistently round to nearly round, most of which are euchromatic. Some pinealocyte variants demonstrate an appreciable amount of nuclear heterochromatin. The former cells are

rich in lysosomes whereas the latter contain very few lysosomes. However, both of these varieties possess the microfilament body, abundant dense core granules, small Golgi saccules and mitochondria are numerous. The endoplasmic reticulum is dilated and forms prominent cisternae and vacuoles particularly in the latter cell variety. The processes of these cells adjoin perivascular space as well as canalicular or lacunar spaces and are rich in dense core vesicles suggesting release of their secretory content in the perivascular space as well as in the residual lumenal system. The universal presence of dense core vesicles and the microfilament body in the parenchymal cells leaves little doubt regarding the identity of the two variants as pinealocytes. The noticeable differences in respect to nuclear chromatin, lysosomal abundance and vacuolation of the cytoplasm would seem to reflect merely variation in the physiological state of a single cell type rather than giving them the status of distinct morphological entities with different functions.

Microfilaments have been described to be abundant in the pinealocytes in *Natrix natrix* as well (Petit, 1971). In this species too, they from dense swirling masses but these are hardly so well organized as a massive body as in the pinealocytes of the saw-scaled viper. The other cells which are known to display a similarly impressive organization of the microfilaments as in the viper are the ganglion cells in the nervus terminalis in some teleost fishes (*Tinca tinca, Leuciscus cephalus, Epinephelus guaza*; Rossi and Palombi, 1969) where they constitute a neurofibrillar body. In these fishes, this body consists of a similar swirling mass of microfilaments (10 nm thick) and microtubules (30 nm thick). The body is nearly as large as the nucleus itself. The microfilaments in this body outnumber the microtubules and maintain a specific spatial relationship where a single neurotubule is surrounded by 9-10 equidistant microfilaments (neurofilaments). In the viper such an arrangement is not detectable. In fact, microtubules are hardly detectable in the pinealocytes of this species. The tangled mass of the microfilaments in the pinealocytes encloses mitochondria, dense granules, lysosomes and lacks a surrounding membrane unlike the case in the ganglion cells in fishes (Rossi and Palombi, 1969). A somewhat similar whorl of microfilaments has also been reported in the spinal ganglion cells in a lizard (*Lacerta muralis*, Pannese, 1963). These observations in the lizard and the fishes show that the neurofibrillar bodies are of unique occurrence in some neuronal elements. The pinealocytes (including the photoreceptors of the third eye and the pineal

organs) too are modified first order neurons. Thus the presence of the microfilament body in these cells is not surprising. What is most surprising, however, is that such a specialized arrangement of microfilaments has not been known for any of the pineal parenchymal cells in other vertebrates. Whereas microfilaments are dense in the pinealocytes of *Natrix natrix* (Petit, 1971), both the microtubules and the microfilaments are sparse in the pinealocytes of the cobra and the krait. Such a paucity of these organelles can hardly be relegated to the fixation technique used in the present study. The pineals of all the three species were fixed in Glutaraldehyde followed by identical procedures of postfixation in OSO_4 but only the viper reveals abundant microfilaments. In regard to the functional significance of the microfilament body, it is interesting to note that whorls of neurofilaments of similar type have been observed during regeneration and degeneration process in the ganglion cells of the crustacean, *Cambarus bartonii* (McCurdy, 1910) and in chick tectum (Gray, 1964). These works and the studies of Glees and Le Gros Clark (1941, Optic tract) have correlated the phenomenon of neurofibrillar densities with degenerative processes. Whether the properties of the pinealocytes together with the propensity of lysosomes in the viper pinealocytes have any relationship with aging process cannot be adequately assessed at this time. The specimens of this viper examined in the present work do not reveal any unusual phenomenon of cell degeneration which could be construed to indicate progressive change in such a direction. Besides, nearly the entire parenchymal cell population in the pineals examined contains this body and appears robust and "healthy". Although pycnotic cells exist here and there, these are rare. Degenerating or atrophic cells and phagocytic activity to a similar extent are a normal phenomena in the pineals of anamniotes (Oksche, 1965; Kelley and Smith, 1964; Hafeez and Merhige, 1977; Vollrath, 1981), other sauropsidan pineals (reviews: Wurtman *et al.*, 1968; Quay, 1979; Collin and Oksche, 1981) as well as in mammals where focal degradation and atrophy have been associated with season (Country rat: Huang *et al.* 1989) or aging (rat: Humbert and Pevet, 1995). In the aged rat decline in pinealocyte number is correlated with decreased production of melatonin and pineal function (See also Reiter, 1994, animals and humans). What precise cell biological role the microfilament body *per se* plays in the pineal of the saw-scaled viper or whether indeed it reflects an aging process can only be resolved further by developmental (age-wise) and experimental or season-based studies. For the present, one may merely argue that the

microfilament body may be, somehow, involved in intramural distribution of the secretory products (Theron *et al.*, 1979: baboon) as well as positioning of cell organelles (Sturmer *et al.*, 1994; Baumann, 1994: locust and honey bee photoreceptors) within the cells particularly that of the mitochondria closer to the cytoplasmic margin and in the cell processes. The position of the organelles has been demonstrated, in the insect photoreceptors, to vary according to photostimulus. Furthermore, the microfilaments are associated with smooth E.R. and abundant mitochondria; features noted in the saw-scaled viper pinealocytes as well.

The pineal of the saw-scaled viper is also unique in regard to the conspicuous presence in the mitochondria of dense granules of the same size as in the dense core vesicles, and they occur in arrays in the mitochondrial matrix. Nothing comparable to this has been reported in the pineal of other vertebrates. Yamamoto *et al.*, (1969) have described a variety of mitochondrial inclusions, globules, vesicular, granular ad filamentous structures in the mitochondrial matrix in various organs (proximal convoluted tubule epithelium, intestinal epithelium, adrenal tissue) in both the hibernating and the arousing striped snake. The homogeneous material in the mitochondrial matrix of the adrenal cortex and testicular cells has been associated with steroid production. Of the various materials, the dense granules (60 nm dia) in the striped snake mitochondria come closest to those seen in the pinealocyte mitochondria in the viper. These granules have been considered to be proteinaceous (Yamamoto *et al.*, 1969). The significance of the granulation in the mitochondria and the close association of the mitochondrial clusters with the dense core vesicles in the viper pinealocytes requires further study. Such intramitochondrial granules do not seem to be restricted to *Echis carinatus* but also characterize the pinealocytes in another viper, the taxonomic identity of which needs confirmation (Hafeez, unpublished). The pineals of the elapid snakes do not display this peculiarity. Is it a viperid feature ? Only further exploration can resolve these considerations.

The morphology of the pineal in the three species of snakes reported in the present study reaffirms the extremely endocrine-like nature of the organ. This stands in sharp contrast to the circumventricular type (tubular, saccular) of organization of the pineal in anammiotes and other reptiles. The highly compact and endocrine type organization of the pineal in the snakes has been evident in nearly all of the species described to date by means of light microscopy

(reviews, Studnicka: 1905; Tilney and Warren, 1919) and electron microscopy (Vivien, 1964; Milcou *et al.*, 1968; Petit, 1971). It is noteworthy, however, that the number of species examined so far, including the species studied presently, is very small if one considers the over 3500 species of snakes known today (Stidworthy, 1974). Two major changes, among others, in the pineal morphology accompany the departure from the circumventricular pineal types to the supraventricular and solid mammalian type. The first of these is the diminution and nearly complete loss of a central lumen along with loss of an epithelial connection of the stalk with the intercommissural area of the brain. The second is the cellular shift from a photoreceptor-cell type to a purely secretory-cell type, the pinealocyte. In *Natrix natrix*, a lumen, continuous with the third ventricle, is prominent during early development which is reduced to a system of minute lumenal spaces in the adult stage. This results in loss of open communication of the organ with the ventricular space (Petit, 1968, 1971). Interestingly also, the epithelial connection of the pineal stalk with the intercommissural area of the brain seems to have been lost in all the three species of snakes studied presently. This parallels the situation in the mouse (Vollrath, 1979), the vampire bat (*Desmodus rotundus*, Bhatnagar, 1988) and also the water snake (*Natrix piscator*, Haldar and Pandey, 1987). Whereas the situation in the cobra, the krait and the viper marks striking convergence with the mammalian situation in respect to lumenal diminution, light microscopic study by Asmatullah and Hafeez (unpublished) on several other snakes reveals wide variations in this respect. Here retention of a small but conspicuous lumen characterizes the otherwise very compact and parenchymatous pineal in *Ptyas mucosus* and *Coluber* sp. (Family: Colubridae). On the other hand and very surprisingly, the pineal in Russel's viper (*Vipera russeli*) is quite saccular with a wide cavity in the proximal part of the vesicle and which communicates with smaller cavernous spaces in its more distal parts. The epithelium itself is thrown into compact and solid cords with few follicles and rosettes. This contrasting situation in members (*Vipera russeli* and *Echis carinatus*) of the same family (Viperidae) makes it difficult to arrive at any unified phylogenetic scheme of change in respect to a trend in lumenal attenuation. The observations on the species examined to date, however, collectively provide sufficient grounds to examine a wider range of species, particularly those belonging to the more primitive families of the Serpentes (Ophidia) to determine if typical lacertilian organization does exist in the less advanced snakes.

The currently accepted departure in snakes (reviews: Quay, 1979; Collin and Oksche, 1981) from the well established norm in all vertebrate groups with respect to cell types in the pineal parenchyma, that is, chief cells (photoreceptors/pinealocytes *sensu stricto*) and supporting cells (interstitial, glia), is another area of profound interest from both ontogenetic and phylogenetic stand point. The demonstration of secretory pinealocytes of the mammalian type, together with arguments favouring supportive cells in the pineal of the cobra and the krait, contrasts with the current view of exclusive presence of pinealocytes in the snakes studied in the past (Studnicka, 1905; Petit, 1971). The saw-scaled viper, on the other hand, is the only species examined here which conforms to the presently generalized pattern in the snakes. While there are sufficient grounds to suggest that the electron dark cells in the pineal of the cobra and the krait are morphologically very different from the pinealocytes and represent supportive elements, their exact identity (glial or other) remains uncertain. In the case of mammals, immunolabelling studies have been quite fruitful in defining glial elements of the pineal (Schachner *et al.*, 1984; Calvo *et al.*, 1988). A similar preliminary attempt using GFAP antigen to characterize the "glial" components in the cobra, and the saw-scaled viper proved abortive (Hafeez *et al.*, 1995). There is hardly any evidence of glia-specific microfilaments in the cells in the cobra and hence the negative results. Additional work is, however, needed using epitopic variants of GFAP as well as other glial markers before making any firm conclusions regarding possible glial nature of the supportive elements in these snakes. Notwithstanding the above, the existence of more than a single cell type has also been proposed for a few additional colubrid snakes (*Ptyas mucosus, Coluber* sp., Asmatullah and Hafeez unpublished) on the basis of light microscopy. Affirmation of this view awaits electron microscopic examination. The present observations on the three species of snakes are indicative of divergent trends within the ophidian group. No valid correlations of such trends with the systematic position of the species can be established at the moment. Contrary to the presently accepted systematic status of Elapidae, McDowell (1987) has placed this family below the level of Colubridae and Viperidae within the Superfamily Colubroidae. Collectively, the results of the present study would then place the duality of cell types in the elapids (Cobra, Krait) as reminiscent of the generalized vertebrate pattern with the viperids displaying the rather unusual and currently accepted ophidian trend of retaining only the pinealocytes. Whereas more work is needed for reaching

definitive judgements in this context, the concept of single and multiple cell types has even deeper implications regarding functional cellular interactions and cellular relationship with the intrapineal perivascular space. It has been commonly argued that the supporting cells ("glia", "interstitial", "ependymal") not only have a supporting or nutritive role but they also isolate the pinealocytes from direct access to the perivascular space implying that release of pinealocyte secretions and exchange of materials with the blood occurs through the agency of the former (reviews: Collin and Oksche, 1981; Vollrath, 1981). In snakes, where pinealocytes alone comprise the entire parenchymal cell population (*Natrix natrix*, Petit, 1971; *Echis carinatus*, this study), such a restriction on release of materials would seem to have been dispensed with. The significance or advantage of such a change remains an open question. Direct access of the pinealocytes to the perivascular space is not unique to the pineal of snakes. In mammals, some or most pinealocytes in a number of species make direct contact with the basal lamina and are free from glial interposition (bovids: Anderson, 1965; monkey: Wartenberg, 1968; rat: Wolfe, 1965; Arstila, 1967).

The secretory pinealocytes of mammals have been most generally considered to be derivatives of the photoreceptor cell line (Collin, 1969; Collin and Oksche, 1981). The existence of cilia, ciliary rootlets and synaptoid structures in the pinealocytes support derivation of these cells by a gradual process of rudimentation of the apical photoreceptive pole and basal synaptic association. Appearance of outer segments during ontogenetic development of the Noctule bat has been demonstrated by Pevet *et al* (1977 a,b). Ciliary rudiments are present in the pinealocytes of the cobra and the krait together with synaptoid elements even though the latter are rare. On the other hand, cilia or ciliary rudiments are not at all detected in the pinealocytes of the saw-scaled viper while synaptic ribbons are present. These observations support that the ophidian pinealocytes too are derivatives of the sensory cell line and, as hinted by Quay (1979), the shift from photoreceptor to the secretory pinealocyte stage or departure from the lacertilian pattern occurred early in the ancestors of the snakes. Immunocytochemical detection of photoreceptor-specific proteins in the snakes (*Thamnophis radix, Thamnophis sirtalis*) places the ophidian pinealocytes closer to the anamniote (trout) pineal photoreceptors than to the mammalian pinealocytes (Kalsow *et al.*, 1991). On the other hand, immunoreactions carried out with polyclonal antibodies against rod-

opsin, cone-opsin and S-antigen on the pineal of the cobra, Russel's viper and saw-scaled viper (Hafeez *et al.*, 1995) have given negative results. This has suggested that the pinealocytes in these snakes are of the type found in primates. Multiple populations of pinealocytes (photoreceptors as well as pinealocytes *sensu stricto*) have appeared even as early in evolution as the lampreys and teleosts (Meiniel, 1981; Ekstrom, 1987; Ekstrom and Meissl, 1990; Tamotsu *et al.*, 1990). Thus, it is difficult to ignore the possibility that the ophidian and the mammalian pincalocytes may represent both lines of evolution, the photoreceptor-cell line as well as the pinealocyte line *Sensu stricto*. This implies a parallel evolutionary process of regression and disappearance of the photoreceptors (typical or rudimentary) and a progressive increase in pinealocytes of independent origin (See Tamotsu *et al.*, 1990) in the snakes. Although the results of immunocytochemistry obtained for the pineal of cobra, saw-scaled viper and Russel's viper (Hafeez *et al.*, 1995) have revealed absence of rod-opsin, cone-opsin, and S-antigen and would partly support the above idea, further work is needed to strengthen the concept of multiple origin of the snake pinealocytes.

There is overwhelming evidence that the pineal of snakes is of exclusively secretory type (review: Quay, 1979). This is also true for the elapid and the viperid species studied presently. This is evident not only from complete absence of typical photoreceptor cells but also from presence of dense core secretory vesicles (100-250 nm) in the pinealocytes and their processes. The abundance of the vesicles varies with the species, at least under the conditions in which the animals were sacrificed. Although the pinealocytes of the krait show fewer secretory granules compared with those in the pineal of the cobra and the saw-scaled viper, their secretory nature is above doubt. Secretory granules have been observed to be generally moderate or few in the mammalian pinealocytes as well (Korf, 1994) (excepting hamster, mouse and golden mole: Pevet and Kuyper 1978) but their intrapineal morphological and perivascular relationships and responsiveness to environmental factors has left no question regarding the neuroendocrine capacity of the mammalian pineal. In the snakes under consideration too there is overwhelming evidence for release of granules in the perivascular space either directly or through the agency of the supportive elements. Since there is no clear evidence of a continuity of the diminutive lumenal or lacunal system in the pineal with the third ventricle in any of the three species of the snakes under report, this remains the principal route of secretion. What then is the significance

of the lacunae or canaliculi in the cobra, the krait and the saw-scaled viper? There is in fact unquestionable evidence of release of granules in the canaliculi in the pineal of the saw-scaled viper. The possibility that these spaces ultimately communicate with the perivascular space cannot be ruled out as has been demonstrated in several mammalian species too (Quay, 1974; Pevet and Kuyper, 1978; Ueck, 1981). Alternatively, this canalicular release may allow some kind of intrapineal intercellular communication.

