Derek John Mechan, B.Sc.

THE PINEAL OF SOME CHONDRICHTHYES

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SUMMARY OF THESIS Derek J. Mechan, B.Sc.

The pineal organs of the two Chondrichthyan species, Scyliorhinus canicula and Raja clavata, were examined by light and electron microscopy.

The Chondrichthyan pineal was found to be a blind ended outgrowth originating from the roof of the brain above the third ventricle. The end vesicle occupies a superficial position applied to the underside of the skull roof. The organ was shown to possess the structural characteristics of a photoreceptor containing small numbers of cone-like cells. The latter, however, were always degenerate and did not appear to have any connection with the pineal tract, since no distinct synaptic structures could be discerned.

Two types of supporting cell were observed which were classified as 'translucent' or 'opaque' depending upon the appearance of the cytoplasm. These cells were sometimes seen to contain dense core granules of unknown composition.

The pineal tract was shown to consist mainly of non-myelinated fibres together with a very few myelinated axons.

Melatonin could not be isolated from the pineal of Scyliorhinus, although it has been reported in other species of fish (Fenwick, 1970).

The effect of continuous light and dark regimes on the pineals of immature specimens was examined. It was found that in the absence of light, proliferation and vacuolation of mitochondria occurred within the various pineal cells. Continuous illumination produced no such effect.

The possible functional significance of the Chondrichthyan pineal structure was discussed with reference to other vertebrate species. The structure of the photoreceptor cell was related to the cell-line theory of Collin (1971).

CONTENTS

I. A SURVEY OF THE RELEVANT LITERATURE

1. Introduction.

The existence of the pineal organ was known to the ancient Greeks and Romans. Galen (A.D. 131-201) observed the pineal in oxen, sheep and apes; describing it as 'scolecoid' or worm-like and having lymphatic functions. He also mentioned that other authors had named it the epiphysis (Singer, 1956). The theory that the pineal possessed a truly glandular function seems to have originated with the Romans (Gladstone and Wakeley, 1940), who described it as the glandula pinealis.

Much later, Rene Descartes (1596-1640) claimed that the epiphysis represented the 'seat of the soul' where the 'spiritus animalis' was formed from blood borne particles. (Ariens Kappers, 1965a).

William Cowper (1666-1709) wrote: "... the glandula pinealis which we take to be a lymphatic gland, receiving lympha from the lymphe ducts which pass by way of the third ventricle of the brain to the infundibulum and glandula pituitara". (Gladstone and Wakeley, 1940).

However, the pineal was shown to be neither the seat of the soul, nor a lymph gland, by the careful anatomists and histologists of the nineteenth century. Leydig (1892) examined the pineal in embryonic lizards and concluded that it was definitely not a sense organ. Later, however, Spencer and Francotte (both in 1887) examined the pineal with respect to a photoreceptive function, since the concept of the 'third eye' had long been established, but neither were able to produce unequivocal results. (Reported by Gladstone and Wakeley). This work did not inhibit later authors from suggesting that the pineal might be a light receptor, however, and in 1892 T.H. Huxley referred to the pineal as 'the remains of a cyclopean eye possessed by some remote ancestor of the Vertebrata'.

The pineal organ has, in this century, been the subject of intensive study which has yielded a large volume of literature. Foremost are the

writings of Gladstone and Wakeley (1940), Kitay and Alshule (1954) and Wurtman, Axelrod and Kelly (1968a).

The widespread use of the electron microscope in the last decade has contributed much to studies of the pineal. This caused Wurtman et al. to write in the preface to their monograph (1968a): 'A description of the pineal written ten years ago would probably have included the following statements: "The pineal body is a part of the epithalamus; it is connected to the brain region in all species by nerve tracts. The pineal has evolved from a primitive photoreceptor, or 'third eye' which is common in extinct species. However, in the modern mammal it has lost all functional relationship to light and persists only as a vestige which mysteriously calcifies at the time of puberty. Pineal tumours are frequently associated with precocious sexual development in young boys. It had once been believed that the human pineal was a gland, and secreted a hormone which inhibited the gonads. Under this formulation, precocious puberty developed because the tumour destroyed the pineal's ability to secrete this hormone. However, experiments designed to test the glandular function of the mammalian pineal have yielded equivocal data. Hence pineal tumours probably produce their endocrine sequellae solely as a result of the pressure that they exert on other brain areas." '

Wurtman et al. point out that much of their hypothetical ten-year old statement was (in 1968) incorrect. The available evidence suggested very strongly that in the great majority of those species which have been studied, the pineal is a functional organ and does not 'produce (its) endocrine sequellae solely as a result of the pressure that (it) exerts on other brain areas.'

Since the publication of Wurtman's book, the pineal has been subjected to further study and has been discussed at a number of symposia. These studies have dealt with the structure, ultrastructure and function of the pineal region in a wide variety of species.

In surveying the literature concerning the pineal, it is convenient

to examine it from two standpoints; firstly, that of histological structure and ultrastructure, and secondly, that of function.

2. Structure of the Pineal Organ in Vertebrates.

The majority of animal species studied so far exhibit pineal organs of differing shapes and sizes. Although an almost bizarre range of morphological diversity is found, a closer examination reveals certain basic similarities from which a pattern begins to emerge.

The pineal, together with the pituitary, choroid plexuses (and others) represents one of the smaller derivatives of the brain wall - one of the so-called 'circumventricular organs'. In essence, it is a simple or complex evagination of the diencephalic roof and may retain a lumen which maintains direct communication with the third ventricle. It is, perhaps, best referred to as the pineal or epiphyseal system, since in many species it consists of a pineal proper (or epiphysis cerebri) together with a parapineal (frontal organ or stirnorgan). The latter, also called the parietal organ, must not be confused with that other diencephalic outgrowth, the paraphysis which lies anterior to the pineal system.

Kelly (1962) has suggested that TWO primordia lying side by side constitute the beginning of the pineal mass; these then fuse to create a single, midline, presumptive area. A secondary doubling might then occur to create the tandem arrangement found in many adults. This does not necessarily exclude the possibility that the pineal and parapineal could have developed from the two primordia and maintained bilateral positions. It is true that the skulls of certain primitive fishes exhibit bilaterally placed indentations suggesting that the pineal and parapineal were located side by side (Edinger, 1956).

A more detailed study of the epiphyseal system reveals that within the various vertebrate classes, two basic types of pineal have developed. The first of these is the primitive saccular structure, characteristic of lower vertebrates; the second being the compact, parenchymal structure found in more advanced forms. (Oksche et al., 1970).

2.(i) Structure of Saccular Pineal Systems.

Although it is not easy to detect a pattern in the evolution of the pineal, it is none-the-less convenient to summarise the literature in a classical evolutionary sequence beginning with the Class: Agnatha.

The pineal system of Cyclostomes has been studied in some detail, particularly in Lampetra lamottei and L.planeri. In the former, Julyan (1964) has described the presence of sensory cells and pigment cells which may have a secretory nature. Ganglion cells have also been shown to be present. He did not, however, confirm the opinion of Knowles (1939) that the nuclei of the pigment cells are capable of changing their position within the cell in response to changing light conditions.

Collin (1969) and Meiniel (1969) working on the pineal and parapineal respectively of L.planeri confirmed the observations of Julyan. They showed that the pineal system consisted of a well-developed epiphysis and separate frontal organ (see fig.1) both of which have a well-defined nerve supply. In the case of the pineal this links to the posterior commissure, while that of the parapineal runs to the habenular commissure.

Both organs contain well-developed pigment cells and sensory cells which bear a strong resemblance to the rods and cones of the lateral eyes. These cells consist of an inner and an outer segment; the latter contain numerous membrane sacs in the form of flat discs piled one on top of the other. These sacs, however, are not as evenly spaced or structurally complete as those of the retinal rod (or cone) cells, neither are the cells as numerous, or as closely packed.

The superficial position of the system, together with the presence of sensory cells and ganglion cells would suggest that perhaps a photoreceptive function is present. This function has now been ascribed to the majority of saccular pineal systems on morphological grounds alone.

The Class Osteichthyes has also attracted considerable interest and the Teleosts in particular have been studied extensively by a large number

of workers. One of the earliest, Hill (1894), studying Salmo fontinalis, S.purpuratus, S.fario, Catastomus teres, Stirzostedian vitreum and Lipomis pallidus showed that the pineal anlage is always an outgrowth of the diencephalic roof rostral to the posterior commissure. He also believed that its earliest stages were symmetrically arranged. An examination of the development of Salvinellus fontinalis has shown that the more dorsal of two diencephalic evaginations develops into the epiphysis cerebri while the more anterior evagination may, in the adult, become situated posterior to the pineal (Holmgren, 1965).

In the developing Toad Fish, Opsanus tau, the parapineal lies to the left of the epiphysis but later comes to occupy a more anterior position (Terry, 1910), while studies of Coregonus macrophthalamus have shown that the pineal may be finally displaced to one side, instead of rostrally or caudally (Friedrich-Freksa, 1932).

In no species has the well-developed parapineal typical of the Cyclostomes been observed (see fig.2).

The compact vesicular pineal structure seems common in most Teleost species and has been reported in Thynnusthynnus (U.Holmgren, 1958). Mugil auratus, Uranoscopus scaber (Rudeberg, 1966), S.pilchardus (Rudeberg, 1968a), Gobius, Dermogenys, Arius, Macrones, Plotosus (Friedrich-Freksa, 1932), and Esox lucius (Owman and Rudeberg, 1970). In the catfish Clarias lazera, the vesicle has been described as 'diffuse' (Rizkalla, 1970, Bose, 1959).

A close examination reveals further similarities. A nervous connection with the brain is present, at least in Onchorynchus (Hafeez and Ford, 1967), Esox (Owman and Rudeberg, 1970), Mugil, Uranoscopus (Rudeberg, 1966), Thynnus (Murphy, 1971) and Phoxinos (Okshe and Kirschstein, 1971). These connections are associated with synapses of varying complexity.

Rod- (or cone-)like 'photoreceptors' are present in all of the aforementioned species, together with supporting cells and ganglion cells which

vary only in detail. There is also some evidence of synapsing between photoreceptors and nerve fibres in Mugil, Uranoscopus, Sardine (Rudeberg 1966; 1968a) and Phoxinus (Okshe and Kirschtein, 1971). These synapses give credence to the idea that the photoreceptors are functional structures, even though they always show some indication of degenerative change. This observation has also been extended to Salmo irideus (Breuker and Horstman, 1965). (See fig.8). This hypothesis is further supported by the fact that in many species the skull immediately overlying the pineal region is markedly thin and probably favours the penetration of light.

The pineal of the catfish Clarias seems to represent an anomaly among the Teleosts. Here the pineal body does not apparently originate as a hollow outgrowth of the brain roof, since in the fry the body is a solid mass, the central region of which degenerates to produce a central lumen continuous with that of the stalk which leads to the third ventricle. (Rizkalla, 1970). It is also poorly innervated and there is no evidence of sensory cells. Instead a glandular epithelial layer is present which exhibits glycogenic activity. For this reason the epithysis of Clarias is considered to have a secretory function.

With the exception of Clarias the Teleosts share a (fairly) common pineal structure, a structure which extends to other bony fish, for the pineal of the Dipnoan, Protopterus has been described as closely resembling that which is characteristic of the more primitive types. (Uek, 1969, Holmgren, 1969).

It is rather surprising, since the dogfish is one of the animals most commonly dissected in schools and colleges, that the cartilaginous fish members of the Class Chondrichthyes have largely been ignored with respect to their pineal organs.

Squalus acanthias was observed by Holmgren in 1918. He described a vesicular structure connected with the third ventricle by a long stalk. He also observed that the epithelium lining the lumen contained cells exhibiting

outgrowths of various shapes and sizes, from thin tendril-like to more bulbous structures. He postulated that these represent part of a regeneration cycle involving the production of outer segment components, but did not mean, as many subsequent workers believed, that they represented stages in a cycle of secretion.

The development of the pineal region of Squalus was described by C. Sedgwick Minot (1901). It develops as a blind ended outgrowth from a region just caudal to the paraphyseal arch, which reaches forward to embed in the skull above the cerebral hemispheres. A familiar stalk plus end-vesicle arrangement is therefore established. (See fig.3). The stalk contains fibres which form a definite tract near to the base. This tract has been followed to the posterior commissure. (Stûdnika, 1905).

The end vesicle is always small and sometimes shows evidence of bilaterality. For example, in Raja clavata and Acanthias vulgaris it may be heart shaped having a small notch on the anterior border, while in Centrophorus granulosus it is T-shaped. Alternatively, it may be round or cone-like and often exhibits intra-individual variation. (Gladstone and Wakeley, 1940).

The pineal region of the dogfish Scyliorhinus canicula has been examined by Balfour (1878), Cattie (1882), Gallioti (1897), Studnika (1905) and Rudeberg (1968b, 1969). The recent studies of Rudeberg have shown that in Scyliorhinus, the observations of Holmgren (on Acanthias) were indeed correct since photoreceptors are definitely present.

These photoreceptors, which are distinctly rod-like, always show signs of degeneration (although the possibility that this is a fixation artefact cannot be excluded) but, as yet, there is no evidence of regeneration. The latter may perhaps be expected since the outer segments of rods and cones in amphibian retina are known to be continuously growing from their base while the topmost lamellae are continuously removed and digested by the pigment epithelium. (Young, 1968).

In addition to photoreceptors, glycogen and granule-containing supporting cells are present, together with ganglion cells which allegedly give rise to the diffuse pineal tract which runs to the posterior commissure. Distinct synapses are seldom seen. (Rudeberg, 1969). Myelinated fibres have not been reported in Scyliorhinus but they have been found in Squalus and Galeus (Altner, 1965).

A distinct parapineal is not observed in cartilaginous fish, although Etmopterus sometimes exhibits a distal pineal terminal vesicle which completely penetrates the cartilaginous skull and resembles that found in Amphibians. (Altner, 1965).

The frontal organ in the Amphibia characteristically lies just below the skin, above the roof of the skull and is joined to the epiphysis by a long stalk. (Kelly and Smith, 1964). (See fig.4). Both the pineal and parapineal exhibit well developed photoreceptors, which bear the familiar degenerating outer segments. (Kelly and Smith, 1964). It is suspected that the latter may be removed by macrophages present in the lumeni of the two structures. There is also evidence of considerable biochemical activity within the cells. (Kelly and Van de Kamer, 1960).

Recent work has shown that discrete particles are present on the lamellae of the outer segments (Uek, 1971). These are about 50 A in diameter and are selectively demonstrated by prolonged osmium tetroxide fixation; it is suspected that they may indicate the presence of photo-pigments.

A complex nerve tract is present which passes down from the epiphysis to a region below the posterior commissure. (Paul et al. 1971).

Developmental observations on Taricha have shown that the cells of the pineal originate in a special cell proliferation zone (Hendrickson and Kelly, 1969). Photoreceptors are always present, but are more numerous prior to metamorphosis (Kelly, 1965) and never account for more that 18% of the total cell population. In Xenopus a meningeal spot continuously

ensures that the frontal organ is exposed to light. (Van de Kamer et al. 1962).

In Amphibia then, there is very strong morphological evidence for a photoreceptive function.

In the Class Reptilia the evidence for photoreceptive function is even stronger, for here the epiphyseal complex exhibits the greatest degree of development among the saccular pineal structures. (See fig.5).

The Reptiles were the first group to be shown to contain well developed pineal photoreceptors, following independent researches by Steyn (1959, 1960) and Eakin and Westfall (1959, 1960) on lizards.

Observations of Lacerta muralis reveal that several noradrenergic nerve endings are present in the pineal at various sites, e.g. between cells and processes in the epithelium and even free in the lumen (Wartenberg and Baumgarten, 1969).

In the turtle Pseudemys scripta elegans evidence suggests that the 'pseudosensory' cells may have a dual function (Vivien-Roels, 1969, 1970) since secretory material appears to be present. It is further suggested that the posterior region of the pineal has a purely sensory function while the anterior region is secretory.

The rôle of the pineal in controlling orientation in the lizard Cordylus polyzonus jordani has been explored by Steyn and Steyn (1965) who suggested that a thermoregulatory function might exist.

It is in Sphenodon that the epiphysis appears to reach a peak of development; for here the extracranial parietal organ has undergone such differentiation as to confer an eye-like morphology. The cells in the roof of the structure have become modified to form a thickened, well-developed transparent lens. The layers immediately above form a transparent cornea and so, together with the retina below, a conspicuous eye-like structure is formed. (Stebbins and Eakin, 1958).

2. (ii) Structure of Compact Pineal Systems.

If the reptiles exhibit the apex of the evolution of saccular pineals, the Class Aves probably provides examples of a structure that is evolving into a more compact organ like that of the Mammals.

The volume of literature relating to Avian pineals is quite large but is confined to relatively few species, with Gallus domesticus and Passer domesticus being singled out for special attention. The relevant literature has most recently been summarized by Ralph (1970). (See fig.6)

Stammer (1961) found that the avian pineal body exhibits a fairly constant structure in several species, consisting mainly of follicular or glandular tissue, being well innervated and with a rich blood supply. However, he found neither nervenor sensory cells to be present. More recent work by Renzoni (1970) has shown that the pineal is only one of several diencephalic evaginations (in doves and pigeons); a second one of which persists as a parenchymal accessory structure (this latter may be a homologue of the parapineal found in the lower vertebrates).

In at least one species, Passer domesticus, photoreceptors have been detected; they possess typical inner segments containing numerous vesicles and granules, associated with irregular complexes of vacuoles or whorl-like lamellae. (Okshe and Vaupel-von Harnack, 1965; Okshe and Kirschtein, 1969). The latter are found in the lumeni of the organ and are generally taken to represent degenerative stages. No strictly rod- (or cone-) like outer segments have been observed.

The presence of dense core granules within the pinealocytes has suggested to Uek (1970) that a dual secretory/sensory rôle might exist. Nerve cell bodies are found, within the parenchyma, whose axons run to the habenular commissure. (Uek, 1970).

A true parenchymal pineal is characteristic of the Class Mammalia. (Quay, 1965a).

The mammalian pineal has been extensively examined by a large number

of workers, with much attention being given to that of the white rat (Arstila and Hopsu, 1964; Arstila, 1967; Gusek et al. 1965), the rhesus monkey (Wislocki and Dempsey, 1948; Wartenberg, 1968), rabbit (Wartenberg and Gusek, 1965) and cow and sheep (Anderson, 1965).

The mammalian pineal differs markedly from that which is found in the lower vertebrates. The familiar (multiple) saccular structure is replaced by a single compact parenchymatous organ containing a number of well-defined cell types. (See fig.9). An exception to this is the hamster which possesses both a superficial and a deep pineal (Sheriden and Reiter, 1970a,b).

Perhaps the prevalent cell type is the so-called pinealocyte (pineocyte, epiphyseal cell, chief cell and others). It contains an endoplasmic reticulum of varying complexity together with a large Golgi apparatus. Lyosomes, mitochondria and possibly lipid droplets are found in the pale, abundant cytoplasm. The nucleus may be so deeply indented as to suggest a polymorphous organisation. Numerous cytoplasmic processes may be present, some of which terminate in dilations near to the pericapillary space. (see fig.9).

Supporting the pinealocytes are small, fat-laden cells with round or oval nuclei and dark cytoplasm containing a very rich endoplasmic reticulum. These glial cells contain discrete lipid droplets and characteristic glial fibrils.

Many non-myelinated nerve fibrils are found among the pinealocytes and glial cells as well as at the surface of the pericapillary space. (See especially Wolfe, 1965 and Anderson, 1965). The fibres end in bulbs containing both granular and agranular vesicles and terminate near to the pinealocytes. (Wolfe, 1965). Clear synaptic junctions have not been observed between pinealocytes and nerve fibres, although somato-somatic junctions between adjacent pinealocytes do exist. (Hopsu and Arstila, 1965).

If, as is supposed, the pineal arises from invaginations and

diverticulation of a primordial membrane, it is not difficult to see how the pinealocytes and astrocytes come to be separated from the blood vessels by a basal lamina and perivascular connective tissue. There is, however, some conjecture as to the exact relationship of the principle cell types to the vascular system (see the arguments of Wurtman, Axelrod and Kelly, 1968a).

If the aforementioned scheme of development is to be believed, then it is likely that the pinealocytes would be drawn out into a number of complex processes. The existence of these processes, together with glial processes and nerve fibres, indicates why it is so difficult to comprehend the various cellular relationships. (See fig.9).

It is apparent in many species that a layer of astrocytes is often associated with the basal lamina, which itself may be intermittent. (Arstila and Hopsu, 1964). The latter may have some significance since it implies lack of a blood brain barrier in the region of the epiphysis.

Developmental studies on the white rat (Wallace, Altman and Das, 1969; Blumfield and Tapp, 1970) have shown that cell proliferation is high in the neonate, continuing at a reduced rate into adulthood, when numbers remain fairly constant. Results suggest that, far from being a non-functional vestige, the mammalian pineal is an actively metabolising, functional organ.

2. (iii) Structural evolution of pineal complexes.

From a purely structural viewpoint, it can be seen that among the lower vertebrates, i.e. the fishes, amphibia and reptiles, the pineal consists mainly of saccular structures of varying types. In almost all cases, there are three predominant cell types; neurons, supporting cells and photoreceptors which bear a striking resemblance to retinal rod (or cone) cells. This cell may depend to some extent upon the supporting cells for its functional maintenance and almost certainly its outer segment undergoes degenerative changes. It is problematical whether this outer segment takes part in a

continuous cycle of degeneration and replacement.

Structurally then, the saccular pineal system gives the appearance of possessing a photoreceptive function; this may, or may not, be associated with a secretory rôle.

The avian pineal may well fit into the above with more accent placed on secretory activity. The mammalian pineal on the other hand has very few factors in common with those already mentioned. Structurally compact, the mammalian epiphysis has many characters of a secretory organ while retaining an extremely abundant nerve supply (Ariens-Kappers, 1965b, 1970). Certainly obvious photoreceptors have been lost.

One small piece of evidence exists which connects the degenerating photoreceptor of the saccular pineal with the pinealocyte of parenchymatous pineals. Dense intracellular lamellae have been reported in parenchymal cells; being studded with small vesicles and having the appearance of synaptic ribbons (Wolfe, 1965). These have been compared to those of the axosomatic synapses in the cerebral cortex and also, perhaps more significantly, to the ribbon containing synapses of photoreceptors.

Using this one tiny piece of evidence it is tempting to consider the possibility that the parenchymal pinealocyte of higher vertebrates is a descendant of the photoreceptor found in more primitive types.

Explanation of text figures

1. Pineal region of the Lamprey after Wurtman, Axelrod and Kelly (1968a). H.C. - habenular commissure, P.C. - posterior commissure.

2. Pineal region of the Teleost, after Holmgren (1965).

3. Pineal region of the Elasmobranch, Scyliorhinus canicula, after Rudeberg (1969).

4. Pineal region of the Amphibian - Anuran, after Kelly (1962).

5. Pineal region of the Reptile, after Nowikoff (1910).

6. Pineal region of the Bird - Gallus domesticus, after Studnika (1905).

7. Pineal region of the Mammal - Rattus, after Wurtman and Axelrod (1965a).

8. Saccular pineal: cell types, after Okshe and Vaupel von-Harnack (1965). S.C. - supporting cell, N.L. - nerve fibre layer, I.S. - inner segment, ellipsoid-granule with inner segment.

9. Mammalian pineal: cell types, modified from Anderson (1965). P.C. - pericapillary space, nerve-myelinated nerve fibre.

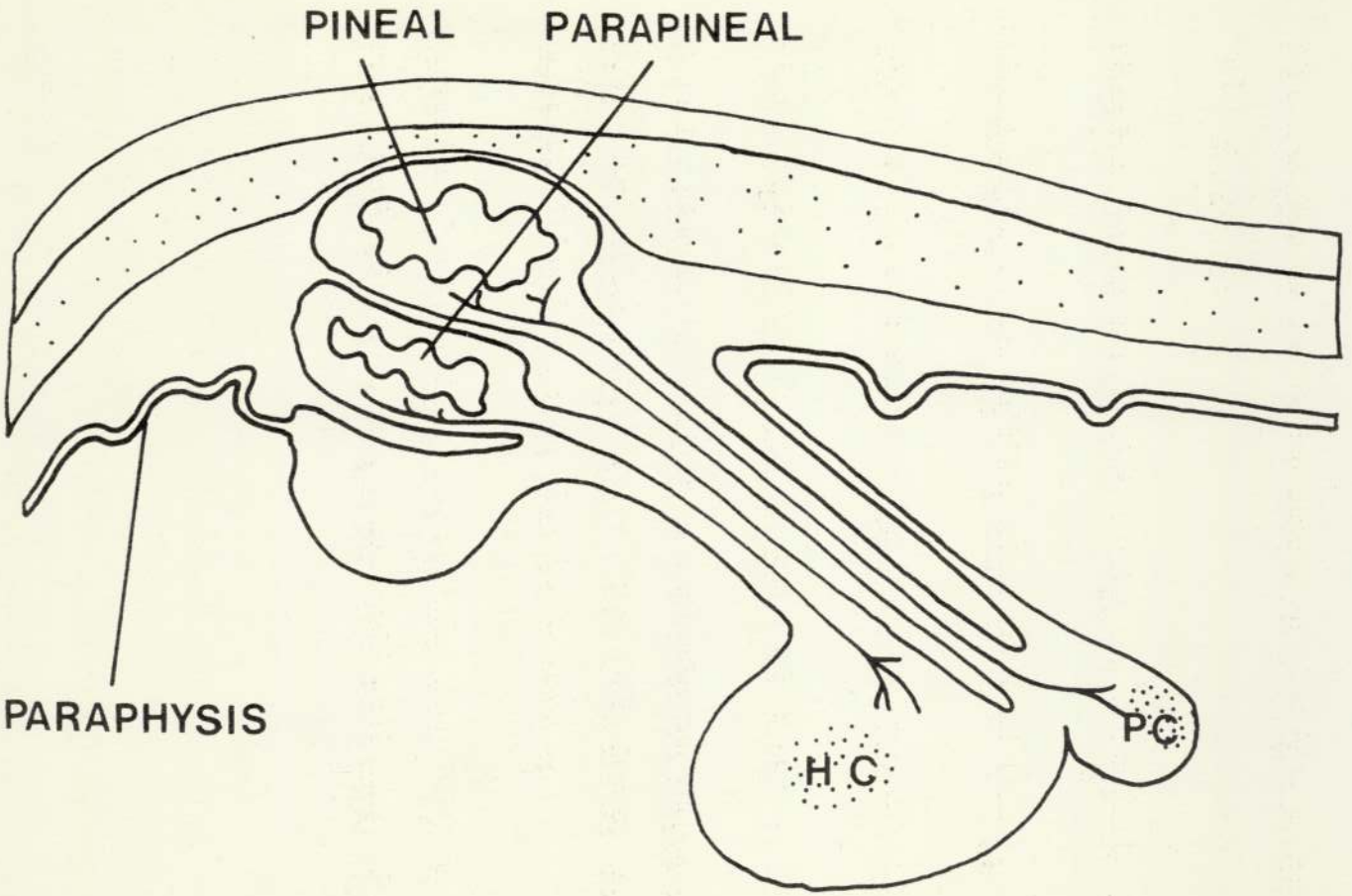


fig. 1.

PINEAL REGION OF THE LAMPREY (WURTMAN et al.)

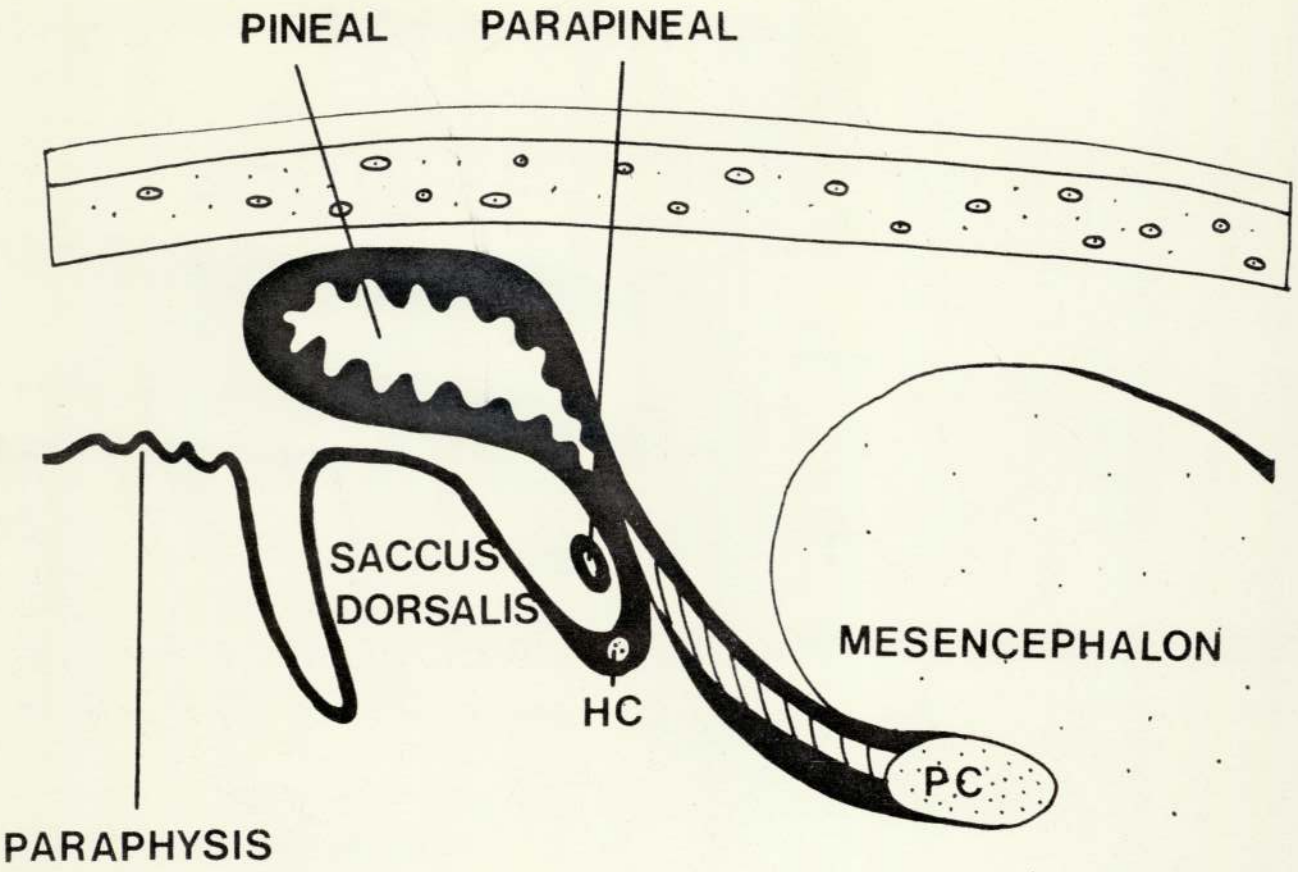


fig. 2.

PINEAL REGION OF THE TELEOST (HOLMGREN.)