The question of metabolic regulation of the pineal as well as its influence on target systems invites attention to its neural associations. The primitive pineal systems of anamniotes are endowed with sensory cells making synaptic contacts with a network of intrapineal neurons (Wake, 1973; Korf, 1974; Ueck, 1979, 1989; Ekstrom and Meissl, 1990; Ekstrom *et al.*, 1990) which ultimately send pinealofugal pathways into various centres in the brain (Paul *et al.*, 1971; Hafeez and Zerihun, 1974; Ekstrom and Van Veen, 1983, 1984; Ekstrom, 1984, 1985). Such a circuitry provides the substrate through which these pineal systems are able to directly relate photic influences (Photoperiodic inputs) with central and peripheral physio-behavioural activities. Already at the reptilian level, there is ample evidence of rudimentation of the basal neural circuitry involving reduction in intrapineal second order neurons and loss of synaptic associations. In this context, the ophidians present an extreme case where intrapineal second order neurons have not been reported in any species studied to date including the three species presently under consideration. Retention of vesicle-crowned synaptic ribbons in *Natrix natrix* (Petit, 1971), the elapid and the viperid snakes (this study) parallels the situation in most sauropsids and the mammals and suggests reduction in direct neuronal connection between the pineal and the brain. A direct neural influence of the pineal on the brain in snakes cannot be envisioned at least in view of the currently available structural evidence especially due to insufficient information on neural relationships of the pineal with the brain. However, caution is warranted in the light of rapidly changing views regarding the neural associations of the mammalian pineal organs which until recently were considered to be endowed with only autonomic innervation (Kappers, 1960, 1965; Quay, 1974; Collin and Oksche, 1981; Vollrath, 1981). A variety of techniques including immunocytochemistry have demonstrated that both pinealopetal and pinealofugal pathways directly relate the mammalian pineal with the brain especially with its habenular area (Korf and Moller, 1984, 1985; Moller, 1992, Korf, 1994,

1995). Whether such neural relationships hold true for the ophidian group as well can only be settled by application of diverse techniques to fully define neural pathways. Whereas there is no clear indication of epithelial continuity of the pineal stalk with the habenular and subcommissural areas in the elapids and the viperid snake, it is possible that nerve fibers run along the connective tissue holding the pineal stalk with the intercommissural area in these snakes. In *Natrix natrix* Petit (1971) has shown numerous unmyelinated fibers in the perivascular space and their terminations in the parenchyma. These terminals are rich in dense granules of variable size (40-160 nm) and are considered to be of sympathetic origin. A sympathetic innervation of the pineal of this species was demonstrated earlier by Quay *et al.* (1968) with the fluorescence method. Both Petit (1971) and Quay *et al.* (1968) have observed a small fiber tract running between the basal part of the pineal and the intercommissural area of the brain in *Natrix natrix* but these fibers are thought to be aberrant. Both myelinated and unmyelinated fibers are prominent in the cobra and the krait. They often contain dense granules especially in axonal profiles seen in the perivascular space. While some of these may innervate the intrapineal blood vessels, others penetrate the basal lamina and reach the pinealocytes. The likelihood that these fibers are of sympathetic origin and that the metabolism of the pineal in these snakes is regulated by autonomic invervation cannot be set aside *a priori*. This may also be true for the saw-scaled viper where terminals of nerve fibers could be detected in the parenchyma only rarely. However, the precise orgin of the innervation in the pineal of the three species studied here, needs to be further elucidated by application of various specific techniques.

The pineal of snakes, including the species examined in this study, is remarkably advanced with features comparable to those of the mammalian pineal. Its ultrastructural details portray secretory activity in the pinealocytes and hence a neuroendocrine role. The best known pineal hormone, melatonin, is elaborated in all vertebrates studied so far (reviews: Wurtman *et al.*, 1968; Quay, 1974; Collin and Oksche, 1981; Vollrath, 1981; Oksche, 1983; Klein, 1985; Reiter, 1991; Korf, 1994). The presence of melatonin has been reported in only one species of snakes, *Natrix tesselata*, by immunohistochemical method (Vivien-Roels *et al.*, 1981). Melatonin is best known to synchronize the endogenous rhythm in vertebrates with ambient light and hence is involved in entraining a number of physiological activites especially

reproductive with the photoperiod (Reiter, 1980, 1991; Korf, 1994). The precise role of the pineal in snakes has yet to be fully elaborated. Temperature appears to be the predominant proximate cue in snakes where it regulates breeding behaviour and gonadal activity (Crews, 1979; Whittier *et al.*, 1987; Crews *et al.*, 1988). Cold temperature, as is characteristic during hibernation period of snakes, is known to suppress rhythmic melatonin production and its release in turtles and some lizards (Vivien-Roels *et al.*, 1979; Vivien-Roels and Pevet, 1983; Underwood and Calaban, 1987; Vivien-Roels *et al.*, 1988). Crews *et al.* (1988) have shown that the pineal in the garter snake, *Thamnophis sirtalis parietalis*, mediates temperature effects on courtship behaviour (see also Nelson *et al.*, 1987). Temperature may also be the only cue responsible for onset of melatonin surge in this species (Joy and Crews, 1987) but a role of subtle photoperiodic cues cannot be entirely ruled out. Like other snakes, cobra, krait and the saw-scaled viper also hibernate during winter season. Their behaviour is also dependent on daily cycle of day and night and the photoperiod may have an equally important influence. There is dire need to conduct cell biological, biochemical, experimental, season-dependent and age-dependent studies on snakes to evaluate not only the functional role of the pineal in snakes but also to assess influences of photoperiodic and non-photoperiodic cues (see also Kappers, 1981) on the morphology as well as biochemistry of the pineal itself.

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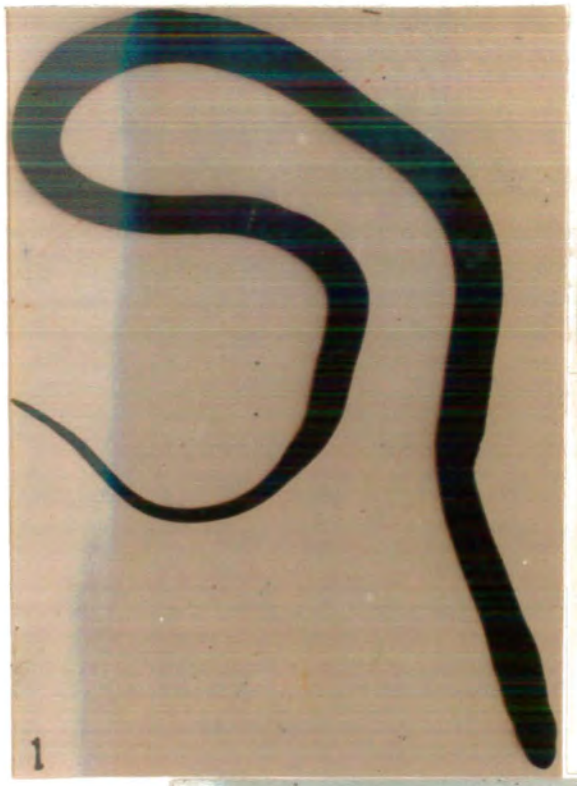
FIGURES