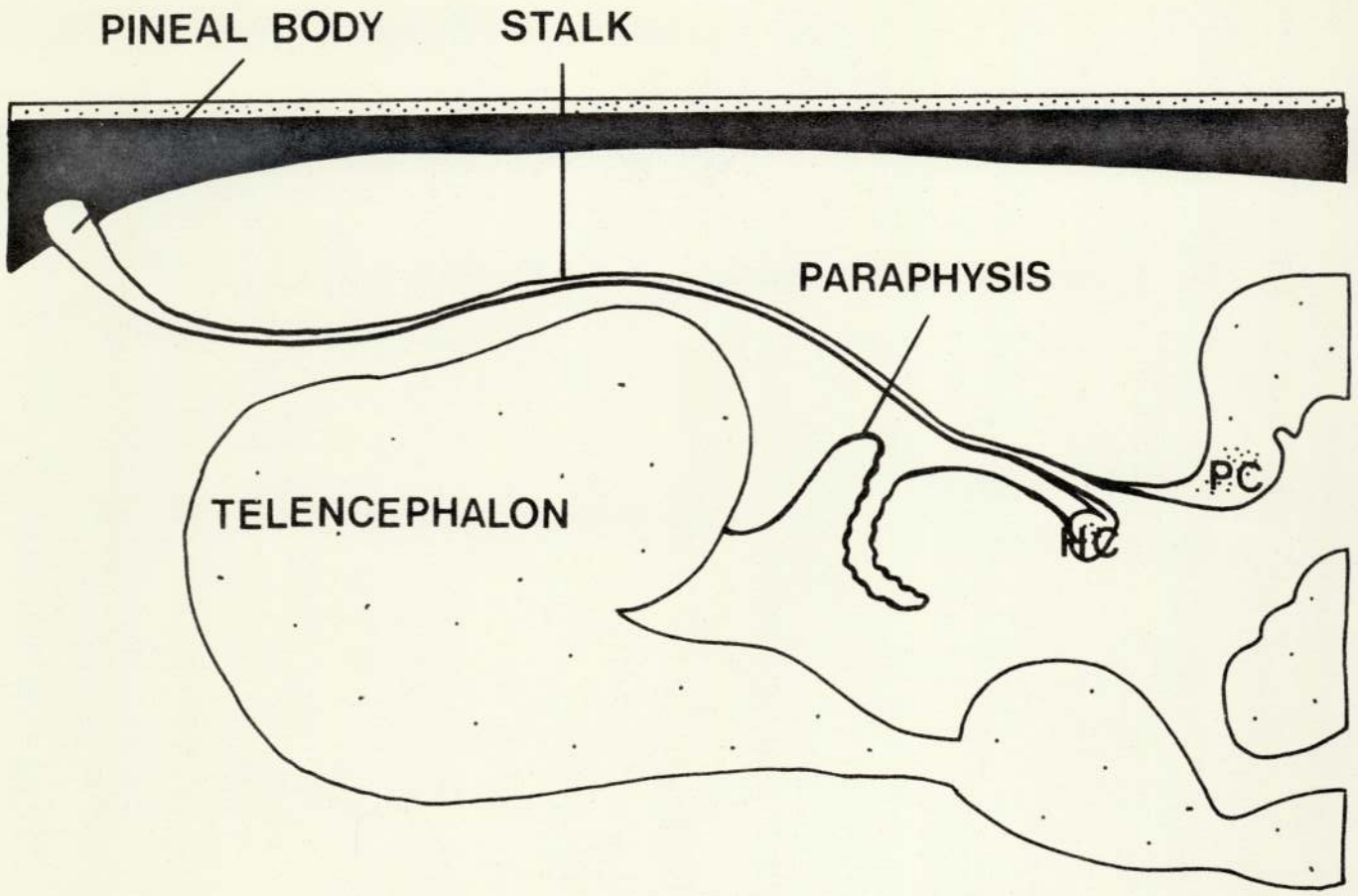


fig. 3. PINEAL REGION OF THE ELASMOBRANCH (RUDEBERG.)

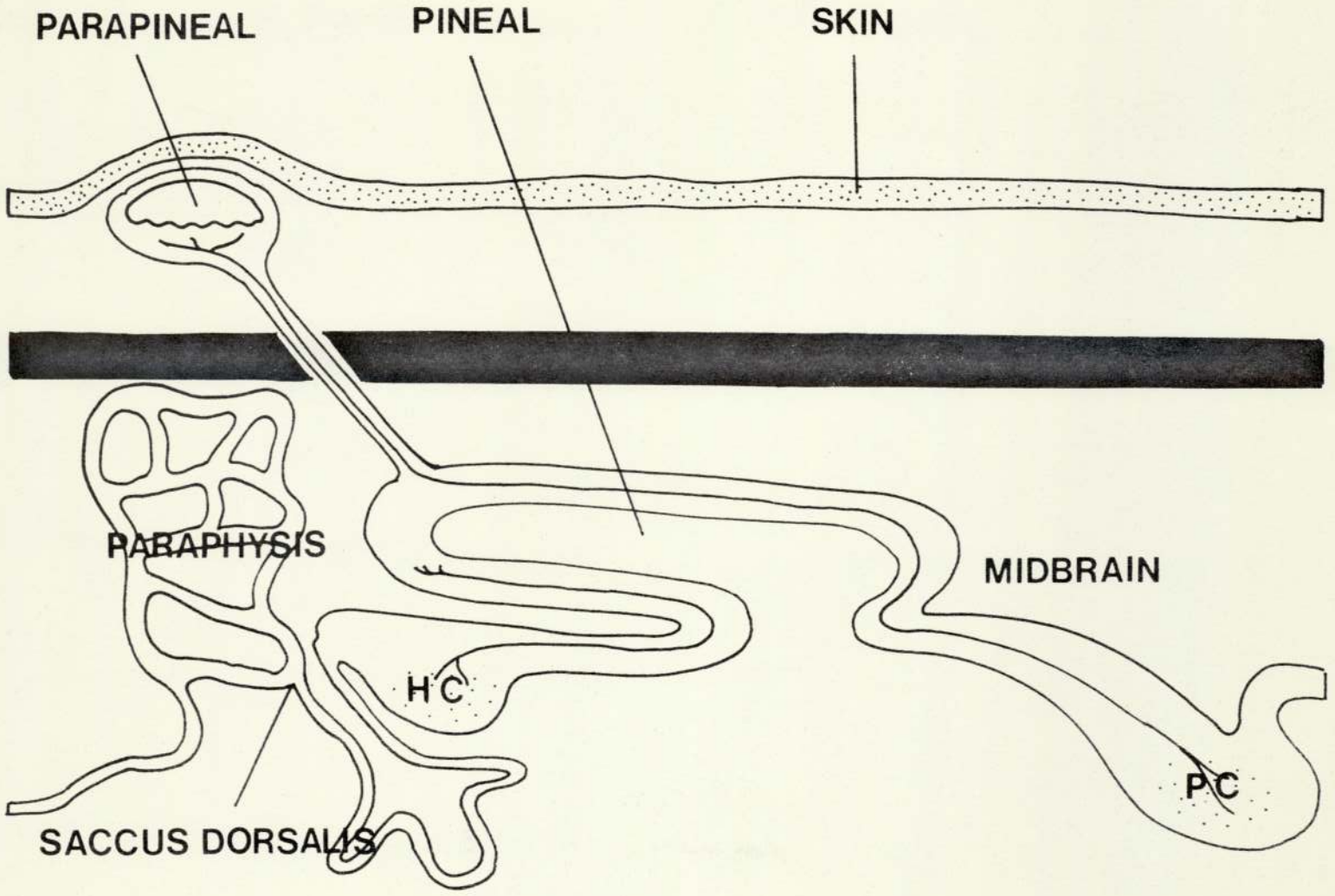


fig. 4.

PINEAL REGION OF THE AMPHIBIAN (KELLY.)

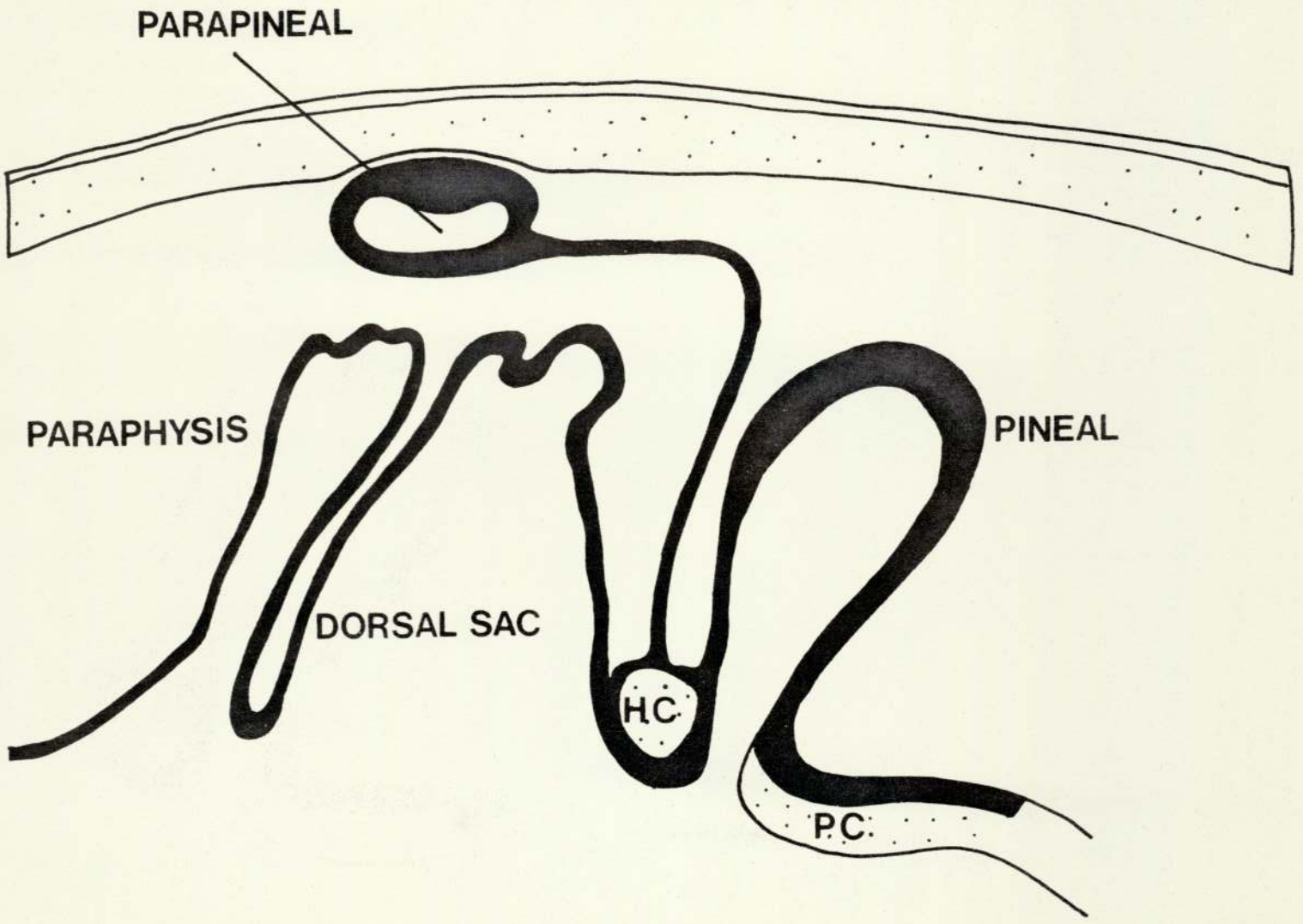
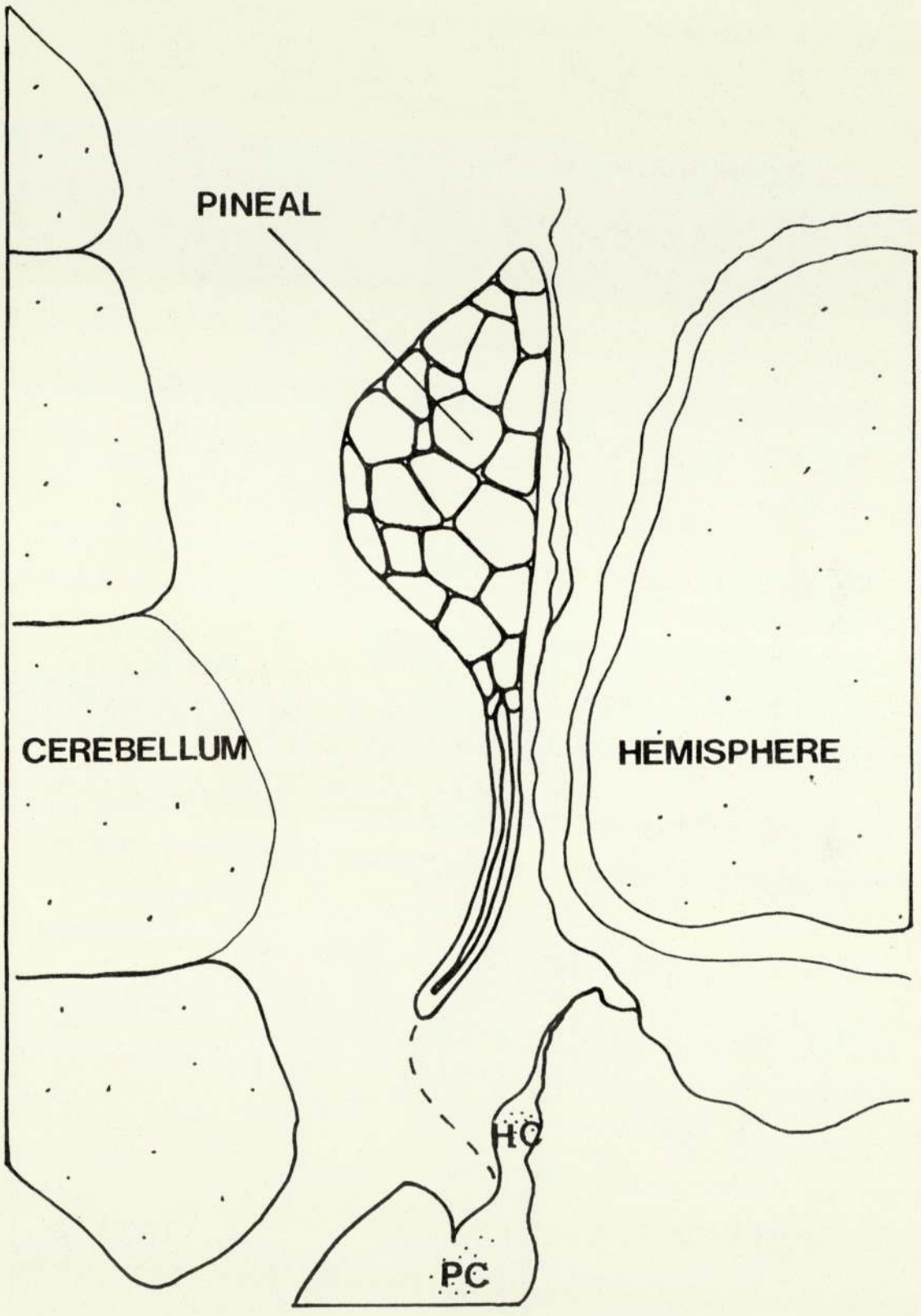


fig. 5.

PINEAL REGION OF THE REPTILE (NOWIKOFF.)



PINEAL REGION OF THE BIRD (STUDNIKA.) fig.6.

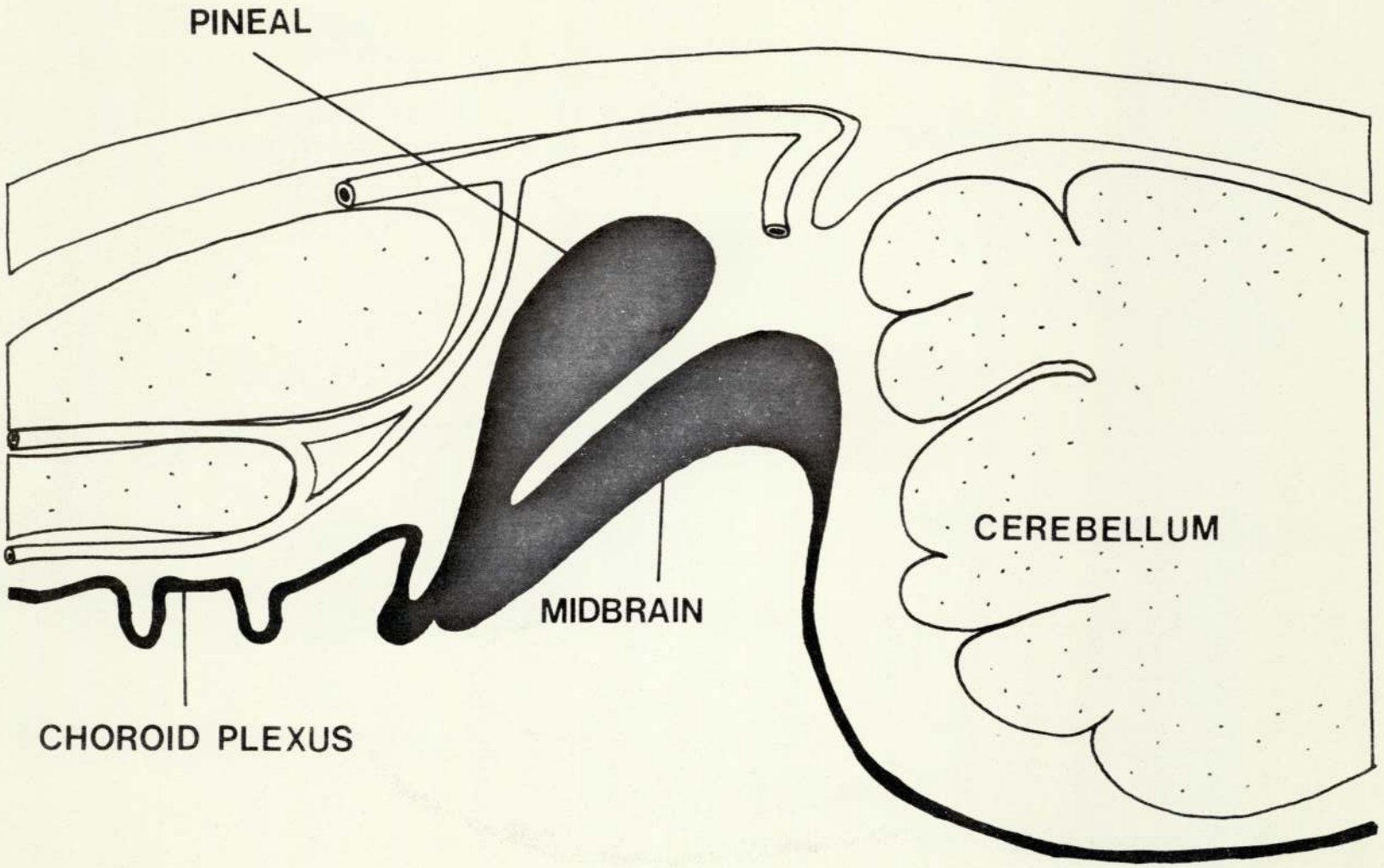


fig.7.

PINEAL REGION OF THE MAMMAL (WURTMAN, AXELROD.)

SACCULAR PINEAL: CELL TYPES

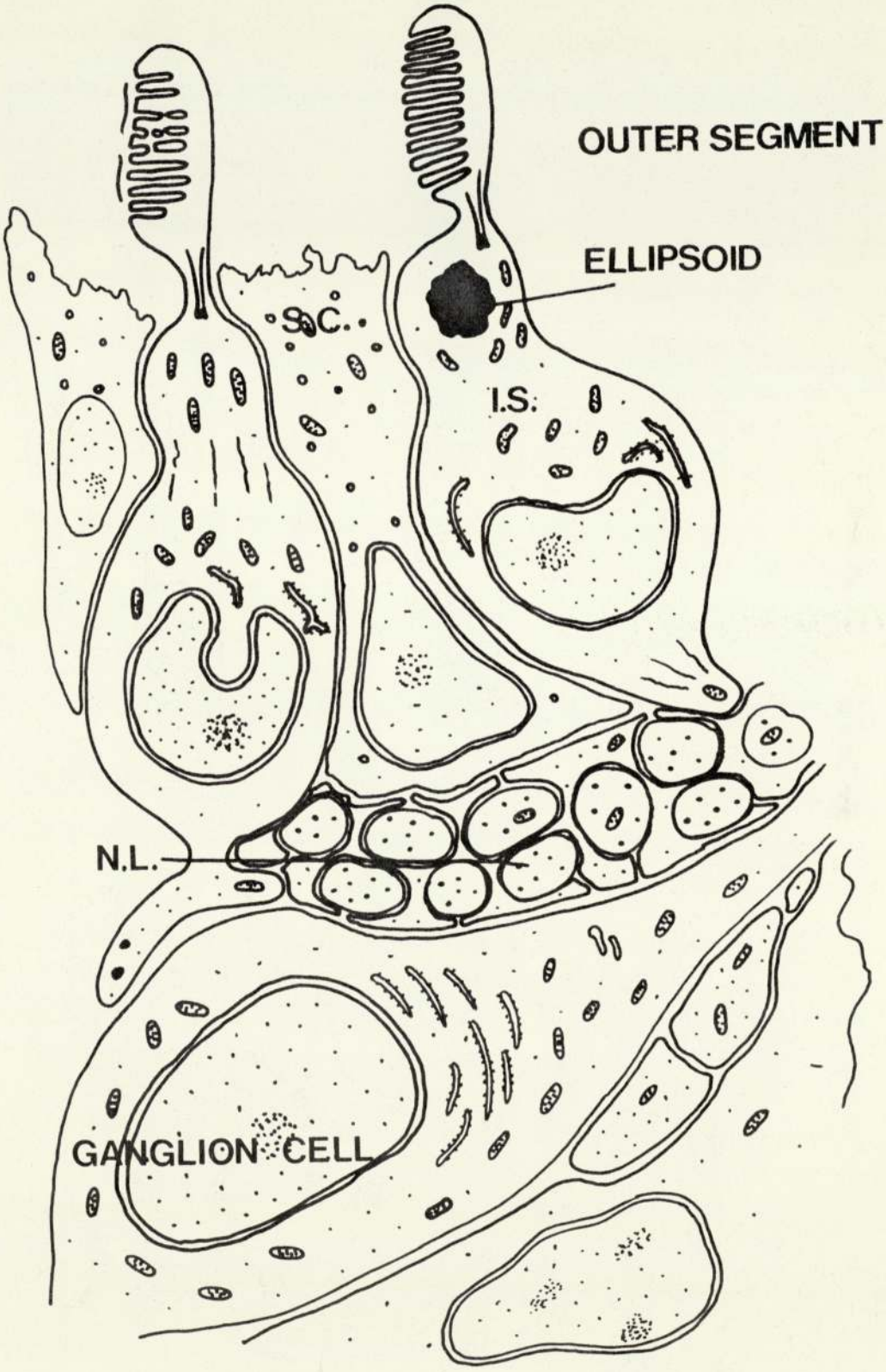
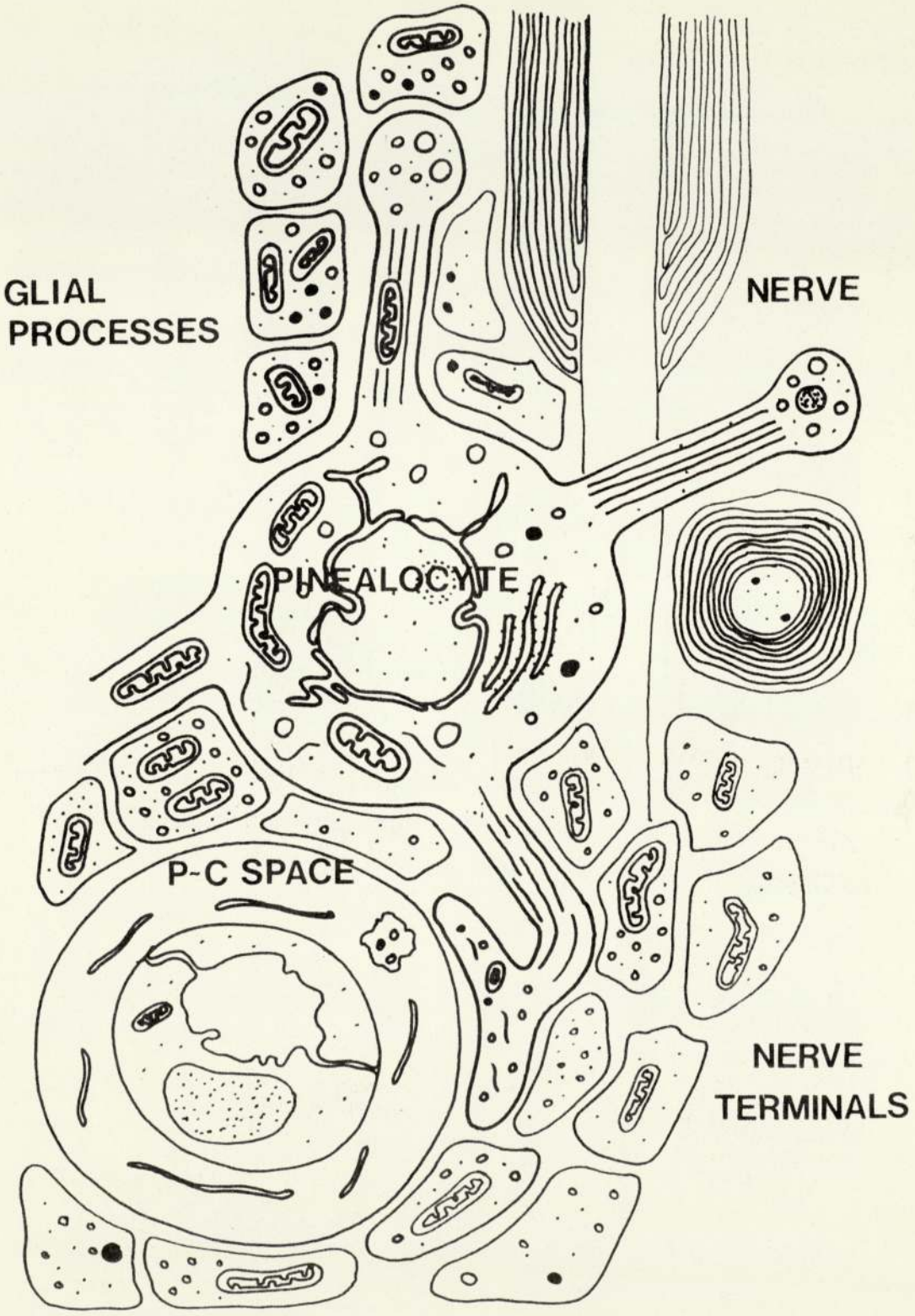


fig. 8.



MAMMALIAN PINEAL: CELL TYPES. fig. 9.

3. Function of the Pineal Organ in Vertebrates.

The lines of research undertaken to investigate the function(s) of the pineal in vertebrates have largely been determined by the structural considerations discussed above. The saccular pineal has been tentatively identified as a 'photoreceptor' and much effort has been made to confirm this hypothesis. The compact pineal of birds and mammals is regarded as having a glandular structure and as a result possible endocrine functions have been examined.

Forty eight years ago, in reviewing the literature, Krabbe (1923) wrote: "In the studies on the pineal gland, one has very often the impression that the author's desire to consider the organ as incretory in function is greater than their critical judgement regarding published results." This statement still has considerable validity, for even today there is little objective evidence to indicate exact function in either pineal type.

Both species of pineal apparatus have been examined biochemically, particularly the parenchymatus types. The latter have also been subjected to implantation, extirpation and replacement experiments. Both saccular and compact pineals have been studied with respect to photoperiodicity and also electrophysiological responses.

3. (i) Biochemistry of the Pineal.

Perhaps the most significant step in the elucidation of pineal function has been the purification and identification of a number of specific indoles in the pineal of mammals.

It has long been known that bovine pineal extracts cause skin lightening reactions when fed to amphibians. (McCord and Allen, 1917). This effect reverses the darkening induced by melanocyte stimulating hormone (MSH) from the pars intermedia of the pituitary and such compounds as caffeine. Lerner et al. (1958) isolated the skin lightening agent 5-methoxy-N-acetyltryptamine or MELATONIN (Lerner et al. 1959a).

Melatonin is a pale yellow crystalline material with a melting point of between 116°C and 118°C (Szmuskovicz and Heinzelman, 1960) and shows characteristic fluorescence in a number of solvents, for example, in hydrochloric acid its maximum by excitation is 300 nm and by emission is 540 nm. This means that it is possible to assay melatonin fluorometrically.

This compound is found only in the pineal[1], but in very small quantities, for example, the bovine epiphysis cerebri contains 0.2 ug/g. (Lerner et al. 1960). For this reason fluorometric methods of assay are generally too insensitive for accurate estimations and biological assays must be employed instead.

A quantitative biological assay was used by Mori and Lerner (1960) who darkened isolated frog skin with either MSH or caffeine and then observed the lightening effect of pineal extracts by measuring the increase in transmission through the skin. This type of procedure was reported to give consistently accurate estimates of melatonin levels (see also Lerner and Wright, 1960). Recent work by Hadley and Bagnara has, however, cast doubts upon the validity of this type of bioassay since melatonin only causes aggregation of pigment within a relatively small number of dermal melanophores and has no effect, for example, on iridiophores (Hadley and Bagnara, 1969).

Having identified melatonin, several workers attempted to determine the pathways leading to its synthesis. It was found to be synthesized by a step-wise conversion from tryptophan; a fact that has been demonstrated in tissue culture (Wurtman et al. 1968). (See fig. 10).

Tryptphan is converted into 5-hydroxytryptophan under the action of tryptophan hydroxylase. 5-hydroxytryptophan is then converted into 5-hydroxytryptamine (serotonin) by L-aromatic amino acid decarboxylase (Shein et al. 1967). Serotonin is then converted into n-acetyl serotonin

[1] Footnote

Minute amounts of melatonin have been detected in some peripheral nerves, e.g. in monkey and cow (Lerner et al. 1959b).

by an n-acetylating enzyme (McIsaac and Page, 1959) in the presence of acetyl CoA (Weissbach et al. 1960) and finally 5-methoxy-n-acetyl tryptamine (melatonin) is formed by the action of hydroxy indole-O-methyl transferase (HIOMT) (Axelrod and Weissbach, 1961). The last mentioned enzyme is apparently formed by a mechanism which is influenced by nor-adrenaline. The later stages of melatonin synthesis at least are therefore influenced by nor-adrenaline. (Wurtman, Axelrod and Kelly, 1968a).

The metabolism of melatonin is less well understood than the biosynthesis. This is partly due to the fact that, generally, studies have necessitated the introduction of large quantities of melatonin into the experimental animals and therefore any pathways identified may not be those typically used. (Wurtman, Axelrod and Kelly, 1968a).