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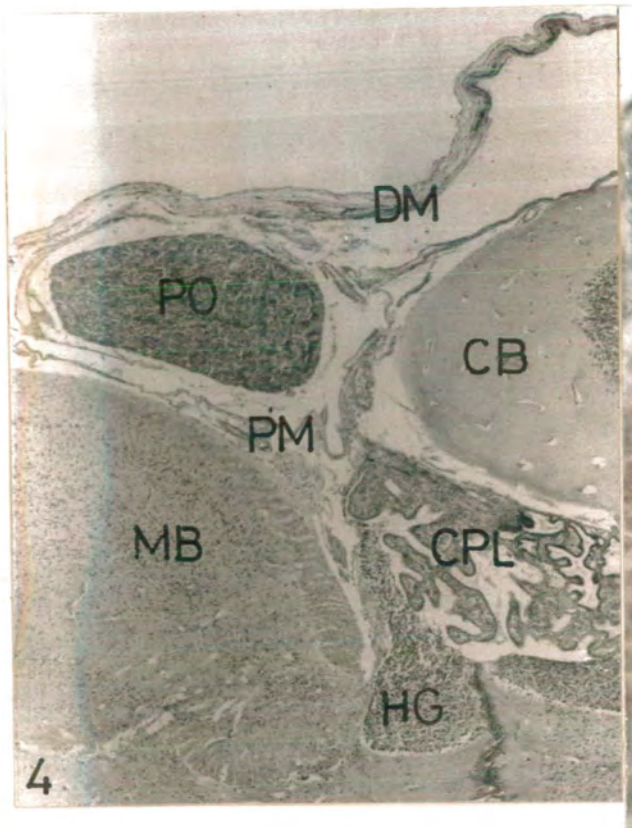
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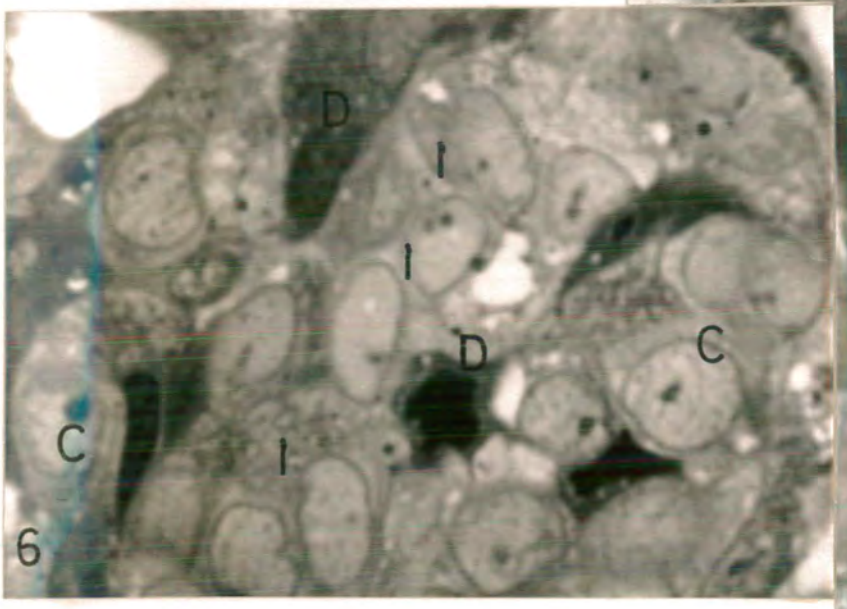
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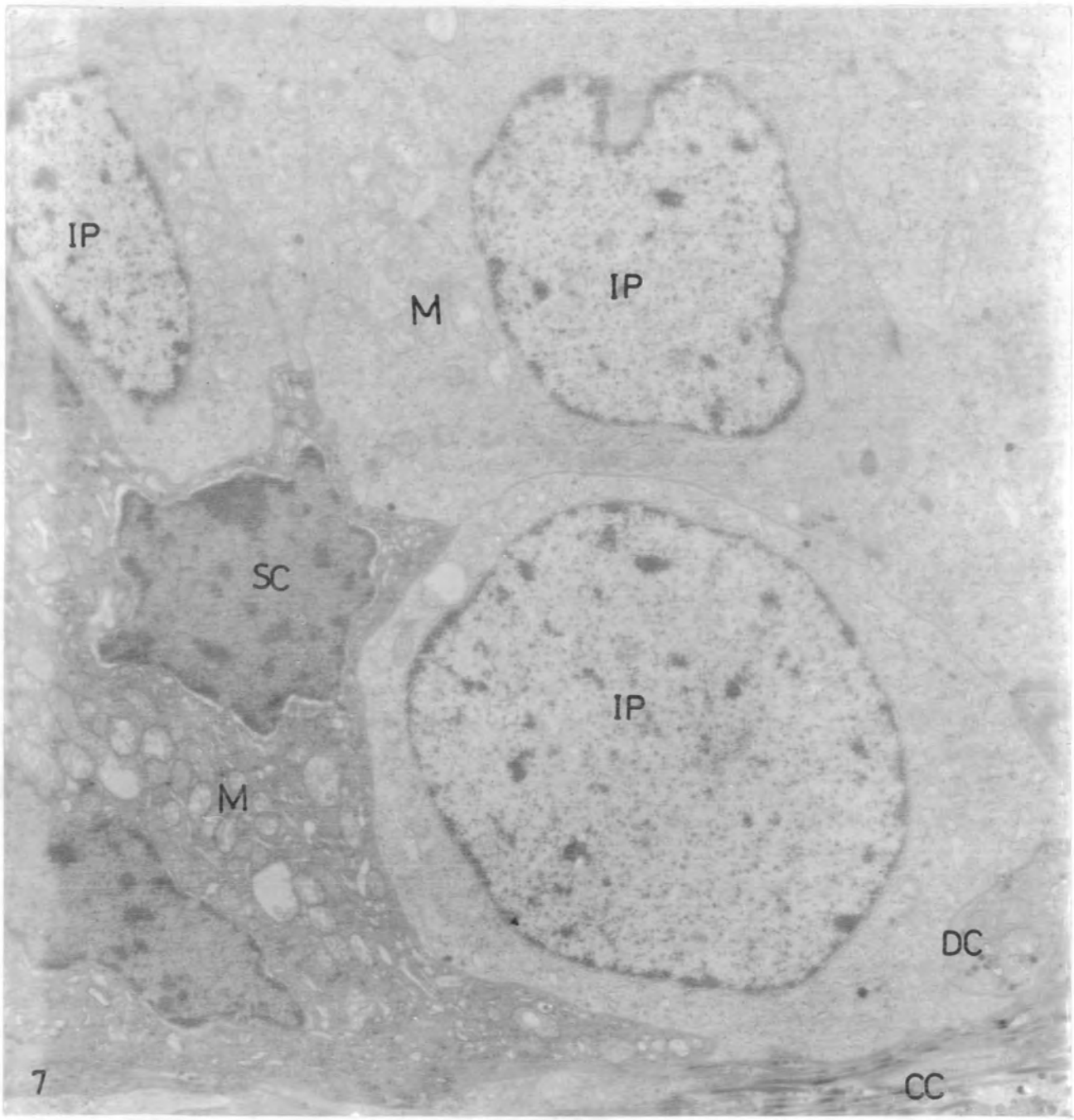
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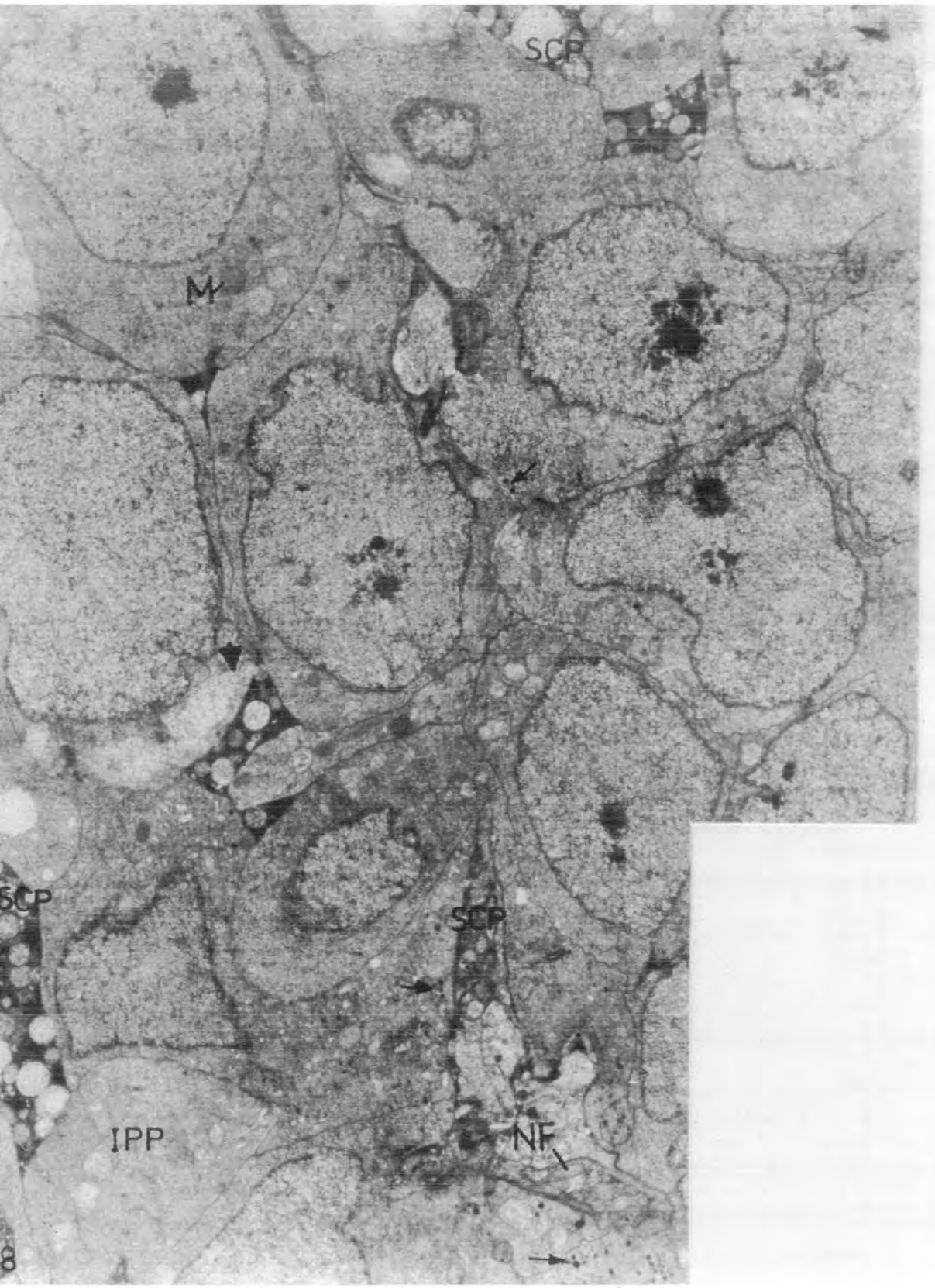
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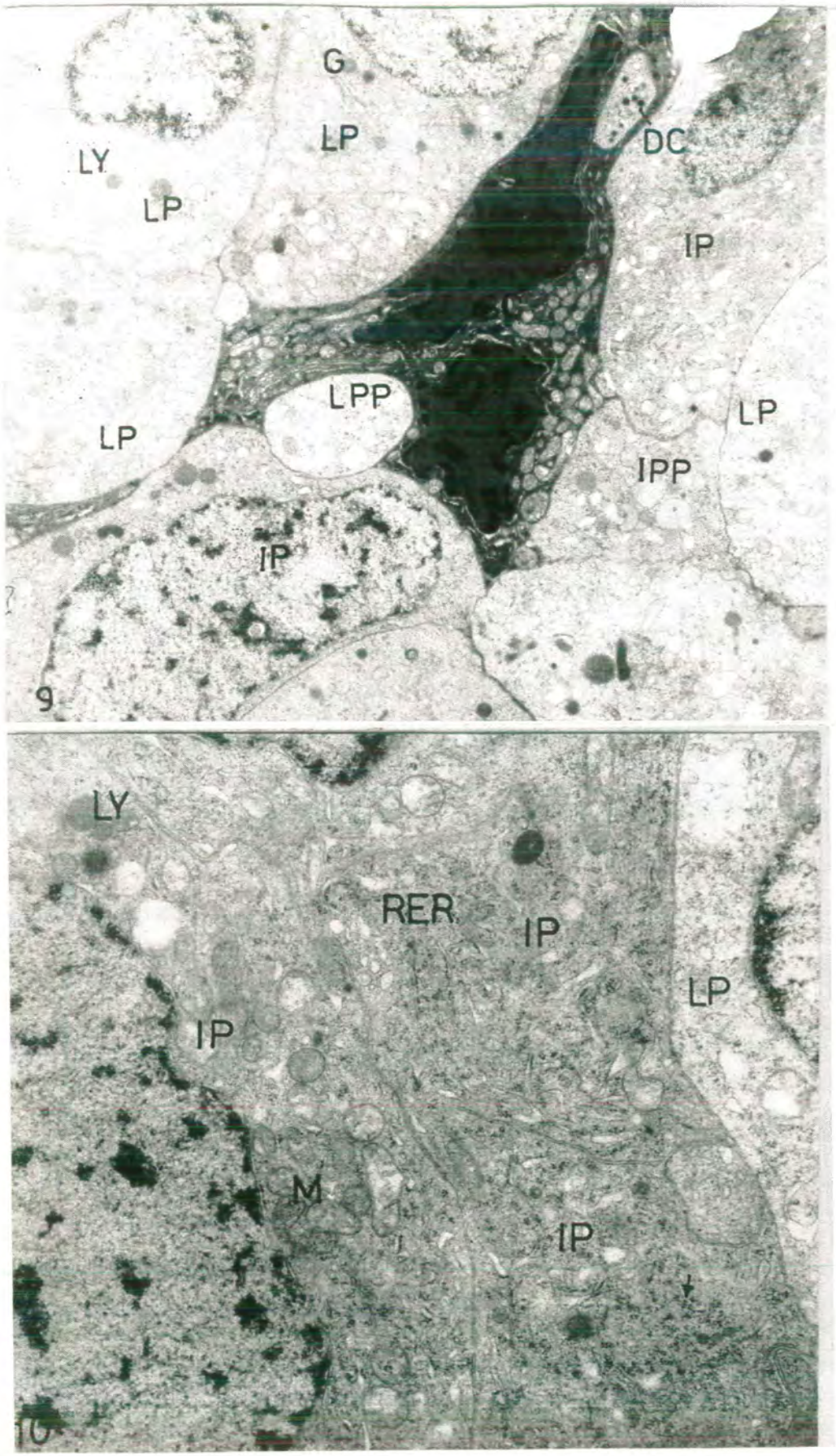
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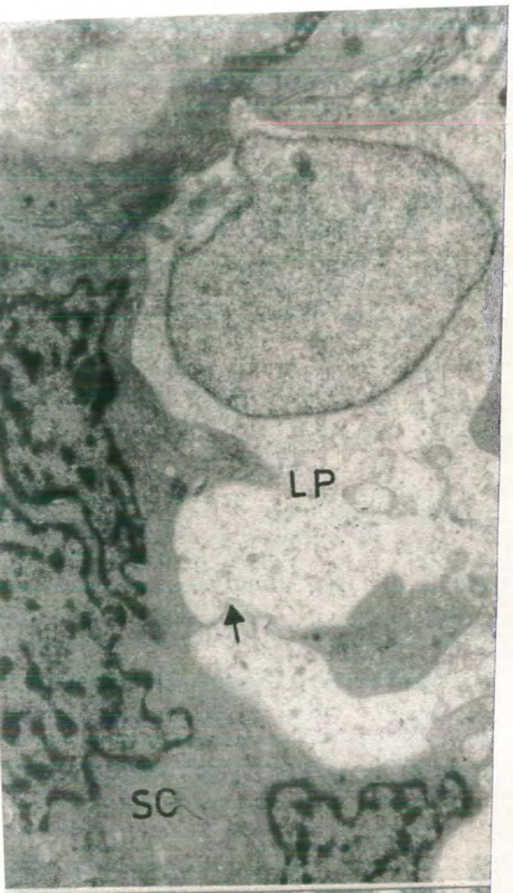