This type of experiment has yielded evidence that melatonin is converted into 6-hydroxymelatonin which is then conjugated. The primary metabolites are 6-hydroxymelatonin sulphate, 6-hydroxymelatonin glucuronide and free 6-hydroxymelatonin. The latter represents only 12% of the excreted material. (Taborsky et al. 1965). Approximately 80% of these metabolites are excreted in the urine and 20% in the faeces. (Kopin et al. 1961; Kveder and McIsaac, 1961).

The pineal organs of many species have been assayed with respect to melatonin, serotonin and HIOMT.

Melatonin has been found in rats (Prop and Kapper, 1961), cow (Lerner et al. 1960), kangaroo (Quay and Baker, 1965), amphibia (Van der Veerdonk, 1965), and fish (Fenwick, 1970a).

Serotonin has been identified in a number of animals including rats (Quay, 1963), cow and monkey (Quay, 1966), sheep and guinea pig (Owman, 1965), turtle, lizard and snake (Quay and Wilhoft, 1964), and birds (Hedlund et al. 1971).

The enzyme HIOMT has also been identified in many species, including monkey, cat, cow (Axelrod and Weissbach, 1961), rat (Wurtman et al. 1963),

hen (Axelrod and Wurtman, 1964), toads, reptiles and fish (Quay, 1965b).

Recent investigations by Bak and his co-workers (1970) have shown that in rats most of the serotonin in the pineal is located in the pinealocytes and nerve endings. In the monkey, on the other hand, serotonin activity is confined exclusively to the pinealocytes and their processes. (Louis et al. 1970).

Other substances of physiological interest found in the pineal include nor-adrenaline (Pellegrino de Iraldi et al. 1965, 1966; Anonymous 1959) and dopamine (Pellegrino de Iraldi and Zieher, 1966). Lipids are present in large quantities forming droplets within the cells (Prop, 1965; Zweems, 1965) and so are lipolytic substances (Rudman, 1970). Significant quantities of certain free amino acids have also been reported, particularly cystathionine and taurine (Krass and Labella, 1970).

3. (ii) Functional Aspects of Saccular Pineal Systems.

The literature relating to saccular pineals has most recently been reviewed by Hoffman (1970) and Bagnara and Hadley (1970).

3. (ii - a) Effects of light on the saccular pineal.

That the pineal itself is sensitive to light has been demonstrated in various ways. Knowles (1939) reported that in lampreys epithelial cell nuclei become scattered in darkness but change their positions to form a regular line in light. This has not, however, been confirmed by later workers. On Astyanax mexicanus, on the other hand, it was reported that the organ exhibits major changes in prolonged light (or dark) regimes. The lumen increases in width, and the epithelium becomes disorganised. In alternate light and dark regimes, glycogen was found to accumulate, a fact that was taken as evidence of apocrine secretion. (Grunewald-Lowenstein, 1956).

Many workers have observed photopigmentary responses in the lower vertebrates and have attempted to relate them to pineal function. McCord and Allen (1917) observed that a temporary blanching was produced in frogs

and tadpoles when fed minced mammalian pineal. It was further observed in Lampetra planeri that diurnal colour changes were interrupted by removal of the pineal so that melanophores remain expanded in light (Young, 1935). It is interesting to note that this result was not confirmed in eyeless catfish where the pinealectomized animal exhibited normal responses. (Wykes, 1938).

This line of research was extended to teleost fish with reference to the degree of pineal exposure exhibited in a number of species. In general it was found that the best photopigmentary responses were exhibited by those animals which had the most exposed pineal. (Breder and Rasquin, 1950).

That the pineal participates in photoregulation is of little doubt; similarly, it seems certain that it plays a part in the control of photo-pigmentary responses. The mechanism of action of the pineal in these rôles is, however, less certain. (See below).

Light has other physiological effects that are mediated through the pineal. In lizards it has been demonstrated that daily and seasonal cyclic activities are governed by the amount of light which the pineal receives. These include reproductive behaviour, since impairment of the parietal eye removes restraint on the recrudescent phase of the reproductive cycle. (Stebbins and Wilhoft, 1966). The relationship between the pineal, the gonads and light has further been explored in Carassius auratus. In this species, Fenwick (1970b) has shown that gonad size is related to pineal activity which is reduced by light.

The photoreceptive rôle of the pineal has been examined in at least two species with respect to oxygen consumption. Clausen and Mofshin (1939) showed that in the lizard Anolis carolineus the pineal plays a part in the regulation of gas metabolism but is less important than either the lateral eyes or dermal receptors. Chugunov and Kispoev (1969), however, were not able to demonstrate any relationship between the pineal and the daily rhythm of gas metabolism in Rana temporaria.

3. (ii - b) Electrical activity of the pineal.

Electrical activity in the pineal nerve has been detected in several species.

Dodt and Heerd (1962) and Dodt (1964) examined afferent impulses from the cut stalk of the frontal organ of Rana temporaria. They found that two possible types of response could be elicited. Some pineals were inhibited by light of all wavelengths, that is they exhibited an 'achromatic response', while others showed varying responses dependent upon the wavelength used. These 'chromatic responses' exhibited maximum inhibition at 355 nm and maximum excitation at 515 nm. A constant reciprocal relationship between the intensity and duration of the stimulus was also found. These results caused them to postulate the presence of a photochemical reaction, an idea that has gained support in other quarters (e.g. Uek, 1971). More recent work by Hamasaki (1970a) has examined the relationship between the excitatory and inhibitory components of the 'chromatic' system. As a result of this he has suggested that perhaps excitatory and inhibitory photoreceptors synapse on a common ganglion cell.

The pineal of Salmo irideus was found to show maximum sensitivity at 507 nm. (Dodt, 1963), while those of a number of lizards exhibit maximum sensitivity at 600 nm., after removal of the lateral eye. In the latter case, the pineal was inhibited by all wavelengths and showed an absolute intensity threshold. (Hamasaki and Dodt, 1969).

This inhibition of electrical activity by light has further been identified in the dogfish, Scylliorhinus canicula. (Hamasaki, 1970b, Hamasaki and Streck, 1971).

3. (ii - c) Biochemical activity of the saccular pineal.

The theory that the epithelium bounding the lumen in saccular pineals is responsible for apocrine secretion has long been held. In recent years Rasquin (1958) studying several species of fish, and Hafeez and Ford (1967)

working particularly on Onchorhynchus nerka have given evidence to support this view.

If apocrine secretion were present, it would seem logical to assume that the secretory product could be cerebrospinal fluid, since in most cases the lumen of the pineal sac is continuous with those of the brain proper. (Rasquin, 1958; Van de Kamer, 1956).

The concept of apocrine secretion has largely become outmoded with the advent of electron microscopic investigations since the 'secretory bodies' have usually proved to be photoreceptive outer segments. This does not, however, totally exclude the possibility that this type of secretion might exist.

There is a general lack of firm evidence relative to a biochemical constituent in saccular pineals. Use of the electron microscope has demonstrated that various types of granule are present, but if these do contain ohysiologically significant biochemicals, then they must be present in minute quantities. More traditional techniques, however, have revealed that several interesting components are sometimes present. (See section 2 (i) above).

Recent work by Hafeez and Quay (1969) has shown that in Salmo gairdneri and Atherinopsis californensis 5-hydroxy trytophan is actively taken up and 5-hydroxytryptamine is actively synthesised. The presence of melatonin in the pineal of Oncorhynchus tshauytscha has been linked with gonad function. It has been suggested that perhaps melatonin inhibits the release of a gonadtropic factor. (Fenwick, 1970a).

The rôle of melatonin as a skin blanching agent in vitro has been extended by many workers to in vivo studies. In the absence of light it seems likely that the pineal is stimulated to release a melanphore contracting agent which may, or may not, be melatonin. This agent causes blanching and when light is reapplied, its release ceases, it is gradually removed from the circulation, and the skin darkens. (Bagnara, 1965).

It may be that the latter is brought about by the release of melanocyte stimulating hormone since MSH and melatonin are known to exhibit competitive antagonism. (Novales and Novales, 1965).

Hydroxyindole-O-methyl transferase activity has also been demonstrated in the pineals of fish, amphibia and reptiles (Quay 1965b). It is of considerable interest that in these groups HIOMT activity is also detected in retinal tissues, a situation which is not met in mammals.

The aforementioned findings may explain the fact that extirpation of the pineal in newt and salamander larvae produced only transitory abolition of the blanching response. In these animals the effect was gradually reversed over the twenty-five day period following pinealectemy. (Kelly, 1962).

If melatonin is indeed the blanching agent in the lower vertebrates it seems likely that the other sites may be able to take over the synthetic processes lost with the pineal.

3. (iii) Functional Aspects of Parenchymatous Pineal Systems.

The literature relating to parenchymatous pineals has most recently been reviewed by Reiter and Fraschini (1969) and Quay (1970) (mammals) and Ralph (1970) (birds).

3. (iii - a) Effects of light on the compact pineal.

Many workers have observed the effects of prolonged light and dark regimes on various aspects of mammalian and avian pineal function.

It was discovered in rat that subjecting animals to continuous light for a period of nine to ten weeks resulted in up to a 25% reduction in the weight of the pineal. If the animals were kept in the dark for a long period, however, no deviation from normal was observed (Fiske et al. 1960). This study was extended to a consideration of normal diurnal rhythms and it was found that the pineal showed a variation in weight over the twenty-four hour period, being lowest at the end of the daily light period.

(Axelrod et al. 1965). This variation may be related to the fact that the metabolic rate also follows a cyclical pattern, being greatest during the hours of darkness. (Roth, 1965). Spontaneous electrical activity is also observed in the pineal during the hours of darkness. This activity is actively inhibited by light, but indirectly, since the light is actually received by the retina. (Newman-Taylor and Wilson, 1970).

Recently the effects of illumination upon the ultrastructure of the pineal in guinea pig and hamster have been observed. In guinea pigs, under constant darkness there is an increase in 'dark cells' and activation of the 'light cells'. Under conditions of constant illumination there is a decrease in the organelles of the 'light cells' associated with an increase in the number of 'vesicle crowned rodlets', which are thought to be synaptic structures. (Lues, 1971).

In golden hamsters light deprivation coincides with the appearance of myeloid whorls which are believed to be a sign of degeneration. (Clabough, 1971). It is worthy of note that in this species light deprivation is also associated with gonadal atrophy.

Continuous illumination has also been found to induce an alteration in mitochondrial structures; they enlarge and lose their cristae (Halaris, 1959). This effect is thought to be due to an excess of serotonin. (See below).

3. (iii - b) Physiological effects of extirpation and implantation.

Early implantation experiments indicated that the pineal exerts an effect on the sympathetic nervous system and therefore could affect many bodily processes. (Grunewald-Lowenstein, 1952). The pineal of the recipient animal is not affected by the transplant, in adults, although some suppression may occur in prepubertal transplants. (Das Gupta, 1969).

Complete removal of the pineal has several effects, which if the view of Grunewald-Lowenstein (1952) were true, is not surprising.

Recently it has been demonstrated that pinealectomized rats exhibit raised blood pressure, but lowered responses of blood pressure to angiotensin and nor-adrenaline, in spite of the fact that the levels of plasma renin are increased. Bodyweight is slightly reduced and serum Na^+ and K^+ are lowered, but not Ca^{++}. No clear alteration in nor-adrenaline, adrenaline or serotonine could be detected. (Karpanen et al. 1970). A reduction in spontaneous wheel running activity in female rats has also been demonstrated. (Kincl et al. 1970), which may be related to the observation that the pineal appears to play some rôle in the regulation of brain composition. (Quay, 1965b).

Some evidence exists that pinealectomy in adult rats impairs the immune potential (Jankovic, 1970); this is perhaps rather difficult to reconcile with the above.

Removal of the pineal induces acceleration of gonadal growth in immature animals, but only a modest enlargement in the adult. Light deprivation, on the other hand, renders the pineal strongly antigonadotropic. (Reiter and Sorrentino, 1970.) The pineal may therefore be important in adjusting the level of reproductive activity relative to environmental change.

It is interesting that in sparrows, testis growth is not affected by pinealectomy, neither is the response to light. It is suggested that while the pineal may be a photoreceptor (in birds), its function is transferred to other areas of the brain when it is destroyed. (Menaker et al. 1970).

Perhaps the latter may be related to the experiments of Van der Wal et al. (1965) who found that pinealectomy did not affect the secretion of aldosterone, but lesions of the subcommissural body did. It is possible that the actual portion of the pineal complex that is removed is important and not the gross act of pinealectomy.

3. (iii - c) Biochemical and endocrinological aspects of compact pineal systems.

The possibility that the mammalian pineal has an endocrine function has been examined by many workers. The literature has most recently been reviewed by Quay (1970), Reiter and Fraschini (1969) and Axelrod (1970b).

Metabolic studies have indicated that, although the pineal is an outgrowth of the brain, it more closely resembles an endocrine organ. (Ford, 1965; Krass and Labella, 1966).

As has already been mentioned, several characteristic indoles are found in pineal tissue, melatonin being the best known. This probably has little effect on pigmentation in mammals but does have an inhibitory effect upon the gonads . (Axelrod, 1970a). Since constant darkness results in a large increase in HIOMT levels (Axelrod et al. 1965) and HIOMT is required for the synthesis of melatonin, then the effect of light on gonadal function is probably mediated via melatonin. (Wurtman et al. 1963). Light does not have a direct effect on the pineal but is received by the lateral eyes and is transmitted via a nerve pathway. Removal of the lateral eyes and both superior cervical ganglia leads to an inability of HIOMT to form melatonin. (Wurtman and Axelrod, 1965b).

Blinding or maintaining animals in complete darkness, or at least with very short light periods, induces a reduction in gonad size. (Reiter et al. 1966; Reiter and Hester, 1966; Hoffman and Reiter, 1965). This effect is removed by pinealectomy.

It is interesting that if hamsters are retained for very long periods in complete darkness, after about twenty-four weeks the gonads spontaneously regenerate, in the presence of an active pineal. (Reiter, 1969). No explanation can be offered for this phenomenon although it is possible that the neurendocrine axis becomes refractory to pineal substance. (Reiter and Fraschini, 1969).

In their recent reviews, Wurtman (1970) and Axelrod (1970a) have confirmed many of the aforementioned observations. Wurtman supposes that the melatonin formed by the pineal exerts its effect upon the midbrain therefore only indirectly affecting the gonads. He also points out that this chemical modifies pituitary activity particularly with respect to MSH and LH. (There is evidence that MSH output is inhibited by melatonin. (Kastin et al. 1969).) It is possible, therefore, that the pineal acts on the gonads via the adenohypophysis. (Thielblot, 1965; Thielblot and Blaise, 1965).

This in itself poses an interesting question; can there exist physiological feedback mechanisms from the ovaries and/or pituitary? Ovariectomy and hypophysectomy both induce ultrastructural changes of the rat pineal, namely, a decrease of clear cells associated with an increase of dark cells. Hypophysectomy also results in a significant decrease in the secretory granule content of the pineal cells. (Satodate et al. 1970).

Evidence has also been put forward by various workers to support the view that the pineal exerts a controlling action on the adrenal gland. (Palkovits, 1965). Farrell (1959a,b; 1960) postulated that the pineal produces glomerulotropin, a substance capable of stimulating the release of aldosterone from the adrenals. In addition to this (1960) he hypothesized that another substance (presumably melatonin) might exist, which was capable of inhibiting the synthesis and release of steroids. It was unfortunate that pinealectomy only had a marginal effect on aldosterone synthesis. (Farrell, 1964). Later workers supported the idea that the aldosterone stimulating hormone might not be located exclusively in the pineal, but could be distributed throughout the central nervous system. The hormone might therefore be serotonin (Jovan and Semperez, 1965).

Gromova et al. (1967) showed that melatonin inhibited aldosterone production in vivo, while corticosterone levels increased. Reiter et al.

(1966) demonstrated that removal of the lateral eyes in female hamsters resulted in a reduction in adrenal size. It is assumed, therefore, that the increased melatonin synthesis found under these circumstances induces a reduction in adrenal activity. Contrary to these results, Miline (1965) demonstrated an increase in adrenal activity in light-starved rats.

Gromova had noted that melatonin treatment brought about a weight gain in the adrenals; Reiter showed that pinealectomy resulted in decreased adrenal weight. These results reflect the confusion that exists in the relationship between the pineal and adrenal function.

It is worthy of note that aldosterone and deoxycorticosterone are both found to deplete granules present in the nerve endings of the rat pineal, suggesting that a feedback mechanism may exist. (Clementi et al. 1965).

The relationship (if any) between the pineal and the thyroid is also extremely difficult to understand. The thyroid evidently does have an effect on the pineal since thyroxine will cause marked dissolution of granules in the basophils of that organ. (Hungerford et al. 1965).

The effect of the pineal on the thyroid has been defined in various ways.

Subcutaneous introduction of melatonin into rats causes a decrease in thyroid weight. (Reiter et al. 1965). Exactly the reverse was found by by Thieblot et al. (1966). Panda and Turner (1968) found that when melatonin was given to remale rats, plasma TSH was increased, while the TSH content of the pituitary diminished. These results are similar to those produced by administration of goitrogenic substances and by thyroidectomy. It is therefore suggested that melatonin acts directly on the thyroid and the reduced thyroxine output results in raised pituitary activity to release TSH. (Reiter and Fraschini, 1969).

Although the presence or absence of light seems to be the key factor in the control of mammalian pineals (indeed Wurtman and Axelrod (1965a)

considered the pineal to be a light-operated biological clock), it cannot be the only one. In studies on the metabolism of pineal indoles, Klein and Weller (1970) demonstrated that a circadian rhythm of N-acetyltransferase existed in which the activity in the dark was fifteen times that in the light. This rhythm persisted in complete darkness but was suppressed by light. In vitro experiments have suggested that nor-epinephrine and not serotonin regulates the activity of N-acetyltransferase through a highly specific receptor.

Melatonin activity in mammals (as has already been mentioned) exhibits a fairly constant pattern. In the hen, however, the rhythms are reversed. Hen pineals show a weight gain in the light and a decrease of HIOMT activity in the dark. (Axelrod and Wurtman, 1964).

3. (iii - d). Summary of the knowledge of endocrine function.

There is little doubt that the parenchymal pineal has a rôle as an endocrine organ. If this statement is accepted then the following questions may be posed.

(1) What is the hormone released by the pineal?

(2) What is the function of this hormone?

(3) Where is the site of hormone action?

The answer to question (1) is probably - yes, Melatonin, but evidence suggests that it is not the only one, else why would pineal extracts not always have an action identical to that of melatonin? Probably some of the other characteristic methoxy indoles share an endocrine rôle.

The answer to the second question is even more difficult to provide. It does have an effect upon the gonads and probably mediates the effects of light. It may play a part in the regulation of thyroid and adrenal function.

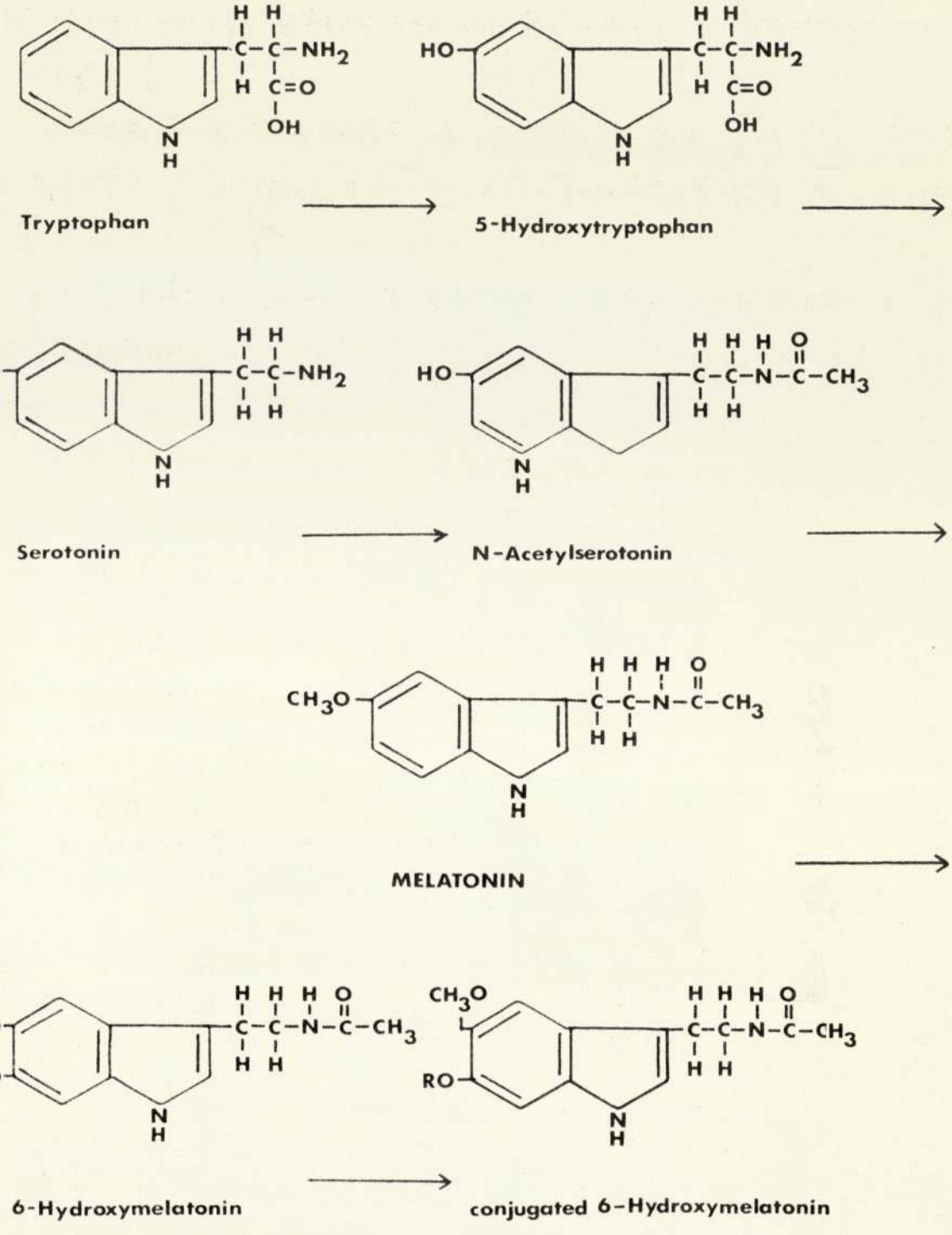
The third question cannot be answered at present.

This hormone, or group of hormones, may act directly upon the target

gland or upon the mid-brain or upon the pituitary. No firm answer can be given.

It seems likely that the pineal system of lower vertebrates, primarily a photosensory structure, has in mammals evolved into a secretory structure (Amens-Kappers, 1963).

The significance of the former is doubtful, the exact function of the latter is unknown.



Biosynthesis and Metabolism of Melatonin

fig.10.

II. MATERIALS AND METHODS

1. Light Microscopy.

Two batches of twelve live, mixed male and female, adult specimens of Scyliorhinus canicula were obtained from the Marine Biological Laboratory, Citadel Hill, Plymouth. All specimens were transported in sea water in closed polythene containers and collected in the evening of the day of despatch. Therefore the fish were not kept in their containers for more than eight hours.

The first batch was obtained in December (winter specimens), the second in August (summer specimens) and both batches contained fish of between fifty-five and seventy centimetres in length.

All specimens were sacrificed by decapitation and the skull was opened to allow penetration of fixative. The heads were fixed in 10% formal saline at room temperature for approximately seventy-two hours. After this time, the heads were washed in distilled water and the skin was stripped from the roof of the skull. Small pieces of cartilage were then cut out containing the pineal end vesicle together with a short length of stalk.

The pieces of cartilage were taken and a number were dehydrated through a series of alcohols and cleared with chloroform. They were then embedded in paraffin wax and sectioned at 6 to 8 microns using a Spencer AO rotary microtome.

Two of the summer specimens were treated rather differently, being dehydrated through a series of alcohols and then embedded in hydroxy ethyl methacrylate according to Ruddell (1967). Sections were cut on an MSE base sledge microtome at a thickness of one micron.

Paraffin wax embedded sections were dewaxed in xylene, hydrated and stained using a variety of methods. Several sections from each specimen were stained by Ehrlich's haematoxylin and eosin or Harris' haematoxylin

and eosin for initial examination. Other sections were examined for the presence of Nissl substance using Cresyl violet (Powers and Clark, 1955) or Thionin (Clark and Sperry, 1945). The tissues were also examined for nerve fibres and myelin with Luxol Fast Blue (Salthouse, 1962), Mahon S (1937) and Bodian (1937). Finally, Pollack's Trichrome stain was employed to study any connective tissue that might be present.

Methacrylate embedded sections were also stained by a variety of methods. Several sections from each specimen were stained by Harris haematoxylin and eosin for initial examination. Others were stained as follows by a modified Dominici method. Untreated sections were stained for ten minutes in a solution containing 0.5% eosin and 0.5% Orange G in distilled water. The sections were then rinsed briefly in distilled water and stained for five minutes in 0.5% aqueous toluidine blue. Following a brief rinse in distilled water, sections were blotted, rinsed rapidly in alcohol, cleared in xylene and mounted in Depex.

Other methacrylate sections were stained by a Solochrome cyanin modification. The staining solution was prepared by dissolving 1.0g of solochrome cynanin in 5 ml. of concentrated sulphuric acid and diluting to 100 ml. with distilled water. The stain was filtered on to the sections and allowed to stand for up to thirty minutes. The staining solution was then washed off with distilled water and the sections briefly rinsed in 10% iron alum. Sections were then rinsed, dehydrated, cleared and mounted in Depex.

Four of the formalin fixed specimens were dissected out to release the pineal bodies and the latter were prepared for histochemical examination.

Each pineal was taken and placed in a groove within a piece of liver from a freshly killed mouse. The liver was then frozen using carbon dioxide and frozen sections were cut using a (Slee) cryostat, at a thickness of 50 microns.

Sections were examined for acetylcholinesterase and alkaline phosphatase.

The presence of acetylcholinesterase was examined by a modification of the method of Karnowsky and Roots. The staining solution was prepared immediately before use by adding 15.8 ml. 0.82% sodium acetate, 0.5 ml. 0.6% acetic acid, 1.2 ml. 2.94% sodium citrate, 2.5 ml. 0.75% cupric sulphate, 2.5 ml. 0.165% potassium ferricyanide and 2.5 ml. of distilled water (in the order stated) to 12.5 mg. of acetyl thiocholine. (making a clear yellow-green solution pH 5.4).

Sections were washed in two changes of distilled water, about one minute each, and then incubated in the substrate mixture for ninety minutes at 37°C. Following a rinse in distilled water sections were counterstained by Harris' haematoxylin, blued, dehydrated, cleared and mounted.

Cold formalin (4°C) fixed sections were examined for alkaline phosphatase by Gomori's method.

Frozen sections were incubated at 37°C for one hour in a solution containing 10 ml. 3% sodium B-glycerophosphate, 10 ml. 2% sodium diethyl barbiturate, 20 ml. 2% calcium chloride, 1 ml. 5% magnesium sulphate and 50 ml. distilled water. The sections were then washed gently in running tap water and placed in 2% cobalt acetate for five minutes. After a further brief wash they were placed in dilute ammonium sulphide for three minutes, counterstained in safranin, dehydrated, cleared and mounted.

A number of wax embedded and methacrylate embedded sections were examined for the presence of PAS material. Wax sections were examined by treatment with aqueous solutions according to Humason (1967). Methacrylate embedded material was examined by a modification of the method of Humason and also by using Best's carmine.

Sections were treated with aqueous periodic acid for thirty minutes and subsequently washed in running water for five minutes. They were then placed in aqueous Schiff's reagent for forty-five minutes and transferred through a series of sulphite rinses (three changes at two minutes each). Following a ten minute wash in running water sections were counterstained

for fifteen minutes by Ehrlich's haematoxylin, blued, dehydrated, cleared and mounted.

A second batch of methacrylate sections were incubated for two hours at 37°C in 1% diastase and then examined for PAS material in exactly the same way.

In an attempt to confirm the presence of glycogen, methacrylate sections were stained by Best's carmine. (Chayen et al. 1969). Sections were treated with periodate solution for fifteen minutes followed by a rinse in 70% alcohol. They were then immersed in the reducing rinse for ten minutes, and taken through a series of alcohols into Ehrlich's haematoxylin for thirty minutes. Following 'bluing' in Scott's tap water, sections were stained for thirty minutes in Best's carmine, differentiated, dehydrated, cleared and mounted in Depex.

2. Electron Microscopy.

Two batches of six adult, mixed male and female Scyliorhinus canicula were obtained as before. In addition, two specimens of Raja clavata were collected from the Marine Biological Laboratories, Citadel Hall, Plymouth and dealt with on site.

All animals were sacrificed by decapitation and the skulls were opened by making two anterior/posterior cuts through the skull, one just above each orbit. The two cuts were then joined by a single transverse cut just posterior to the orbits. The skull flap was then raised and ice cold 3% gluteraldehyde in phosphate buffer (Sabatini et al. 1963) was introduced into the cavity above the brain.

The pineal stalk was severed and the roof of the skull containing the pineal vesicle was removed completely and trimmed to eliminate excess cartilage. The fragment of skull was then immersed in buffered gluteraldehyde for three to four hours at $4^{o}C$.

Following gluteraldehyde fixation the specimen was washed in isotonic sucrose buffer overnight and then post-fixed in osmium tetroxide (Caulfield, 1957) for two hours. The specimen was further washed in cold isotonic veronal acetate buffer for two hours during which time excess cartilage was trimmed away to completely release the pineal vesicle. The specimen was dehydrated through a series of (ethyl) alcohols and embedded in Epon 812 (Taab laboratories) according to Luft (1961).

All specimens were flat-embedded in small polythene Auto Analyser cup caps (Technicon) in order to facilitate orientation within the resin. Following hardening (for two days in a $56^{o}C$ oven) the polythene container was cut away and the block hand trimmed.

Sections were cut on an L.K.B. Ultratome III, using triangular glass knives with a knife angle of 45^{o}, prepared on an L.K.B. Knifemaker, at 60-150mμ according to the Peachey (1958) scale. Sections were floated out on a solution of 2% ethanol in water and stretched by allowing trichlorethylene

vapours to settle on them. The sections were then collected on copper grids pre-coated with a carbon support film. (or a simple Formuar film).

Following drying on filter paper in a petri dish, the grids were then stained according to a variety of methods.

Grids were stained by floating them on the surface of the stain either on a glass slide ringed with wax or on a perspex staining 'plate' bearing half centimetre depressions.

Most sections were stained with a freshly prepared saturated solution of (Analar) uranyl acetate (Taab Laboratories) in absolute ethanol. The staining solution was centrifuged immediately prior to use and the working solution was removed from below the surface of the fluid in the centrifuge tube. Sections were stained for up to one hour and then washed by repeated immersion in a series of alcohols. They were then allowed to dry on clean filter paper in a petri dish.

Many grids were double stained in uranyl acetate and a lead stain. Two lead stains were utilised; a modification of the lead hydroxide stain of Karnovsky (1961) and the lead tartrate method of Millonig (1961).

Karnovsky lead hydroxide involves the use of a high pH (and therefore stable) solution of lead salts (plumbite ions). The particular modification utilised was carried out as follows.