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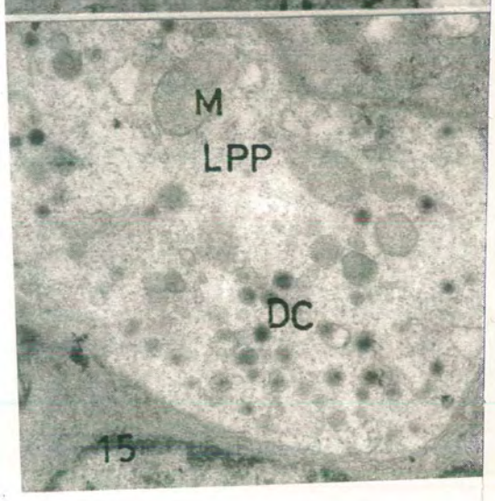
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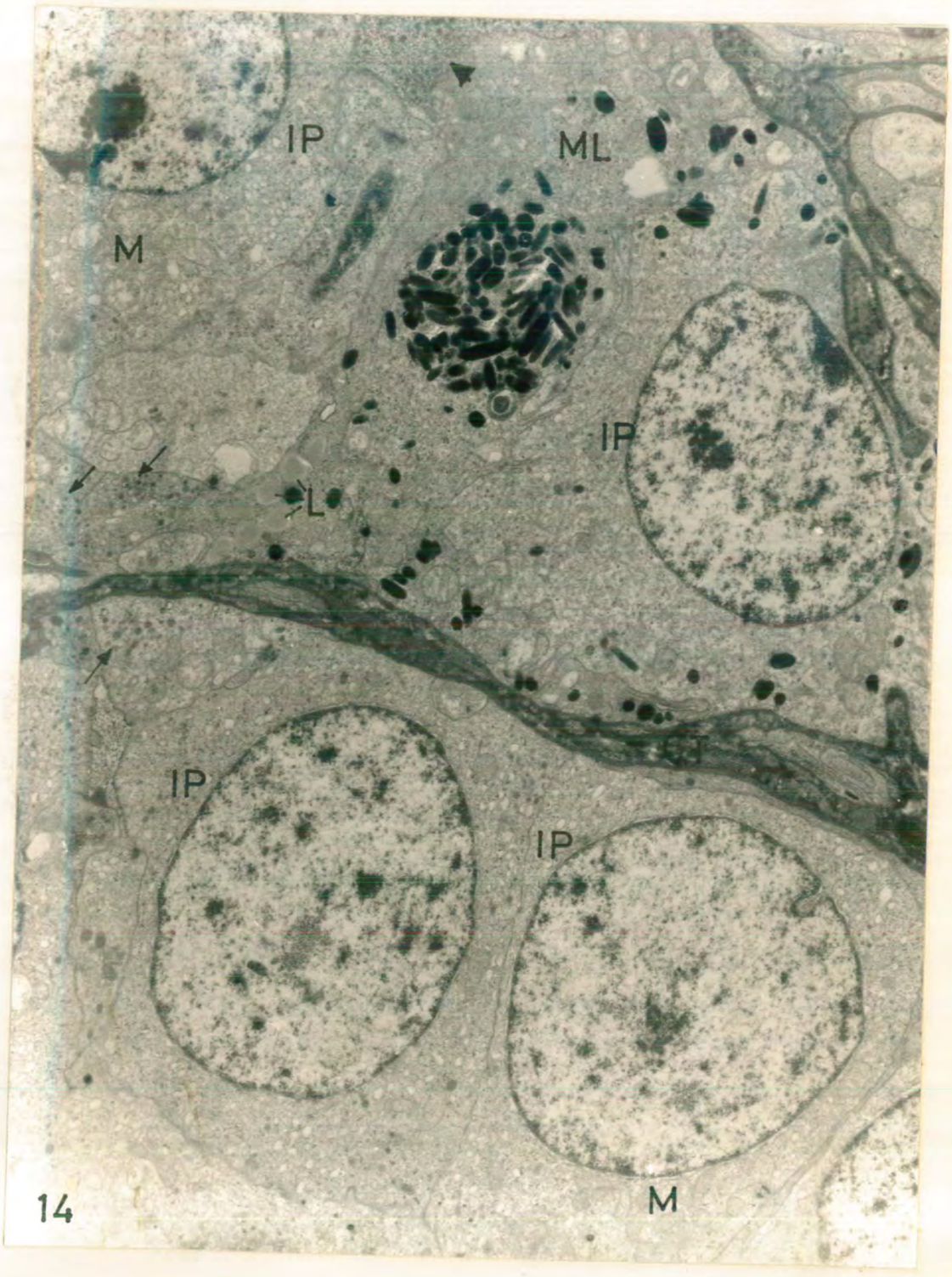
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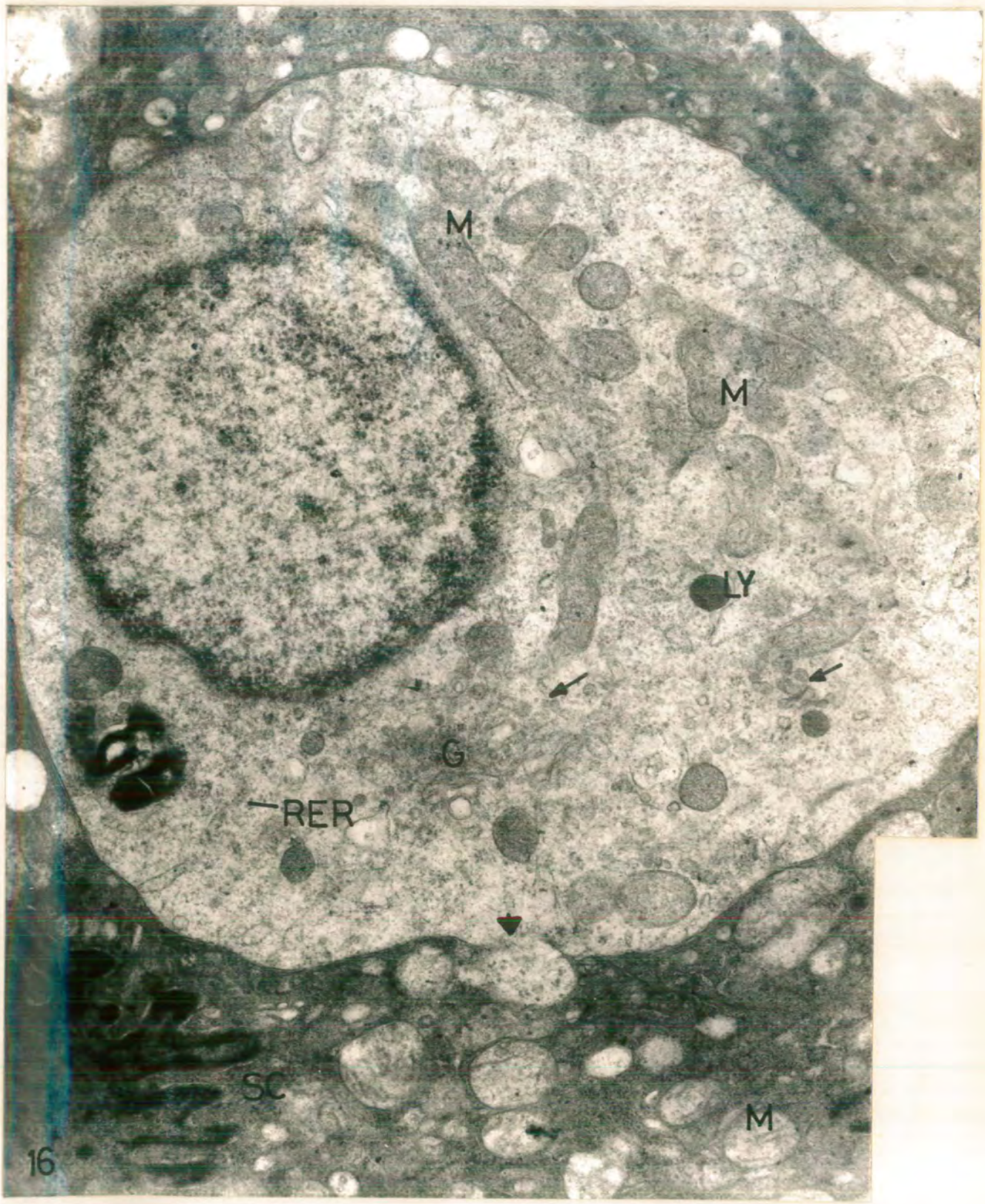
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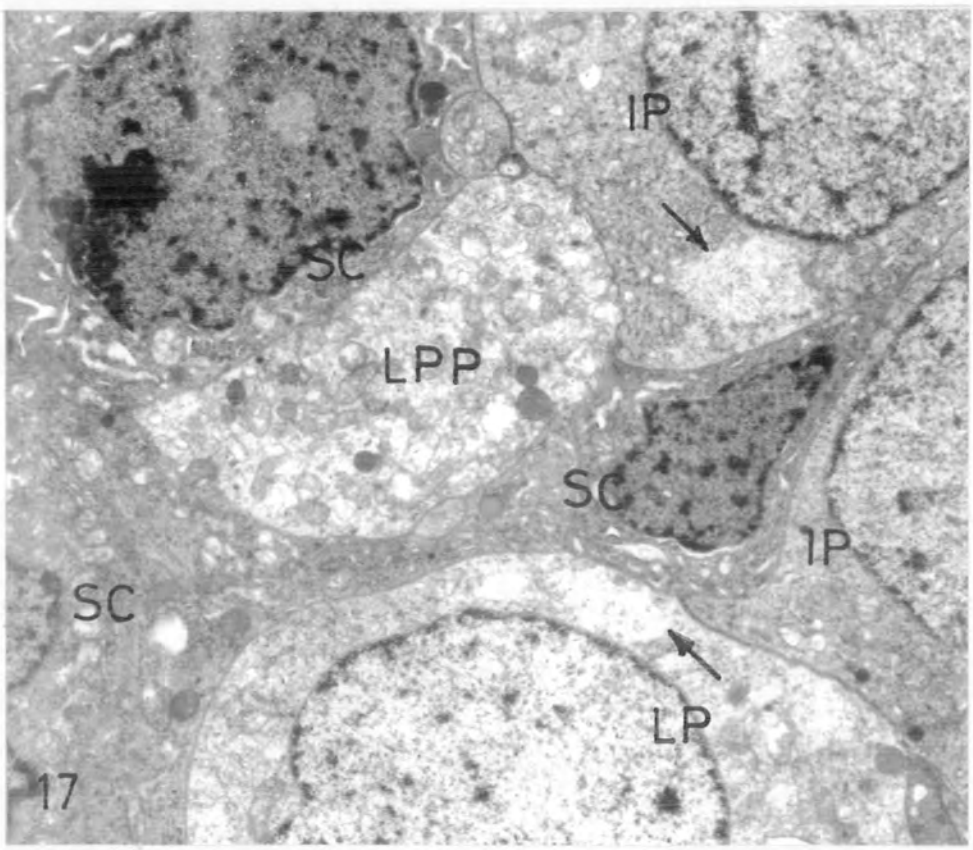
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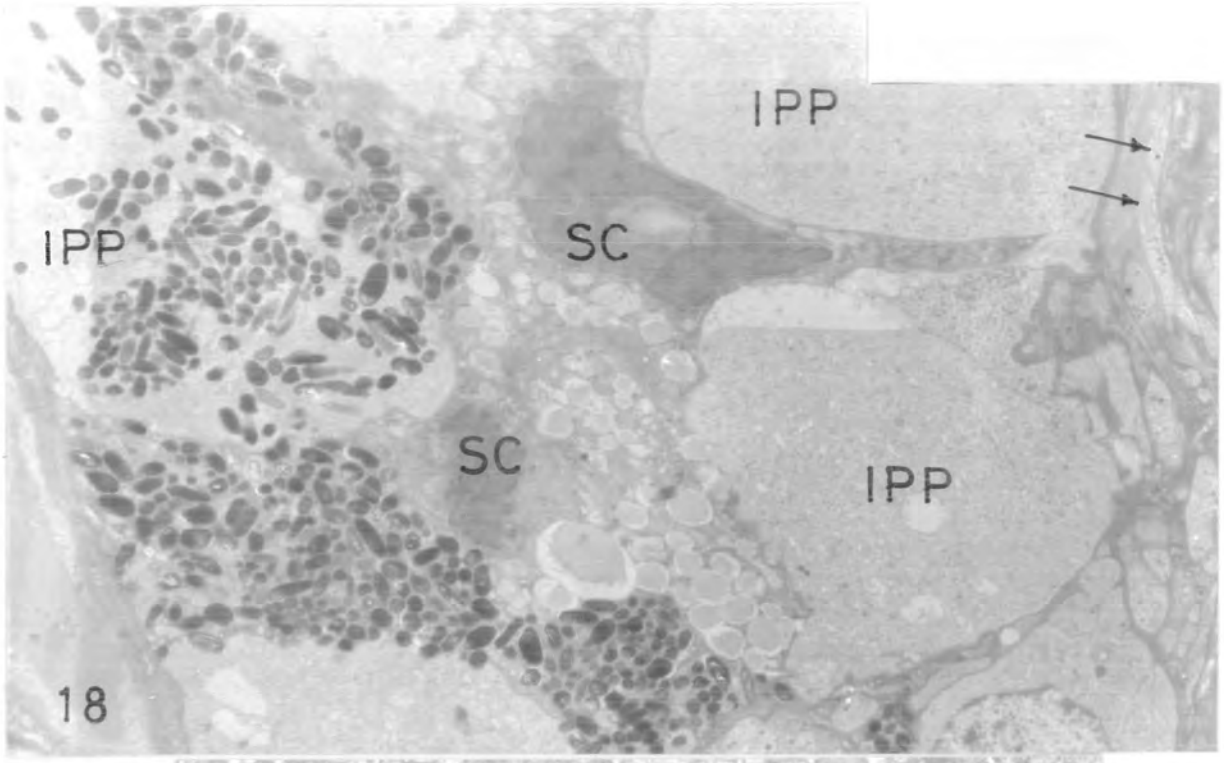
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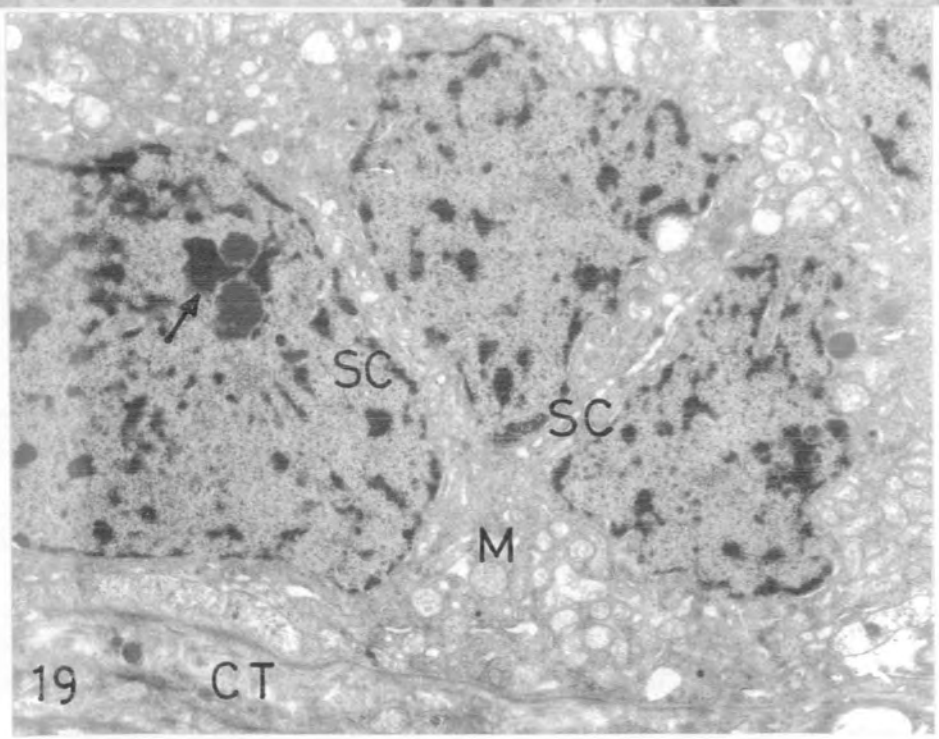
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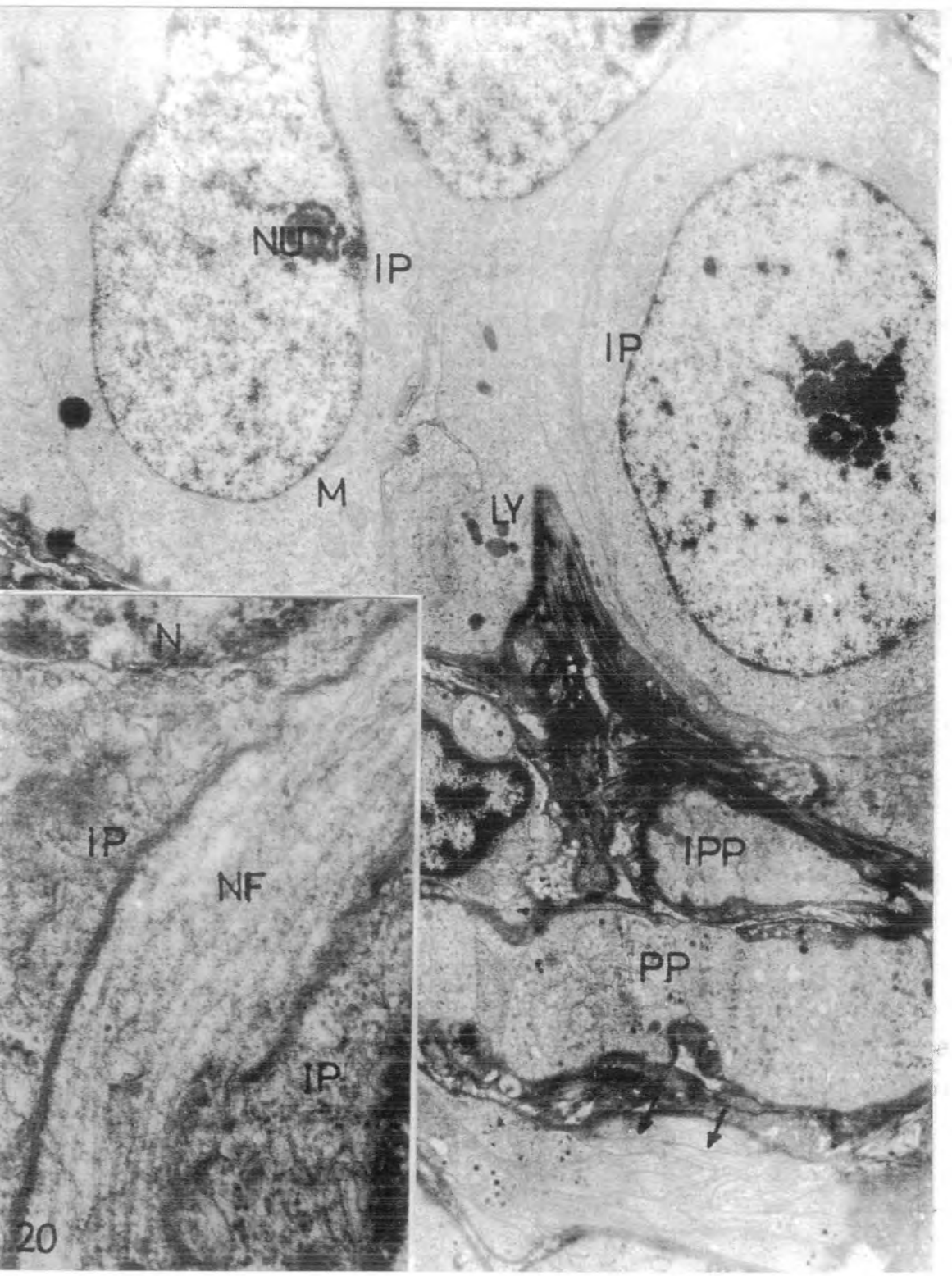