Lead monoxide was added in excess to 15 ml. of hot normal sodium hydroxide. The solution was boiled gently for fifteen minutes, then cooled and filtered. The filtrate was retained and 1 ml. aliquote was diluted to 50 ml. with distilled water. This, the staining solution, was then centrifuged and a sample removed from below the surface and placed on to the surface of a sheet of dental wax placed in a petri dish. The latter also contained a few pellets of sodium hydroxide. Grids were stained for not more than thirty minutes and were then thoroughly washed as described previously.

The lead tartrate method of Millonig (1961) was carried out as follows.

20.0g. of sodium hydroxide and 1.0g. of sodium-potassium tartrate were

added to distilled water to give a final volume of 50 ml. 1 ml. of the aforementioned (stock) solution was then added to 5.0 ml. of 20% lead acetate in water, with stirring. The dense white precipitate thus formed was diluted ten times with distilled water, mixed thoroughly and filtered using a very fine paper. (Whatman 542).

Sections were stained as previously described and were washed thoroughly with distilled water .

A small number of grids were stained with 1% vanadatomolyblate according to Callahan and Horner (1964) but since no special advantage was obtained this method was abandoned.

All sections were examined using an A.E.I. E.M.6.B. electron microscope at 60 kilovolts and photographs taken by the standard plate camera.

3. Examination of Immature Specimens.

Two batches of twelve and eighteen egg cases of Scyliorhinus canicula were obtained from the Marine Biological Laboratories, Plymouth.

In order to maintain the developing embryo an apparatus was constructed consisting of a flat, heavy duty polythene tank 90 cm. by 120 cm. by 20 cm. into which was placed a flat refrigerated plate (specially made by Pioneer Refrigeration Ltd., Birmingham). The tank was three-quarters filled with tap water containing a small amount of Chloros to inhibit bacterial/fungal growth and the plate thermostat set to give a temperature of 13°C. (see fig. 11b).

A number of small glass tanks were placed on the refrigerated plate and filled with sea water (Tidmans Sea Salt 35.5g/litre) to just above the water level in the outer tank. A Nuora filter pump containing a glass wool filter was also placed in the larger tank and filtration of water in one of the tanks was commenced. (The pump was switched from tank to tank at three-daily intervals). All tanks were continuously aerated.

Above the tank was suspended a Philips streamlight with a white tube to provide continuous illumination (900 lux/m^2 at the bottom of the tank) for the first batch of fish.

Three of the first batch of fish did not develop at all, while the other nine developed a fungal infection from which three died. At this stage the embryos were from 30 to 32 mm. in length and too small to allow easy removal of the pineals for electron microscopic examination. It was therefore decided to sacrifice all six for histological examination .

The six remaining egg cases were opened to release the embryos with their yolk sacs attached. The latter were severed from the bodies and the length of each embryo was ascertained. The embryos were sacrificed by decapitation and the heads fixed for twenty four hours in 10% buffered formalin. They were then washed, dehydrated, cleared and embedded in paraffin wax.

Four of the heads were sectioned transversely and two longitudinally at 5 to 8 μ. Sections were dewaxed and stained by Ehrlich's or Harris' haematoxylin and eosin.

The second batch of egg cases was divided into groups and maintained as before, but one half was kept in constant darkness while the other half was subjected to continuous illumination. (900 lux/m^2 at the bottom of the tank).

After approximately three months the embryos, between 45 and 60 mm in length, were sacrificed and the pineals removed by microdissection under a Vickers Zoomax microscope. The pineals were then fixed in ice cold gluteraldehyde (Sabatini et al. 1963) and osmium tetroxide (Caulfield, 1957) Sections were cut, mounted and stained as previously described.

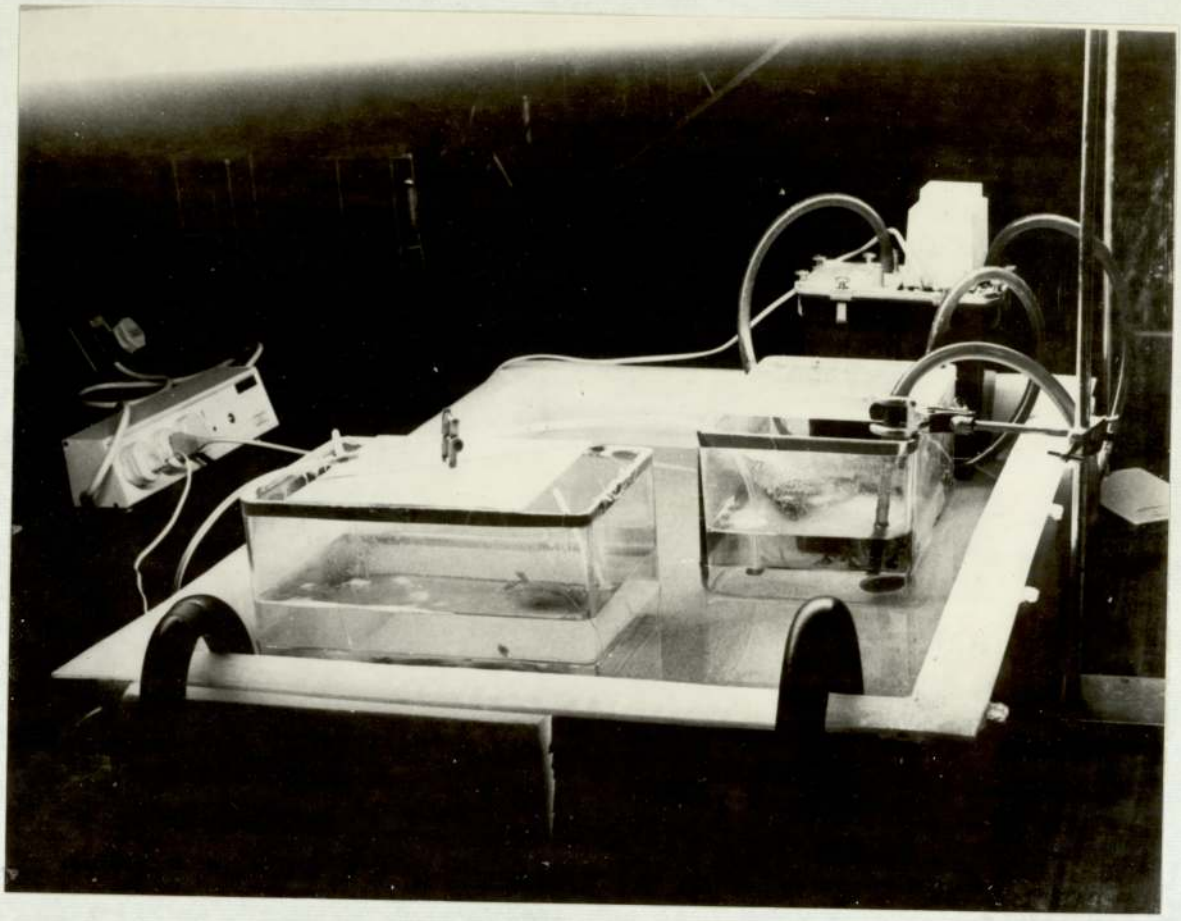


Fig. 11b. Apparatus used in studies on the effects of light on the pineal of S. canicula.

4. Assay of Melatonin and Serotonin.

4a. Chromatographic Isolation of Melatonin and Serotonin.

Two batches of 6 adult, mixed male and female Scyliorhinus canicula were obtained (as before) and examined for the presence of Melatonin and Serotonin, according to the method of Fenwick (1970) but with some modifications.

Fish were killed by decapitation and the pineals were immediately dissected out. Those of the first batch were placed immediately into ice cold 0.1N anhydrous sodium phosphate (monobasic), while those of the second batch were placed into a small polythene container immersed in a vacuum flask of liquid nitrogen.

Both batches were treated in an identical fashion after the initial collection. They were homogenized in 10 mls. of ice cold 0.1N anhydrous sodium phosphate (monobasic) using a glass-teflon homogeniser. The indoles were then extracted by prolonged mixing with ice cold ethyl acetate. Following separation the latter was dried with 10g. of anhydrous sodium sulphate and the mixture was filtered. The dried filtrate was then evaporated to dryness in a rotary evaporator under an atmosphere of Nitrogen at 20°C. The residue was taken up into 2 ml. of absolute ethanol containing 0.1N HCl in the proportions 4:1 and the resulting solution was evaporated (as before) to a final volume of approximately 0.5 ml. The alcoholic extract was then spotted on 20 cm x 20 cm glass plates coated to a depth of 0.25 mm with Merck Silica Gel G. The plates had been air dried and liberally sprayed with a solution of 0.5% ascorbic acid in methanol prior to use.

The plates were then developed in an alkaline solvent system containing methyl acetate, isopropanol and 25% ammonia in the proportions 45:35:20 until the solvent front had reached halfway up the plate. The plates were removed and placed in a chromotank containing warmed concentrated sulphuric acid in order to remove excess ammonia.

After thirty minutes the plates were rotated through ninety degrees and

developed in an acidic system containing chloroform and 96% acetic acid in the proportions 95:5.

Following development the solvent fronts were marked and the plates were sprayed copiously with modified Ehrlich's reagent (prepared by dissolving 1 g. of 4-dimethyl-aminobenzaldehyde in 50 ml. conc. HCl and adding 50 ml. of absolute ethanol).

The test extracts were run against standards prepared by dissolving commercial Melatonin and Serotonin (Sigma Laboratories) in acid alcohol. The extract procedure was checked by dissolving known amounts of commercial preparation in ice cold 0.1N anhydrous sodium phosphate.

The Rf values were obtained from each solvent system for each experimental run and were compared with the literature.

4b. Frog Skin Bioassay of Melatonin.

The pineal extract was also examined by means of a bioassay technique, a modification of the method of Mori and Lerner (1960).

The alcoholic extract was evaporated to dryness and the residue redissolved in Frog Ringer. (6.5g. sodium chloride, 140 mg. potassium chloride, 120 mg. calcium chloride and 200 mg. sodium bicarbonate made up to one litre with distilled water).

A frog was pithed and the skin removed from the median dorsal surface of each thigh. Both pieces of skin were placed in a beaker containing 1% caffeine in Frog Ringer in order to produce maximum darkening. After one hour, one piece of skin was removed, washed and placed in a clean perspex tissue culture slide and a stainless steel ring was placed on top in order to stretch the piece of skin out flat. Fresh Frog Ringer was introduced into the cavity created by the ring and a coverslip was placed on top to eliminate evaporation effects.

The tissue culture slide was placed on the stage of a Vickers MI5C microscope with a quartz iodine light source and flat field objective. Into one eyepiece of the microscope an OCP 71 photocell fitted into the base of

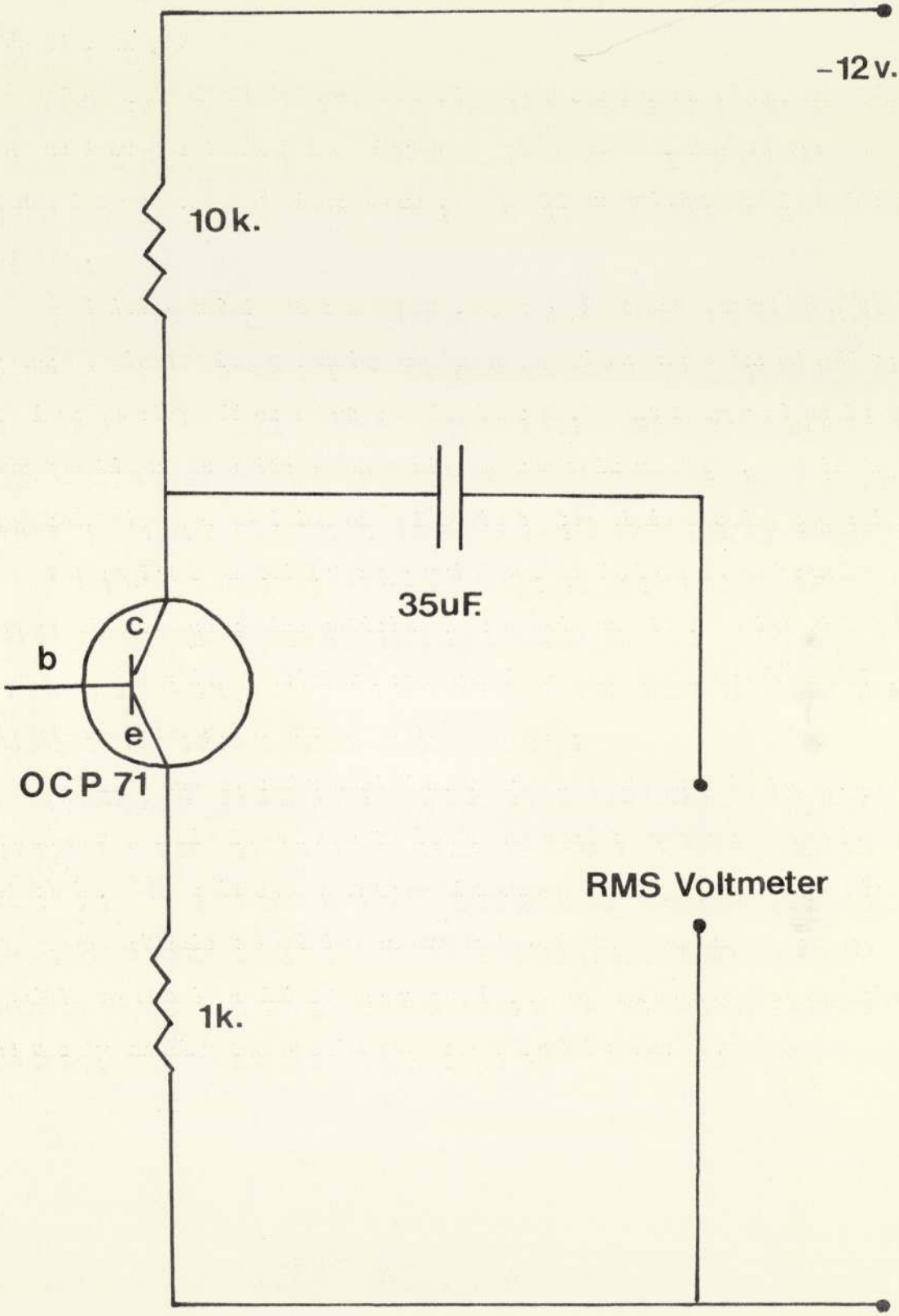
a light-tight bung was inserted. This was connected to an RMS voltmeter. (See fig. 11a).

Since the OCP 71 is more sensitive to intermittent light, an electric fan was introduced below the microscope stage so that the movement of its blades interrupted the light source. In all experiments a x10 objective was used.

In initial trials of the apparatus, the light was turned down to produce a given voltage reading and then the light was switched off to eliminate heating effects, but the fan left on. After approximately one hour the light was switched on again and the voltage noted. This was repeated, with the addition of melatonin to the fluid bathing the skin.

A second set of trials was carried out as follows in an attempt to remove effects due to inequalities in the skin samples. Five fields were chosen on the skin, initial voltage readings were taken from each field and the experiment carried out as described above.

Finally, due to the unsatisfactory results produced in the above experiments, a simple melanocyte index was used to asses the presence of melatonin. In this case a Watson Microscope was employed fitted with a Pye closed circuit television camera and monitor. It was therefore possible to obtain a fairly accurate picture of melanophore contraction by accurately tracing the outlines on the television screen.



APPARATUS FOR MELATONIN ASSAY fig.11a.