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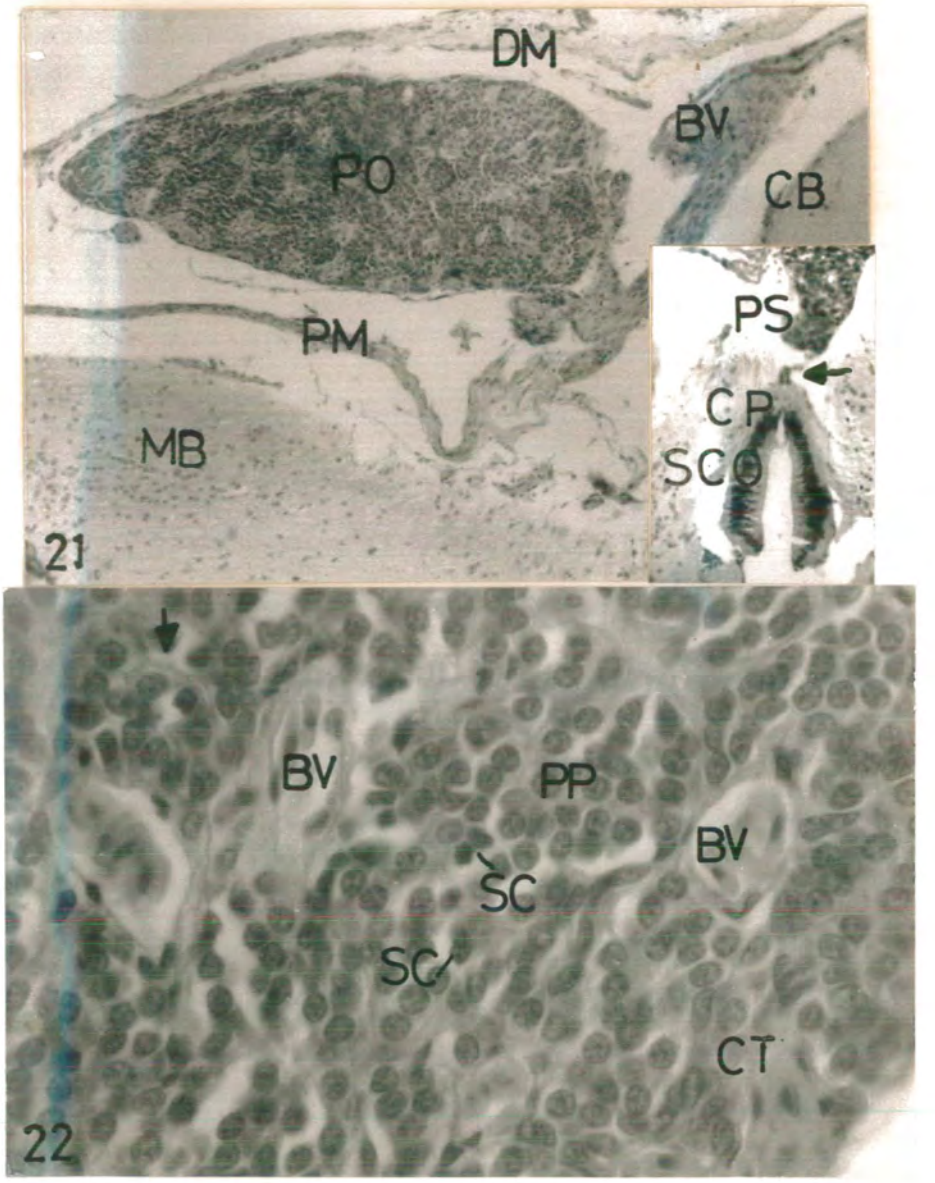
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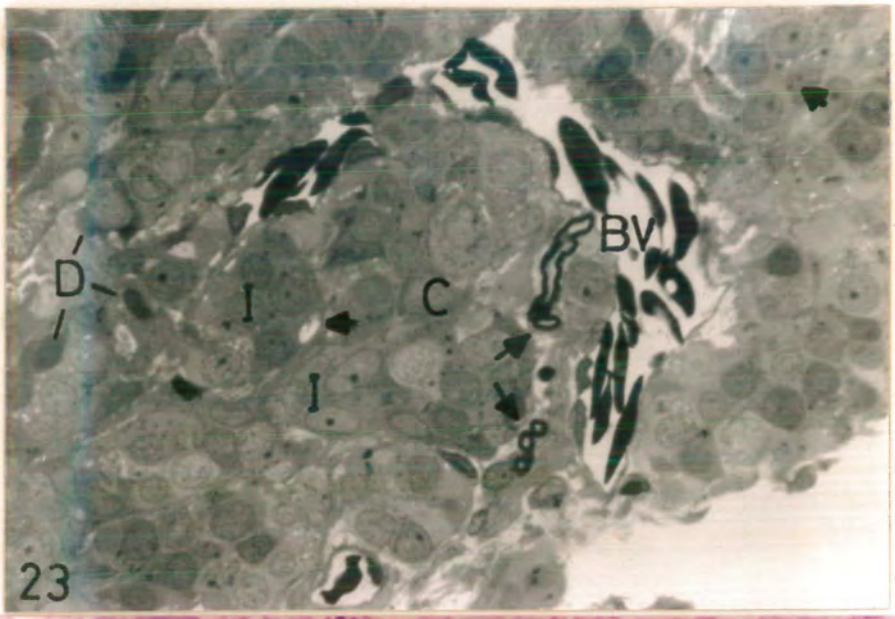
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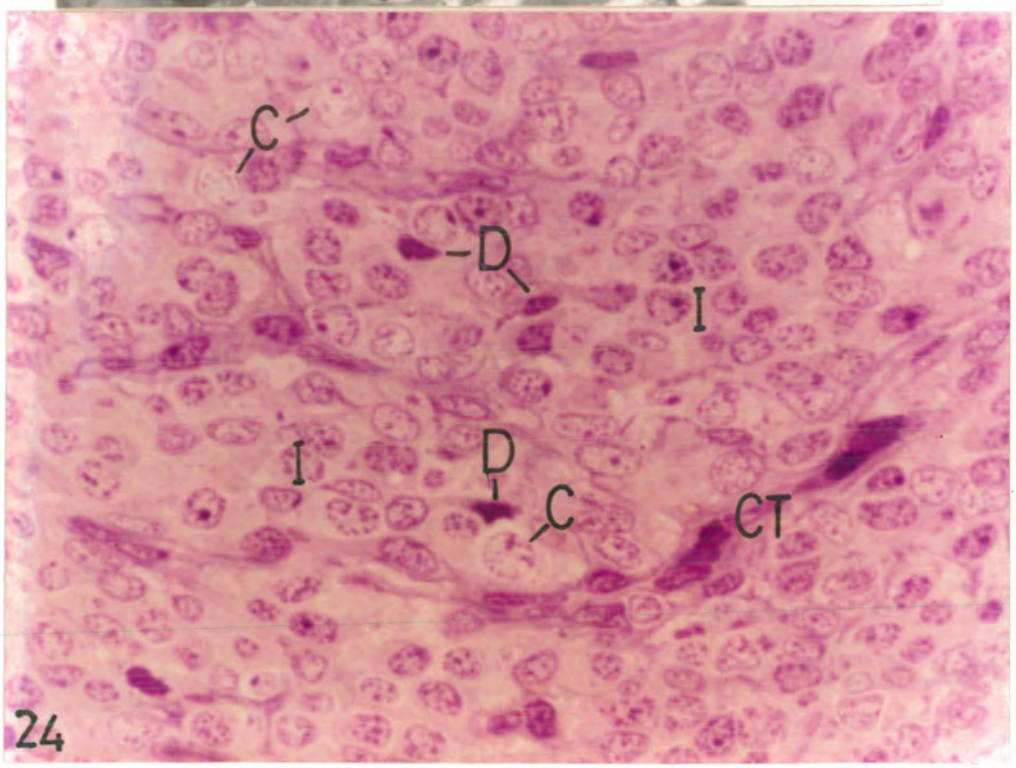
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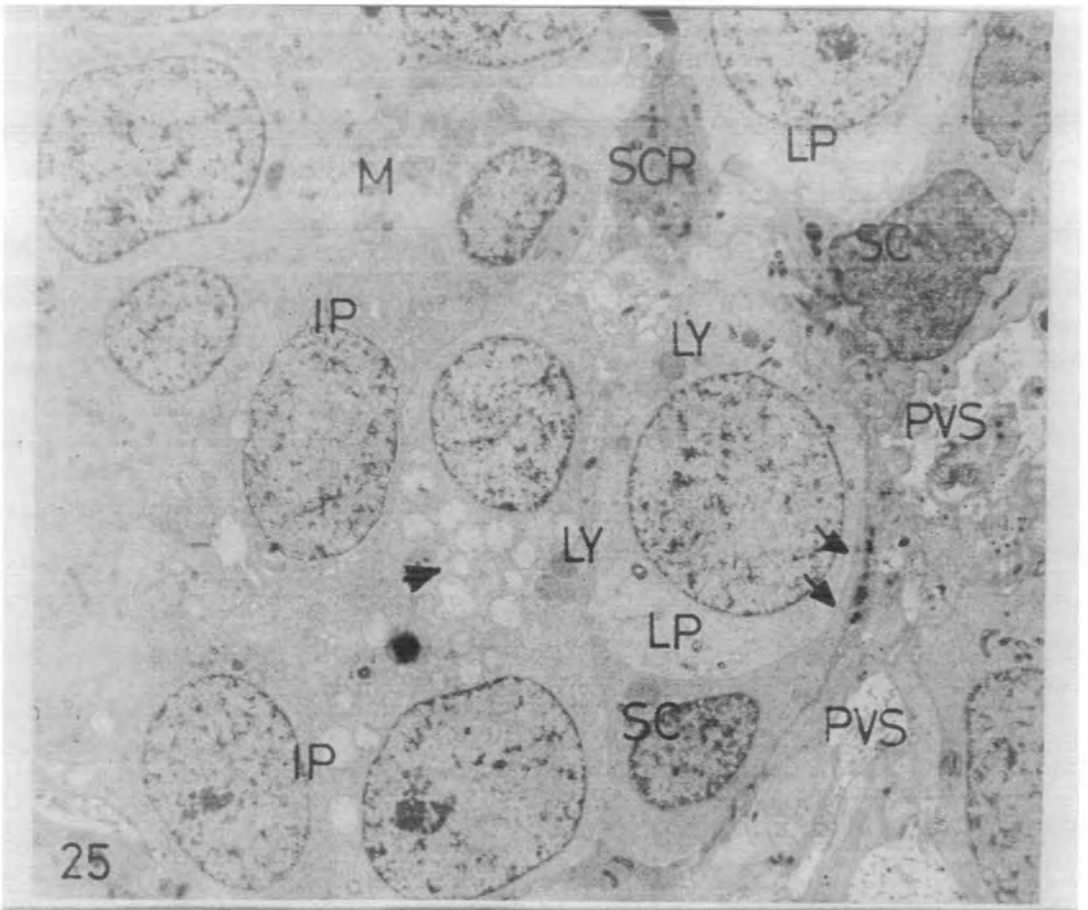
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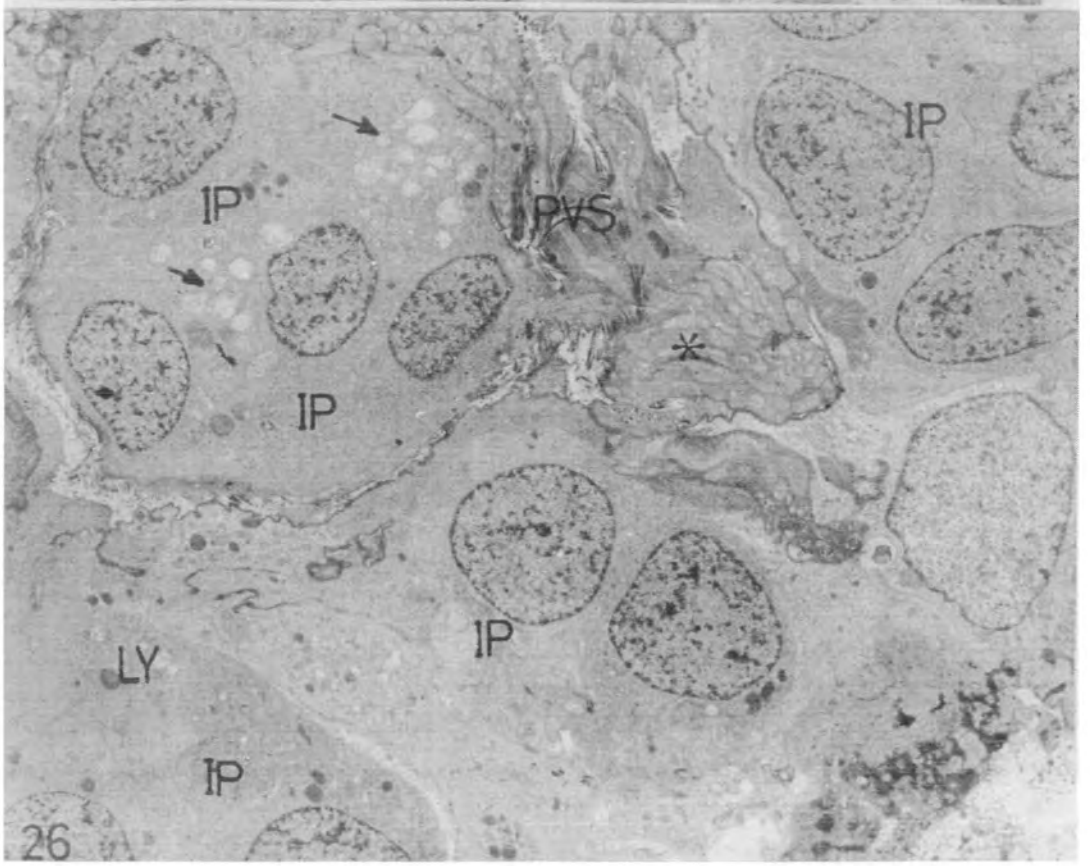
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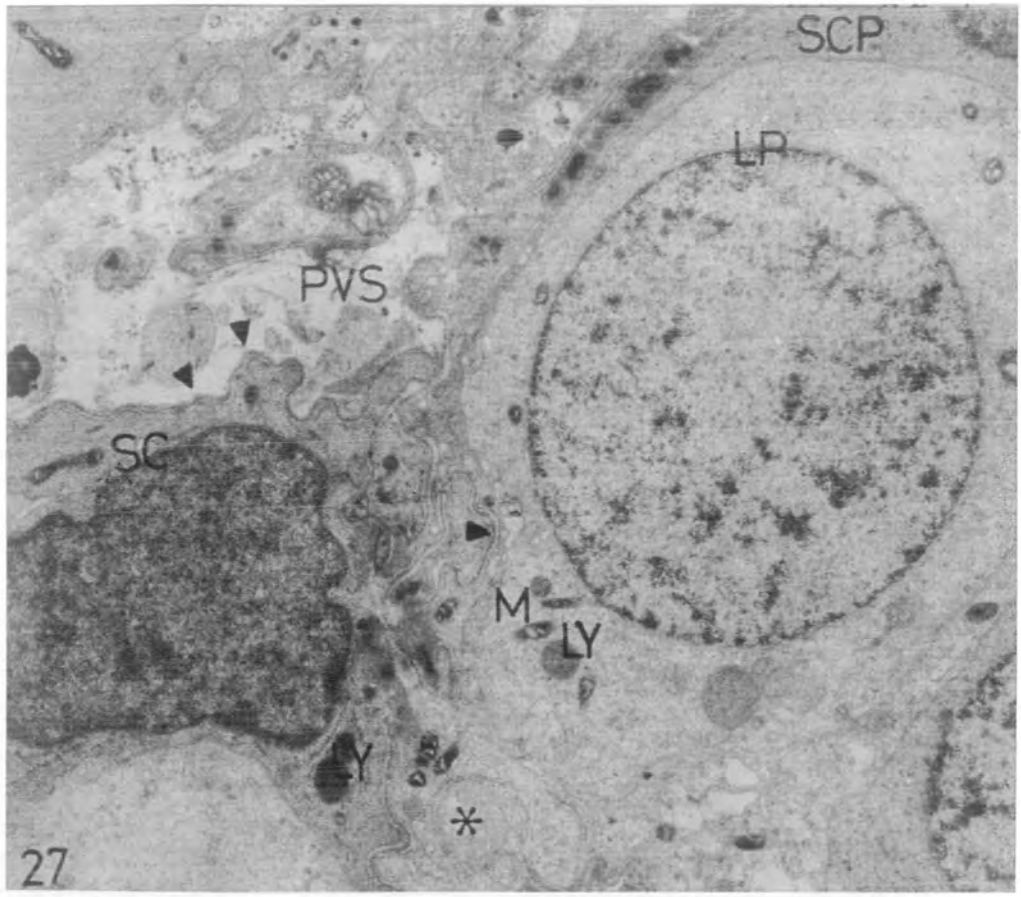

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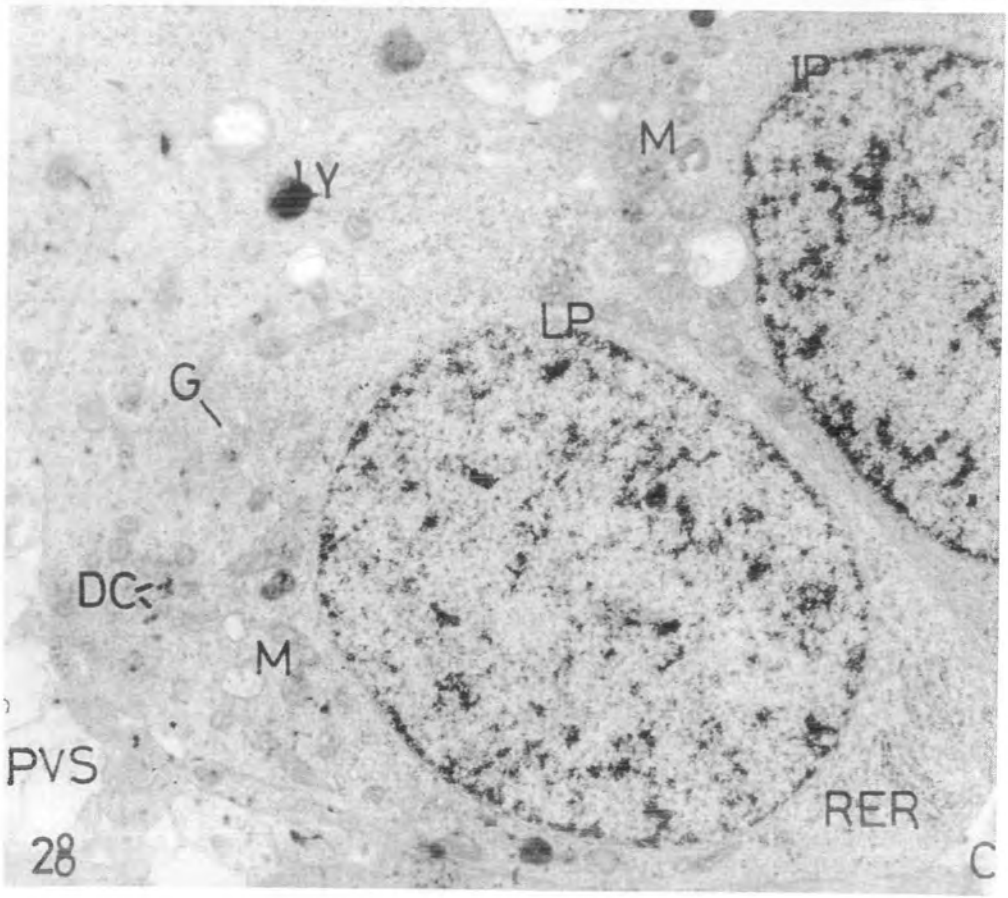

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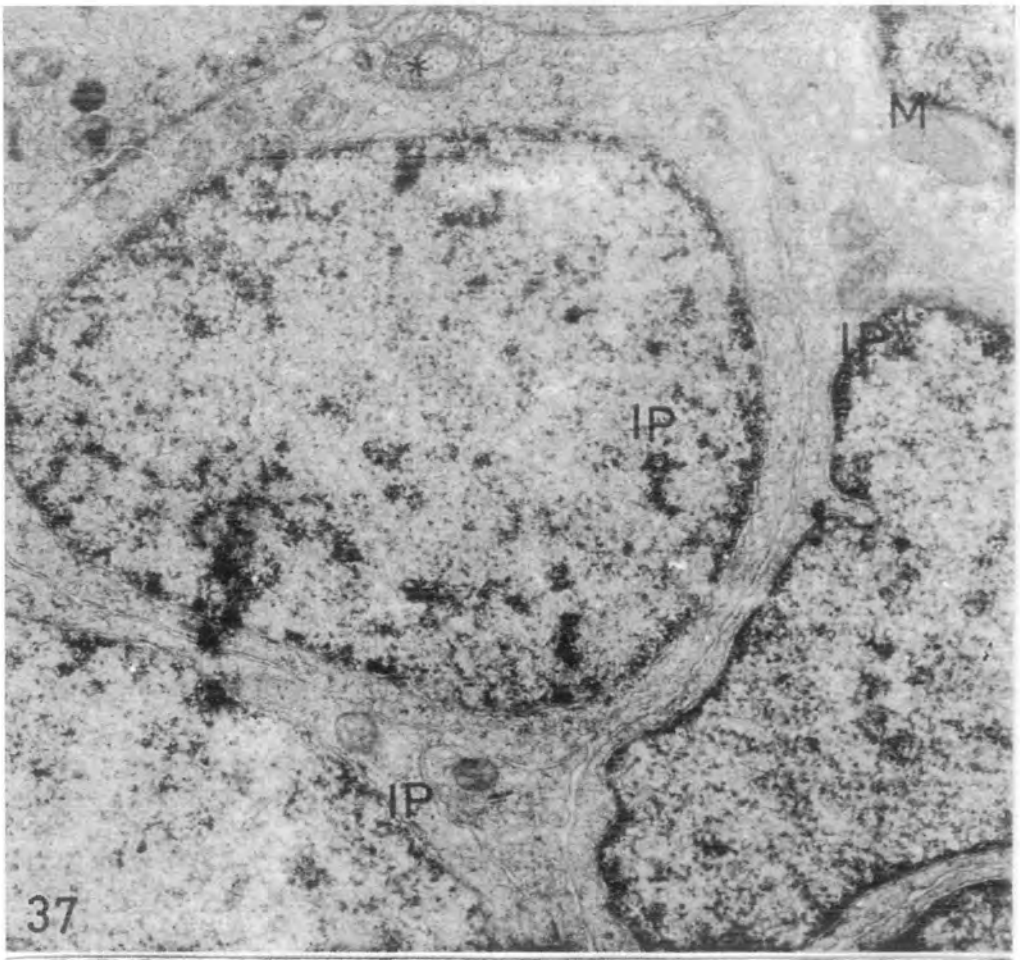
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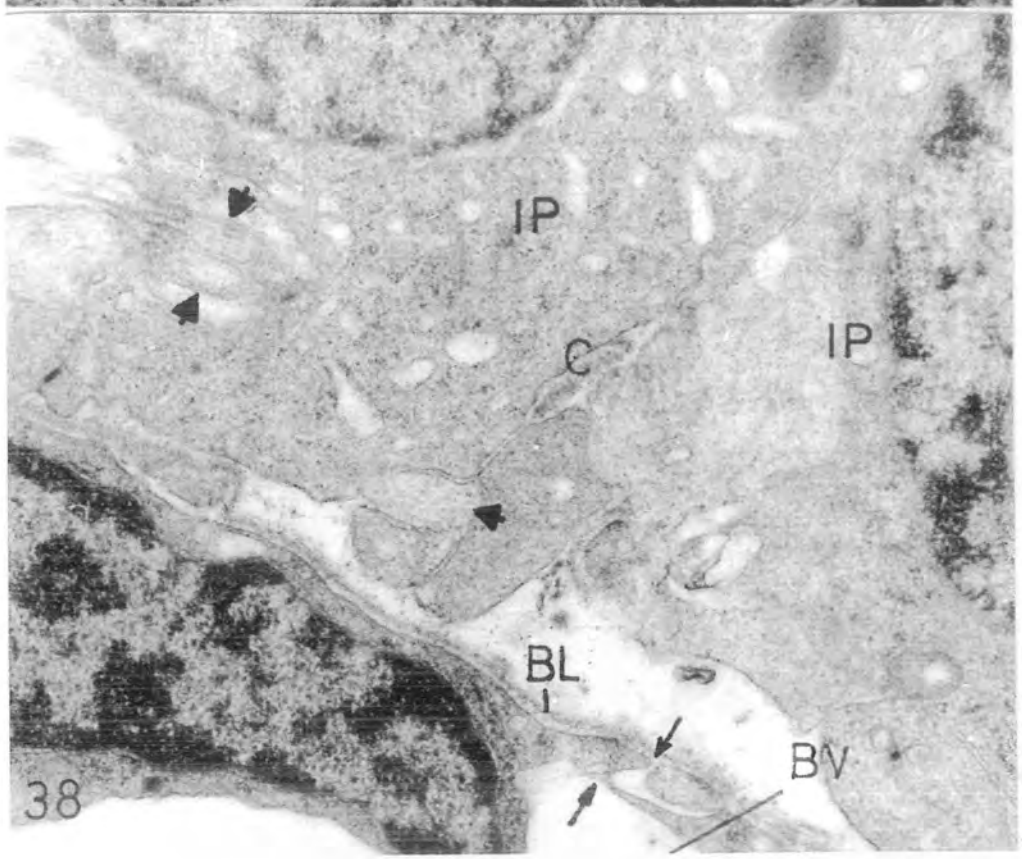
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BL
BV
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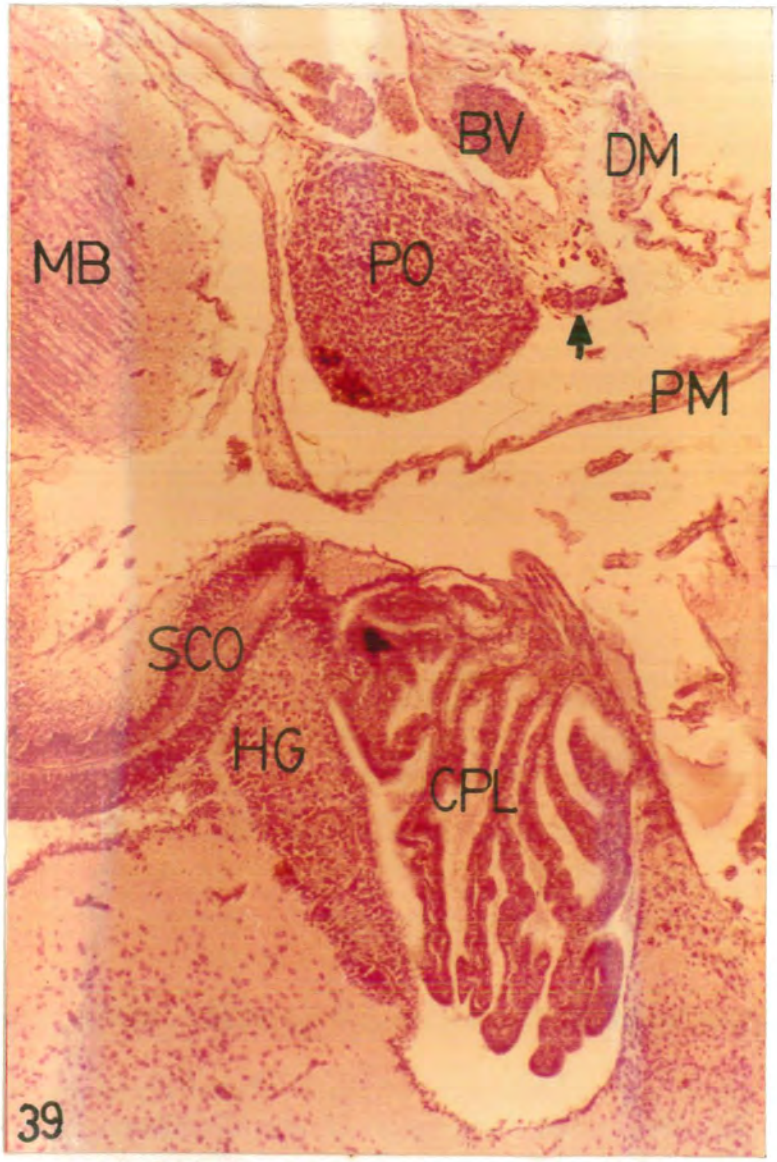
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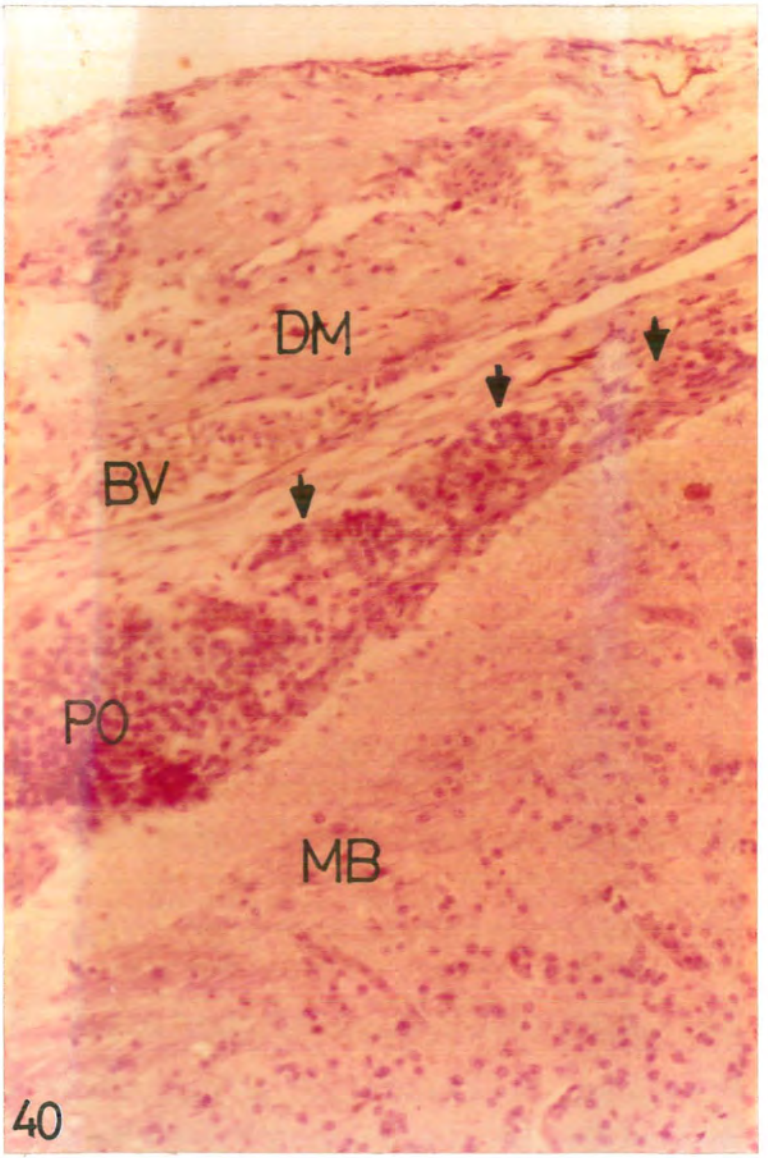
PO
PM
SCO
HG
CPL
39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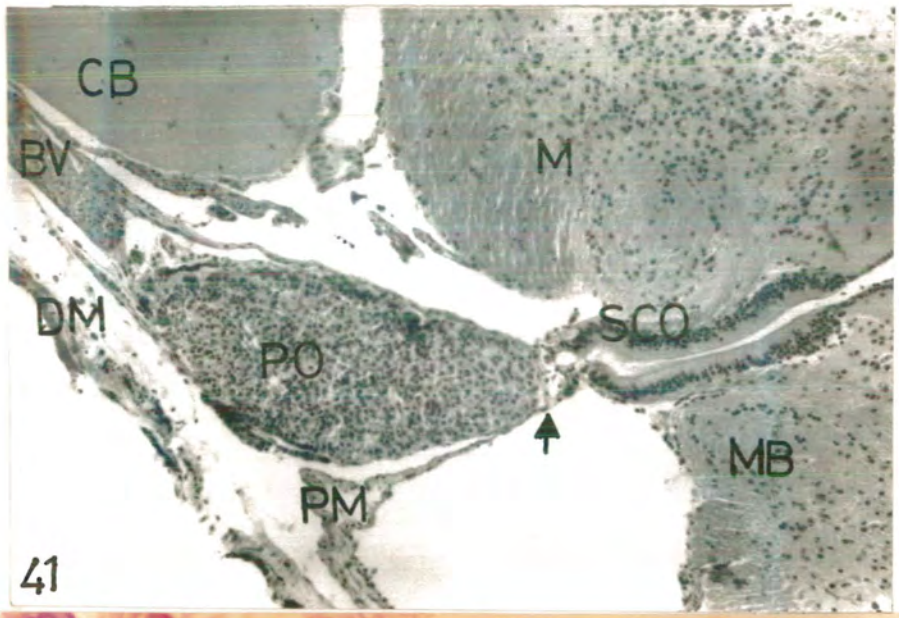
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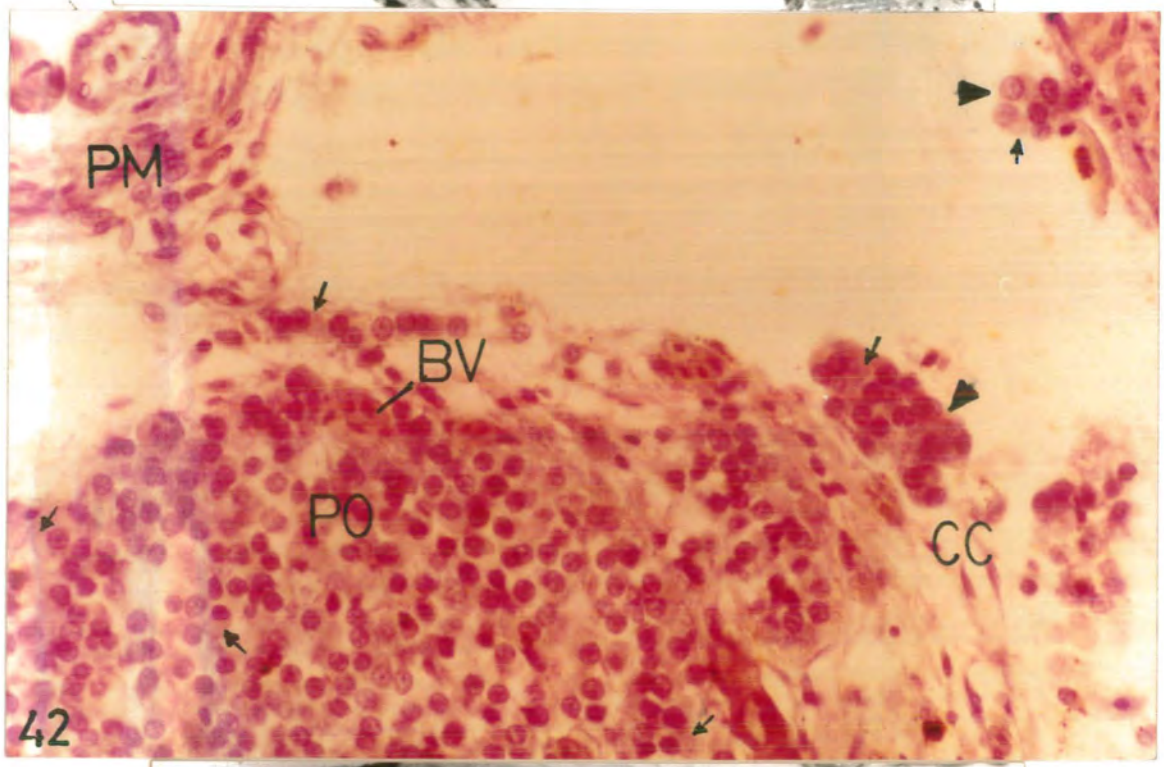
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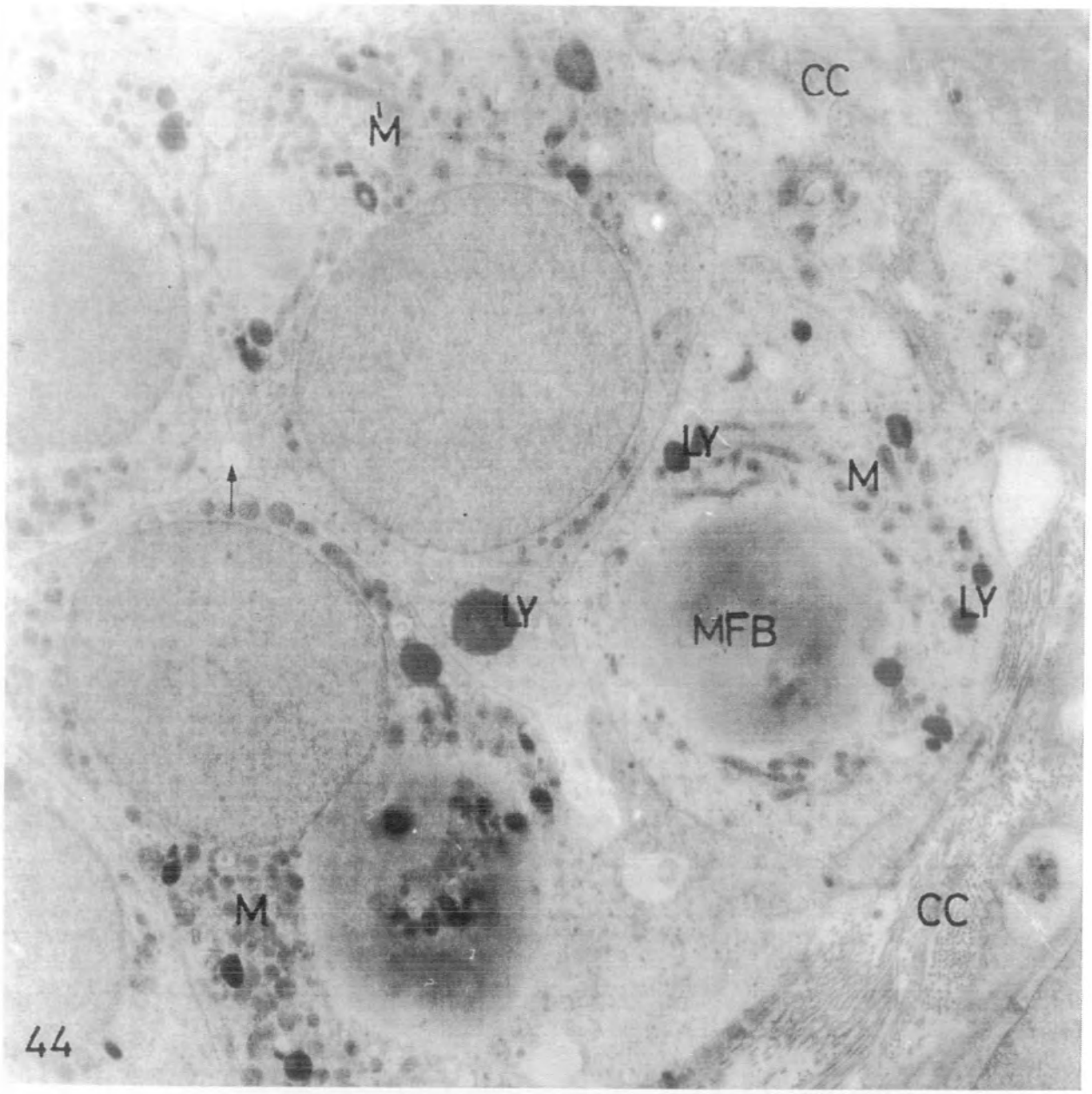
42


43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LY
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LY
MFB
LY
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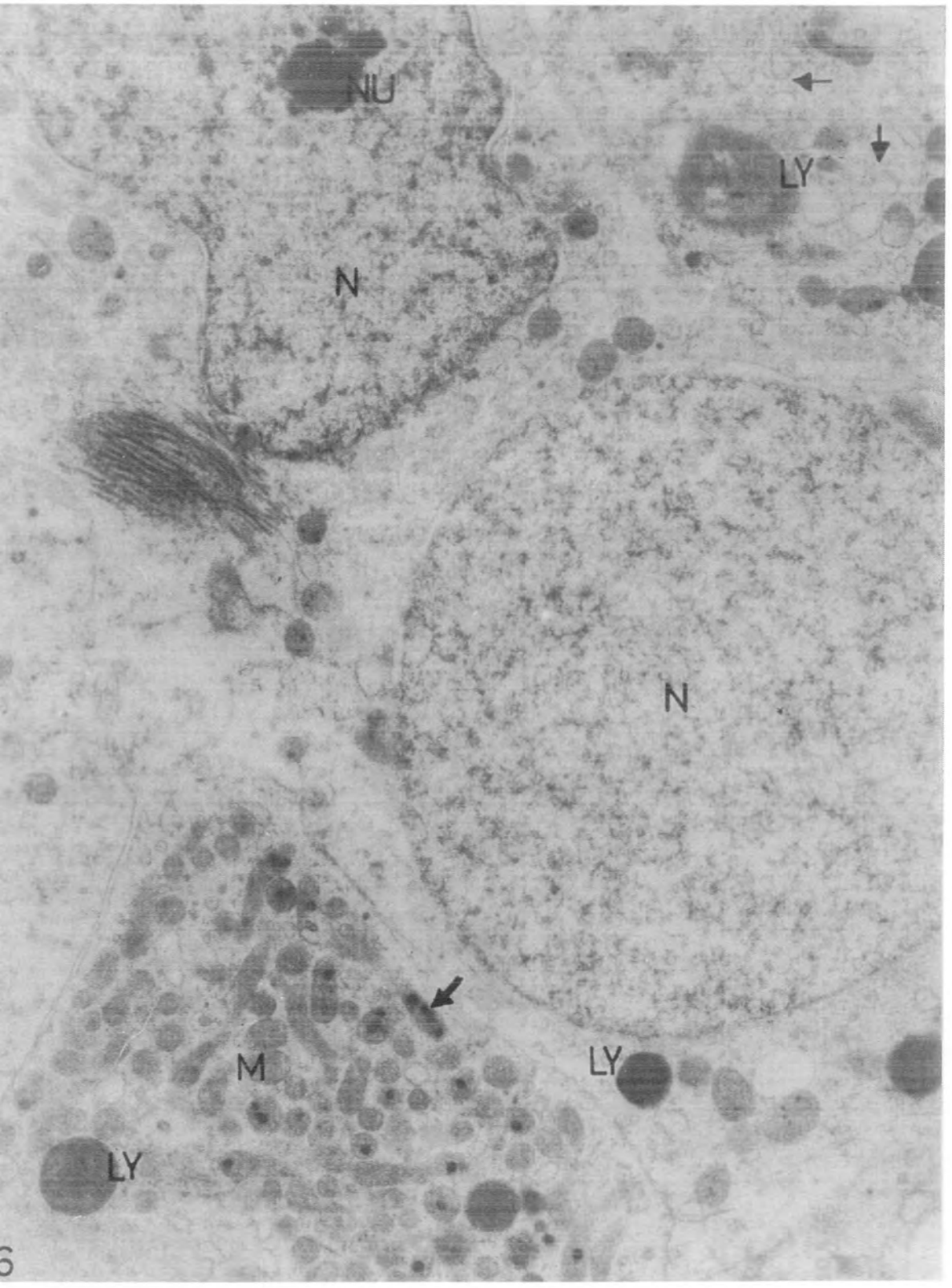
CC
44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LY
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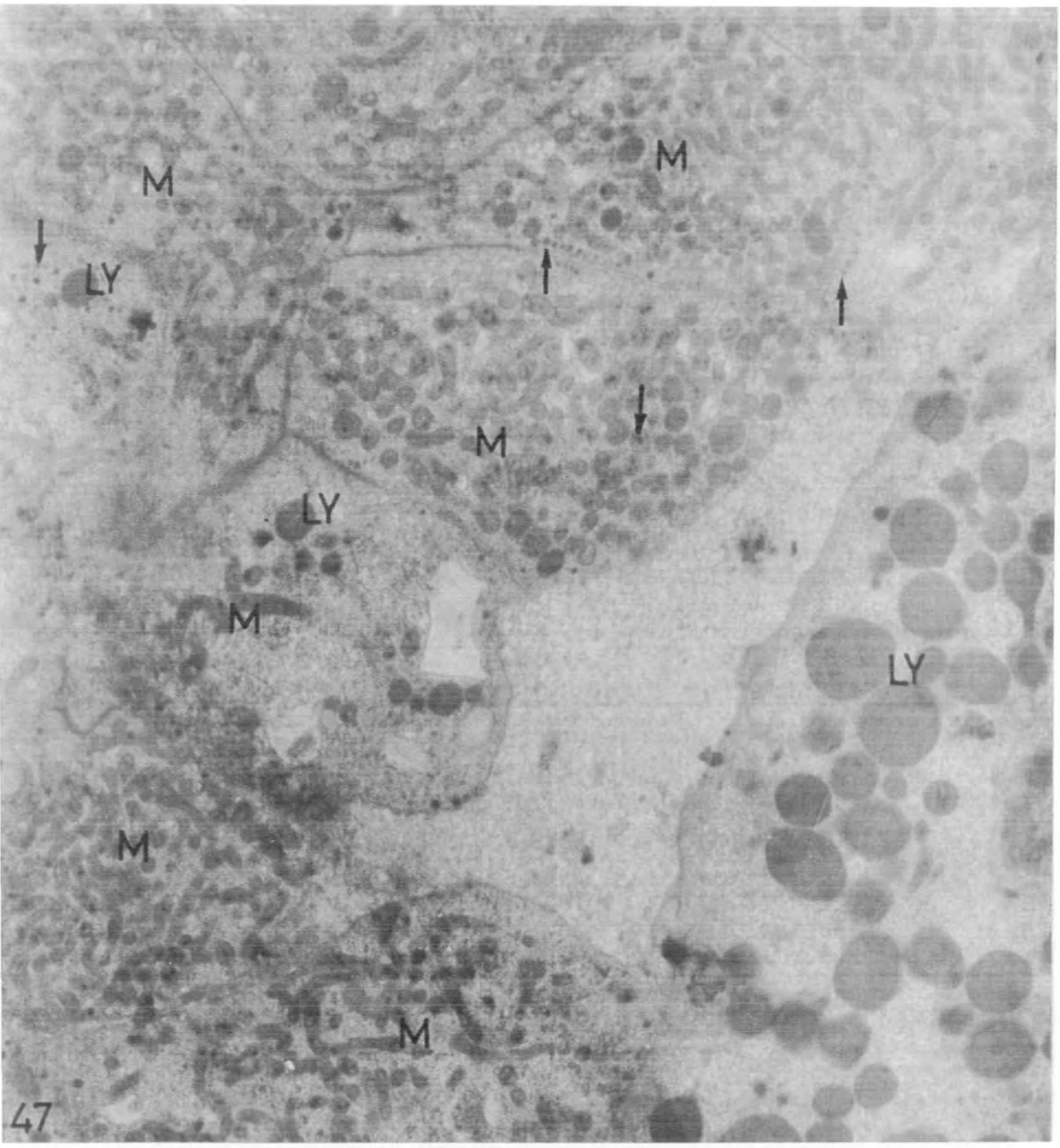
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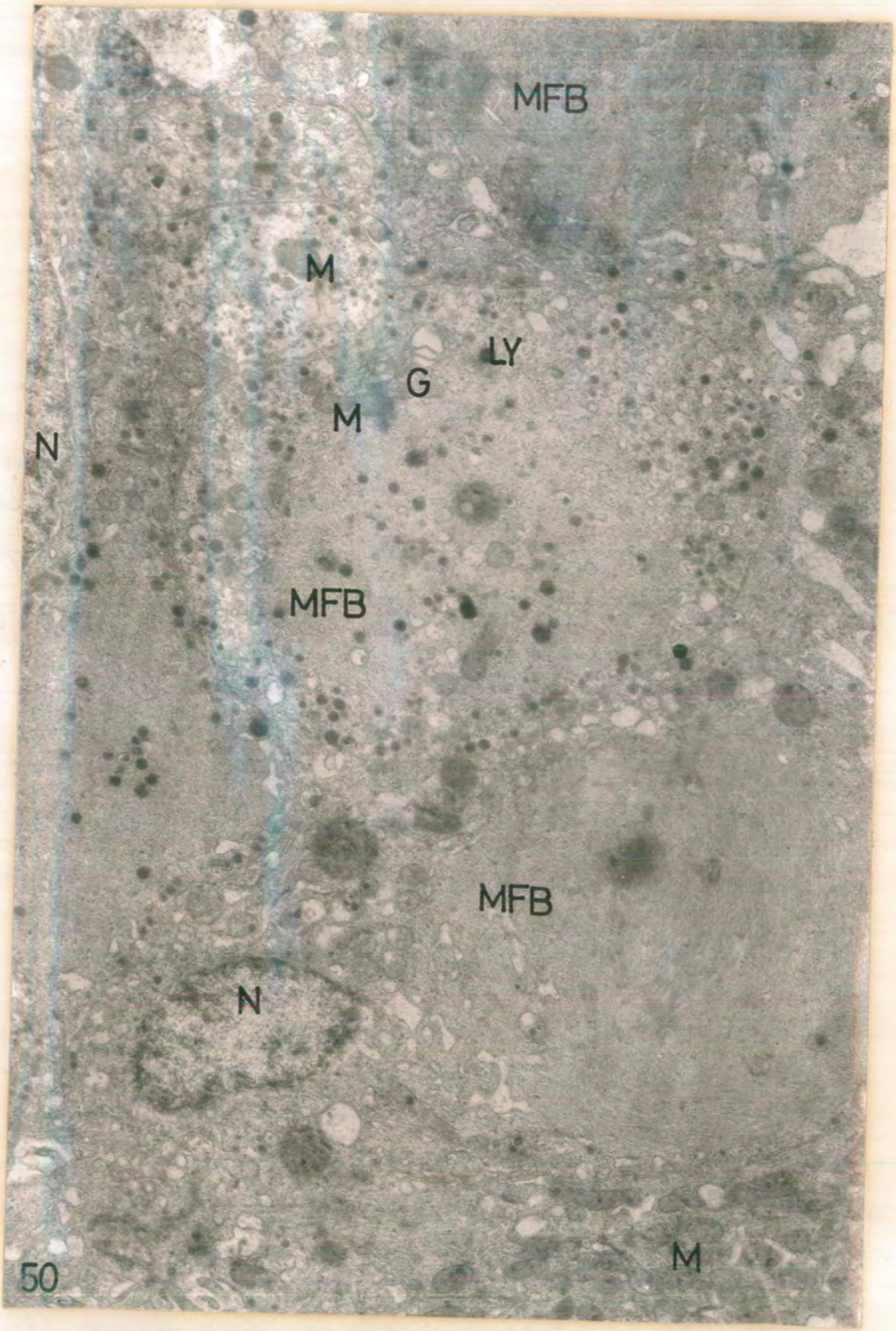
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MFB
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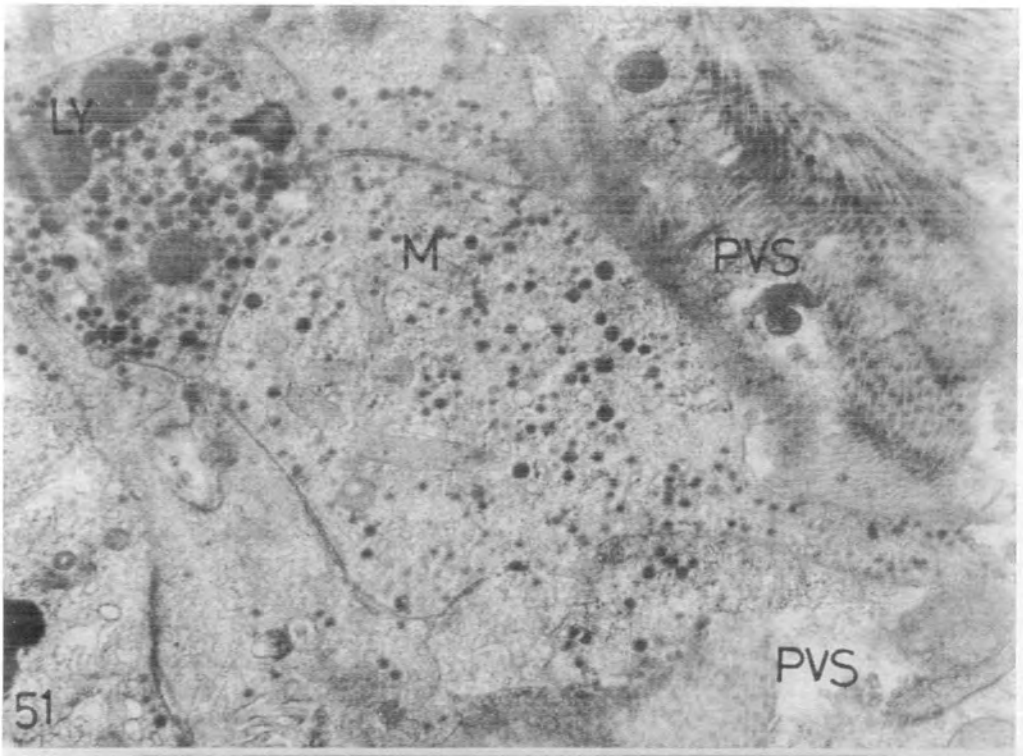
M
50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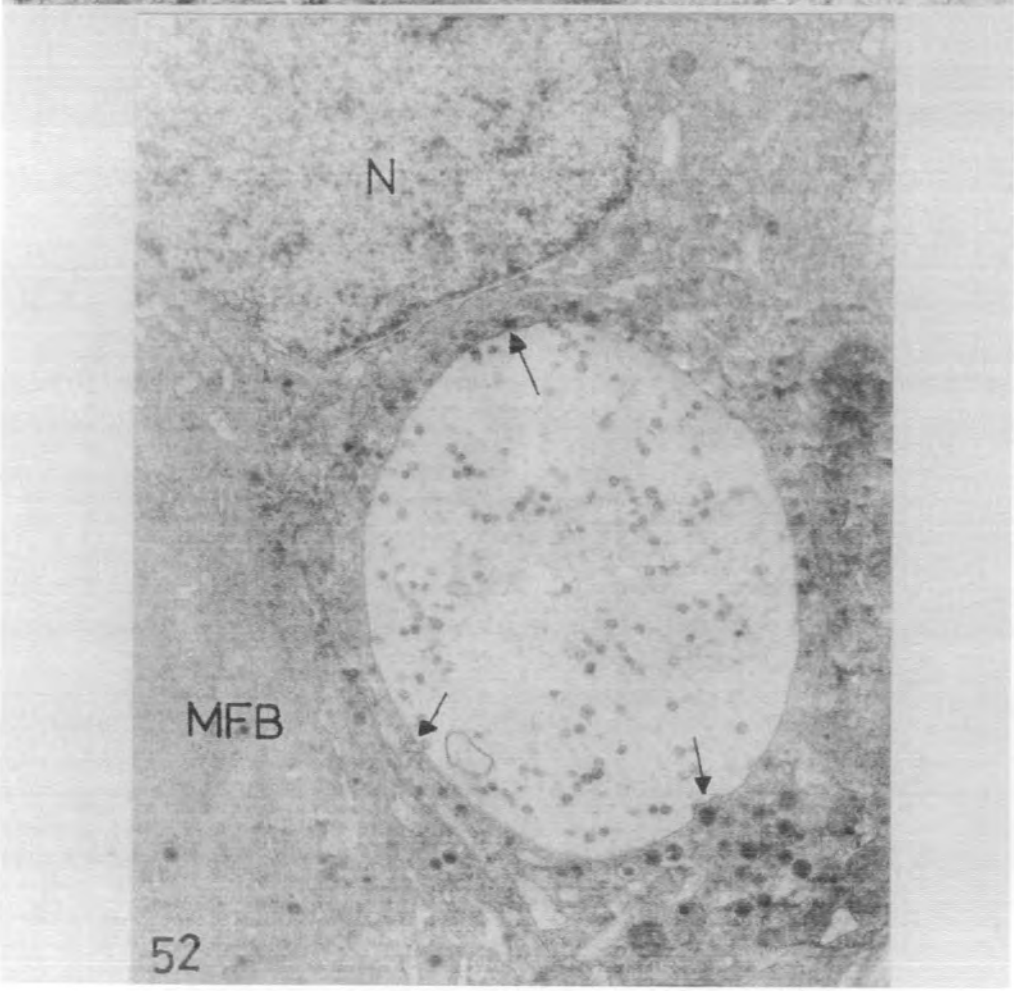
51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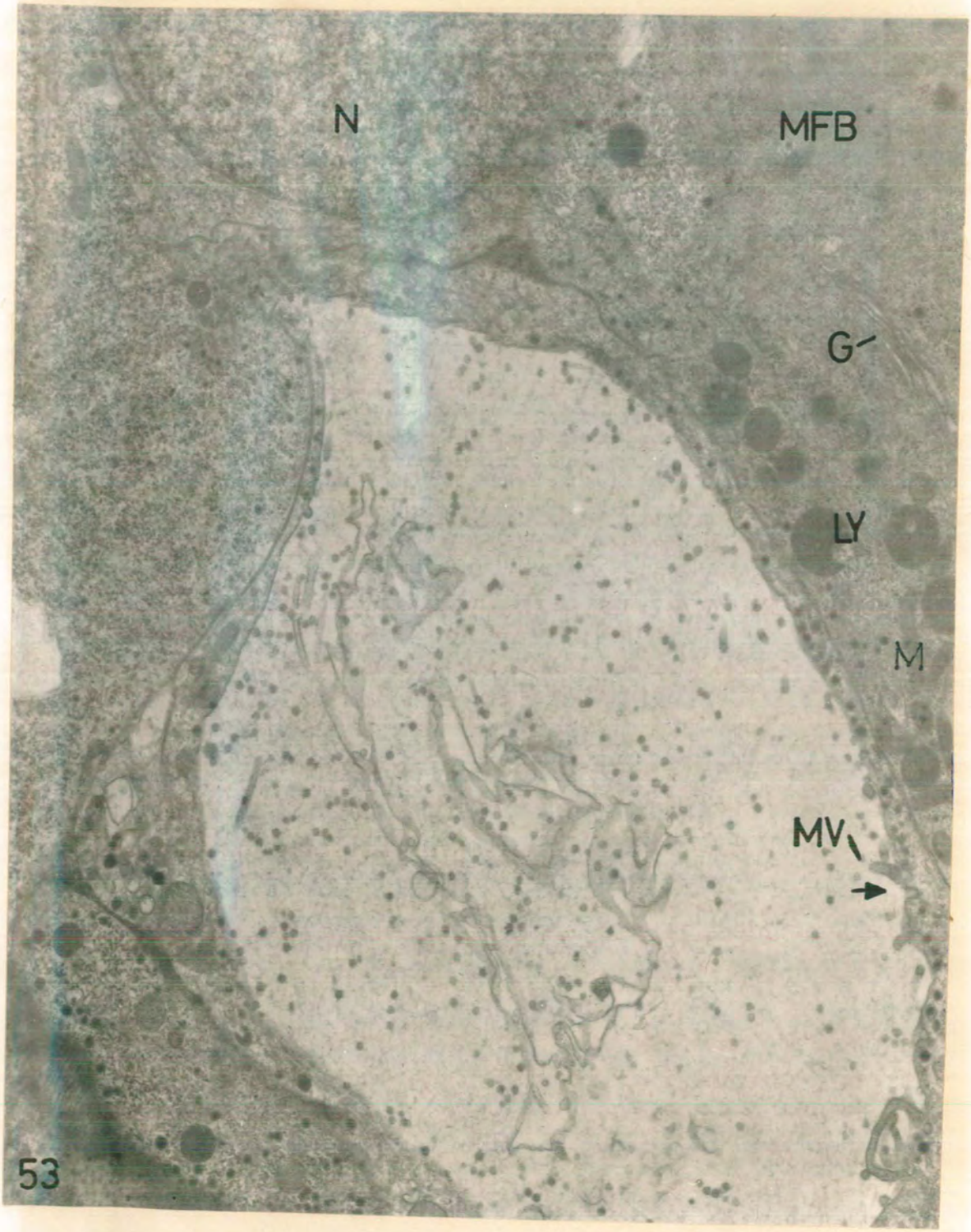
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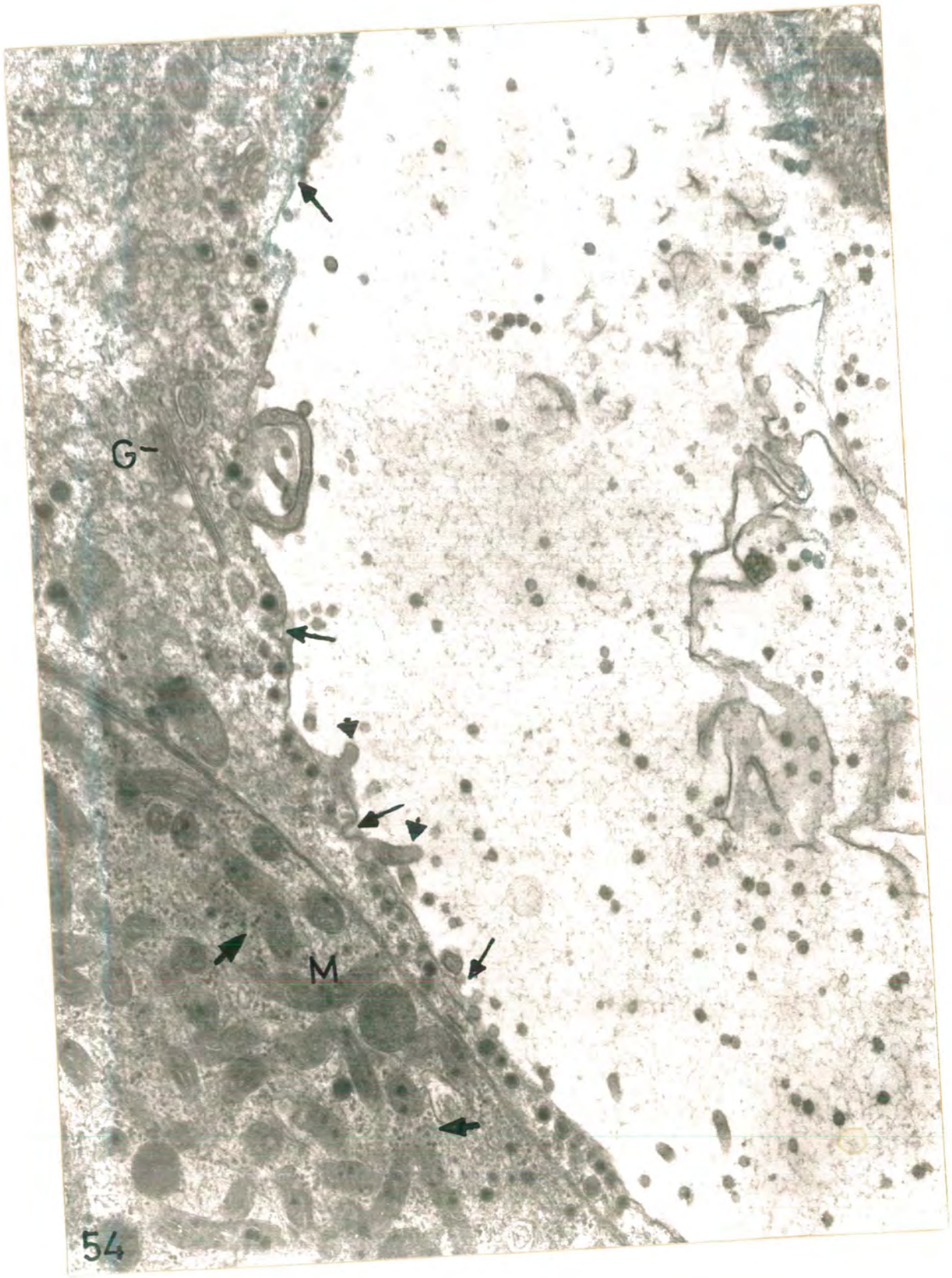
MFB
G
LY
M
MV
53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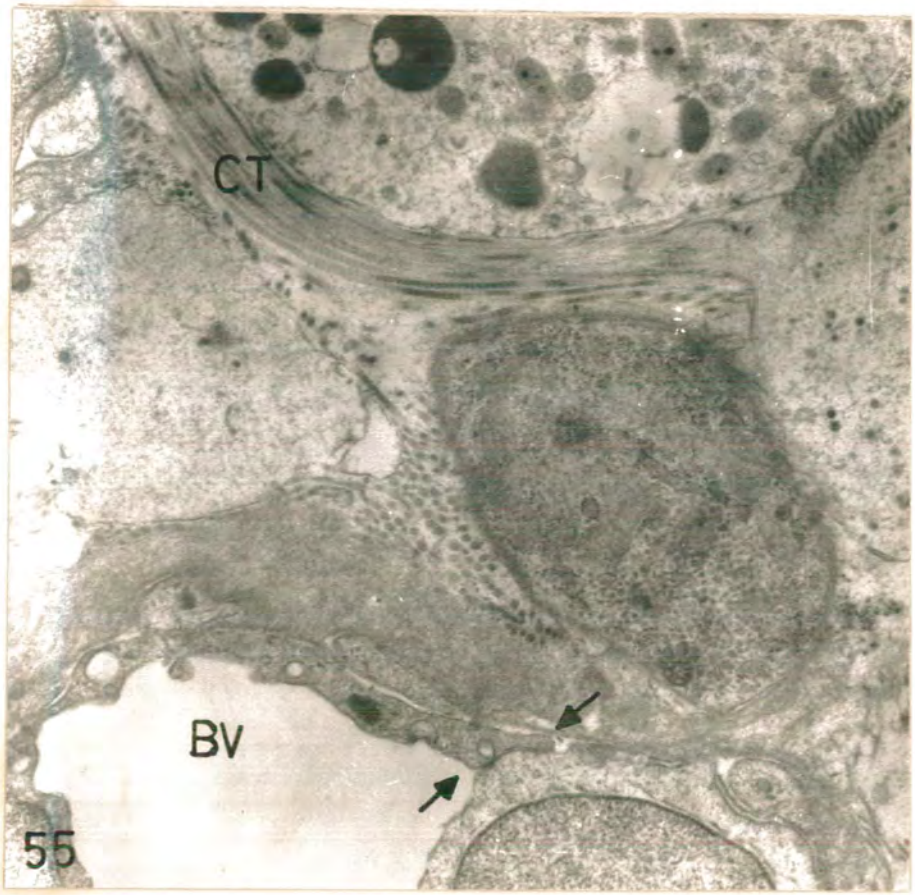
M
54


CT
BV
55