III. RESULTS.

1. Light Microscopy.

The pineal organ of Scyliorhinus canicula arises as a blind ended outgrowth from the diencephalic roof at a point just anterior to the tectum opticum and posterior to the velum transversum. This is illustrated in the larval animal in longitudinal sections figs. 12a and 12b. It consists of a long thin tube, the pineal stalk, which enlarges at the distal end to form the pineal body. The latter has a distinct lumen, continuous with that of the stalk and leading into the third ventricle. (Figs. 12a, 12b).

In the adult, the pineal body is attached to the roof of the braincase at a point in front of its origin and therefore the pineal stalk passes over a major part of the telencephalon. There is, however, no connection with the latter and in dissection the pineal stalk may be lifted free without difficulty.

The distal end of the pineal is always attached to the underside of the connective tissue layer which forms the roof of the braincase. The degree of attachment varies within the species. In some cases the pineal occupies a very superficial position with only the dorsal surface of the pineal sac in contact with the lower surface of the braincase. In other individuals the pineal body is completely embedded in a deep trough in the braincase and therefore in transverse section can be seen to exist within the skull. (Fig. 13). The pineal never completely penetrates the braincase. The cartilaginous skull is covered by a dermis and epidermis, neither of which gives any indication of the position of the pineal, i.e. no pigment free spot is to be observed.

The total length of the pineal organ is 15-17 mn., while the pineal vesicle occupies 1.5-2 mm. The latter is always dorso-ventrally flattened even during development. (Fig. 14a, 14b). In the adult the horizontal diameter is 0.6-0.8 mm. while the dorso-ventral diameter is 0.3-0.5 mm.

There is some variation in the shape of the end-vesicle; in the amount of dorso-ventral flattening and in the angle of entry into the braincase. It is interesting that there is a conspicuous difference in shape between the larval pineal and that of the adult. In the former the pineal vesicle appears to be disc-like, the stalk entering just posterior to the centre of the disc (figs. 14a, 14b). In the adult, on the other hand, it is generally found that the vesicle represents a straight-forward enlargement of the pineal stalk; the stalk enters the vesicle from behind and, in fact, it is difficult to discern a distinct junction. (Fig. 15).

The adult pineal in section is seen to consist of three distinct areas. (Figs. 16a, 16b). These areas are identified in schematic L.S. fig. 18 as 1, 2 and 3.

The first layer (1) is the innermost layer which abuts on to the lumen and is identified as the nuclear layer. Next to this is the middle (2) or fibrous layer which is coated on the outside by (3), the connective tissue capsule.

The outer layer (3), the meninx primitiva, covers the pineal throughout its whole length. This layer is stained green by Pollack's Trichrome stain and may thus be identified as collagen. The density of tissue in this layer appears to vary, for example in fig. 16a it is very compact, while in 16b it is very loose. (This may, of course, be a fixation artefact, although since the two samples were fixed as part of the same batch, this is doubtful). Similarly, the depth of this layer varies; sometimes it is fairly even (fig. 16a, while in other individuals the lower wall is considerably thicker than the upper. (Fig. 13). The degree of fusion with the braincase also varies, and fig. 13 illustrates a situation where there is complete fusion at certain points around the rim of the pineal. In fig. 15 the junction is so loose that the pineal has become separated from the skull wall. This outer layer is richly supplied with blood vessels of varying sizes. In some individuals two larger vessels are present on the underside of the stalk which fuse

together in the region of the vesicle. In others, no such regularity of structure is seen and an apparently random arrangement of blood vessels is observed. These blood vessels were never observed to penetrate into the fibrous layer. Indeed there appears to be a distinct junction between the meninx primitiva and the fibrous layer, so that the presence of a basement membrane may be suspected.

The middle (2) or fibrous layer stains pink with Haematoxylin and eosin techniques, pale green with Pollack, and light blue with Solochrome. From the last result it became evident that it does not consist of connective tissue since the latter gives a dark blue reaction. However, this layer gave negative reactions to cresyl violet, thionin, luxol fast blue Mahon and Bodian's stains. (Rudeberg (1969), however, reports that Bodian's stain gives a positive reaction.)

This area of the pineal, then, could not be identified by optical technique although, from its position in relation to the brain and its optical appearance, it was suspected that nervous elements might be present.

The innermost layer (1) or nuclear layer gave uniform nuclear reactions to the various staining techniques employed. The cytoplasm similarly gave uniform reactions with the conventional counterstains, e.g. pink with eosin, pale green with Pollack's Trichrome.

This innermost layer consists of a large number of nucleated cells which are, in the pineal vesicle, apparently arranged haphazardly in a number of layers. In development, the nuclear layer has a constant density throughout, i.e. an equal depth on the dorsal, ventral and lateral surfaces. (Figs. 14a, 14b). In the adult, however, this is not always true, and often the ventral nuclear layer is significantly deeper than the corresponding dorsal layer. (Fig. 13). Also, the depth may not be even, since the nuclei are often seen to exist in 'bunches' pushing out the inner wall of the pineal structure into the lumen. (Figs. 15, 16a).

The depth of the inner layer may be reduced to a single nucleated cell

in the centre region of the stalk, but it becomes deeper again at the proximal end.

It is difficult to determine any structural organisation in the nuclear layer, using optical techniques. As has already been noted, the cells are arranged (apparently) randomly and therefore their shapes are difficult to discern. As a result of the cellular distribution, any one section will cut the cells (and nuclei) at different points (particularly in methacrylate sections) and therefore classification of cells (and nuclei) is doubly difficult. An examination of a large number of sections, however, seems to indicate that at least two nuclear types are present.

The first nuclear type, present in the smallest numbers, are round (or oval) and often possess a distinct nucleolus. These nuclei are found especially in the cells at the innermost edge of the nuclear layer, next to the lumen. (Figs. 17a, 17b). The cytoplasm of those cells is often drawn out into long strands which pass out into the lumens. The nucleus is nearly always basal within the cell. (Fig. 17a).

The second nuclear type is slightly smaller and less regular, often being (almost) spindle-shaped and containing a nucleolus. They are found in greater numbers than the first type and form the bulk of the deeper lying nuclei, although many are also found at the top of the layer next to the lumen. An added distinguishing factor is that the nucleoplasm of the second nuclear type is rather more uniform than that of the first type.

The degree of cytoplasmic preservation varies considerably from individual to individual, but is particularly good in fig. 17a. This section also shows that material is present in the lumen; this may represent degenerative material or possibly secretory material from the cells abutting the lumen. (See below).

Histochemical examinations, in the main, do not reveal any specific activity in this layer. Alkaline phosphatase activity is not present, neither is cholinesterase. (The latter might be expected if the fibrous

layer were nervous tissue, since synapses between it and the nuclear layer might be present.

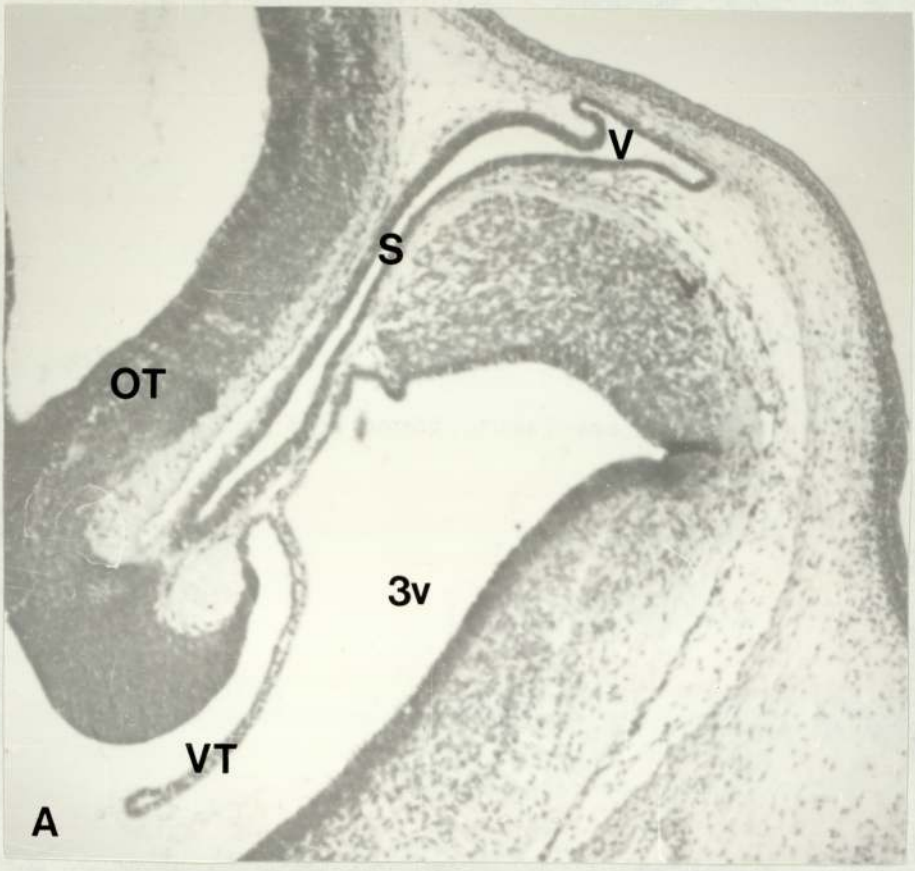
PAS positive reactions, however, are obtained from the topmost cells. Fig. 17b shows that PAS positive material is present in the cytoplasm of many of the cells next to the lumen. This material appears to be specifically situated in the apices of these cells, in the cytoplasmic processes, as distinct granules. They are particularly obvious in methacrylate sections. The identity of this PAS material was determined by diastase testing of paraffin sections. (Diastase tests do not work well on methacrylate). It was shown to be glycogen. Virtually all of the cells on the lumen of the pineal vesicle possess glycogen granules. (Fig. 17b).

The lumen of the adult pineal vesicle often contains small amounts of extraneous material, particularly at the edges. However, no complete cells are present (e.g. macrophages), neither is there any distinct trace of (neuro)secretory material. It would seem probable that this material simply represents cytoplasmic processes lost from the lumenal cells.

Figs. 12a, b. Sagittal sections through the pineal region of S. canicula (30 mm. specimen), formalin fixed paraffin sections stained by Haematoxylin and Eosin.

a. Section through whole pineal region at a slightly oblique angle showing the pineal stalk (S) and vesicle (V), but not the connection with the third ventricle (3V). The optic tectum (OT) and velum transversum (VT) may also be seen. x 85.

b. Basal portion of the pineal stalk (S) showing that it does lead into the third ventricle. The habenular commissure (HC) and posterior commissure (PC) can be seen, in addition to the origin of the velum transversum (VT). x 150.


V
S
OT
3v
VT
A

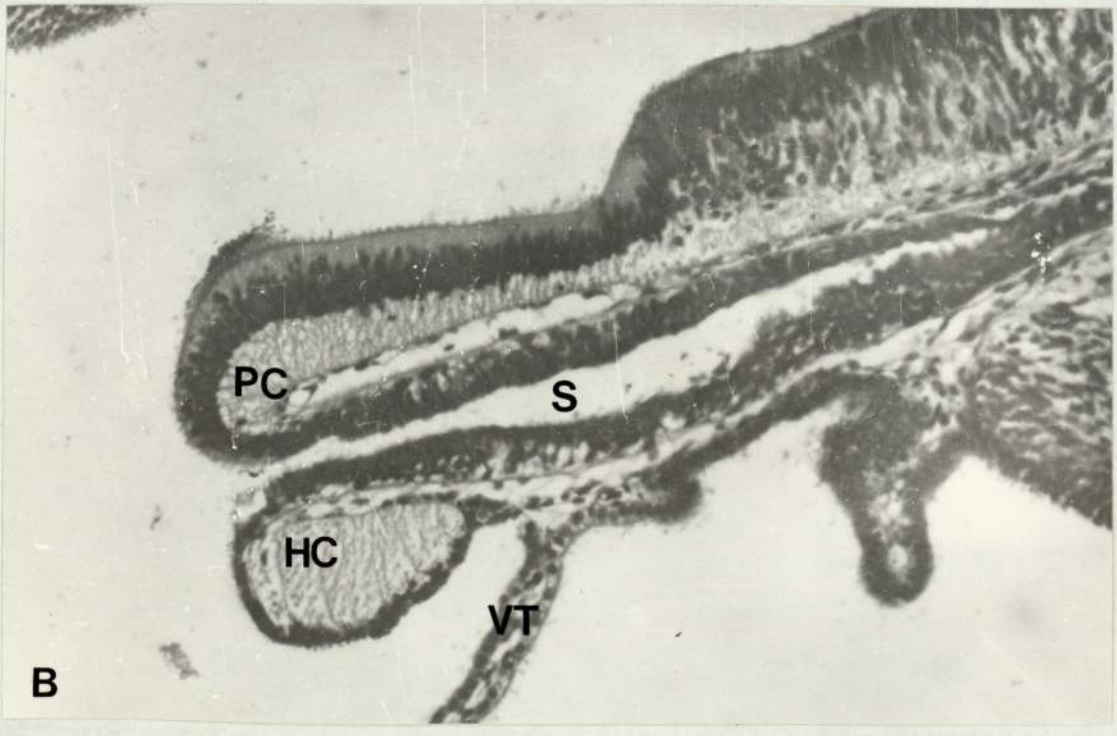
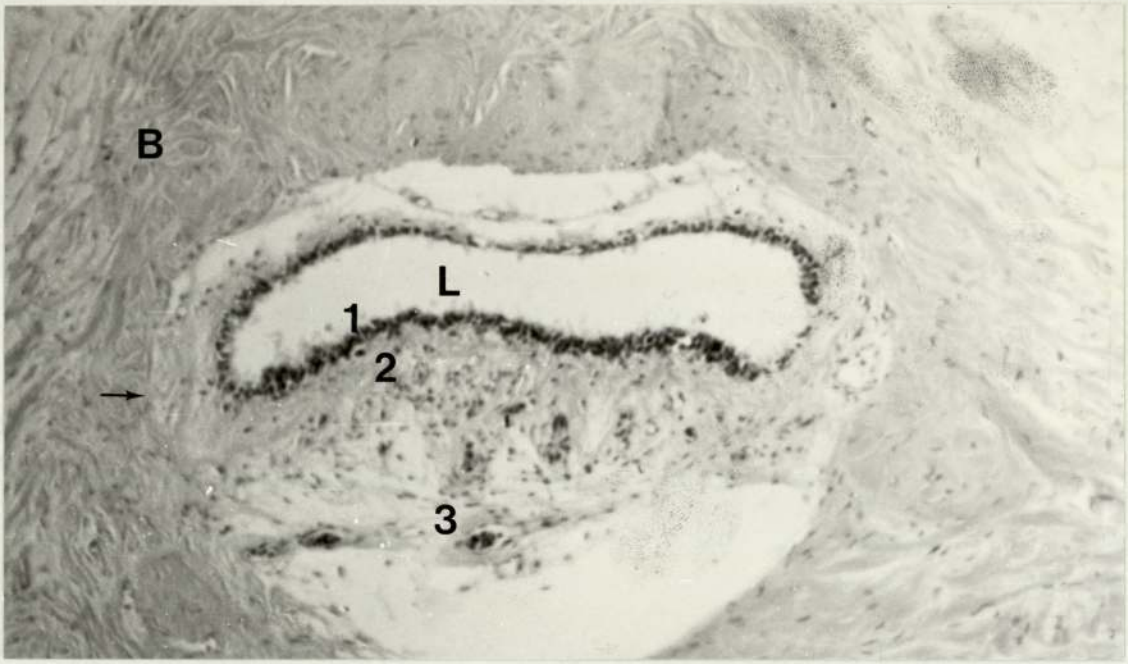

PC
S
HC
VT
B

Fig. 13 Median section through the pineal of adult S.canicula, formalin fixed paraffin section stained by Haematoxylin and Eosin.

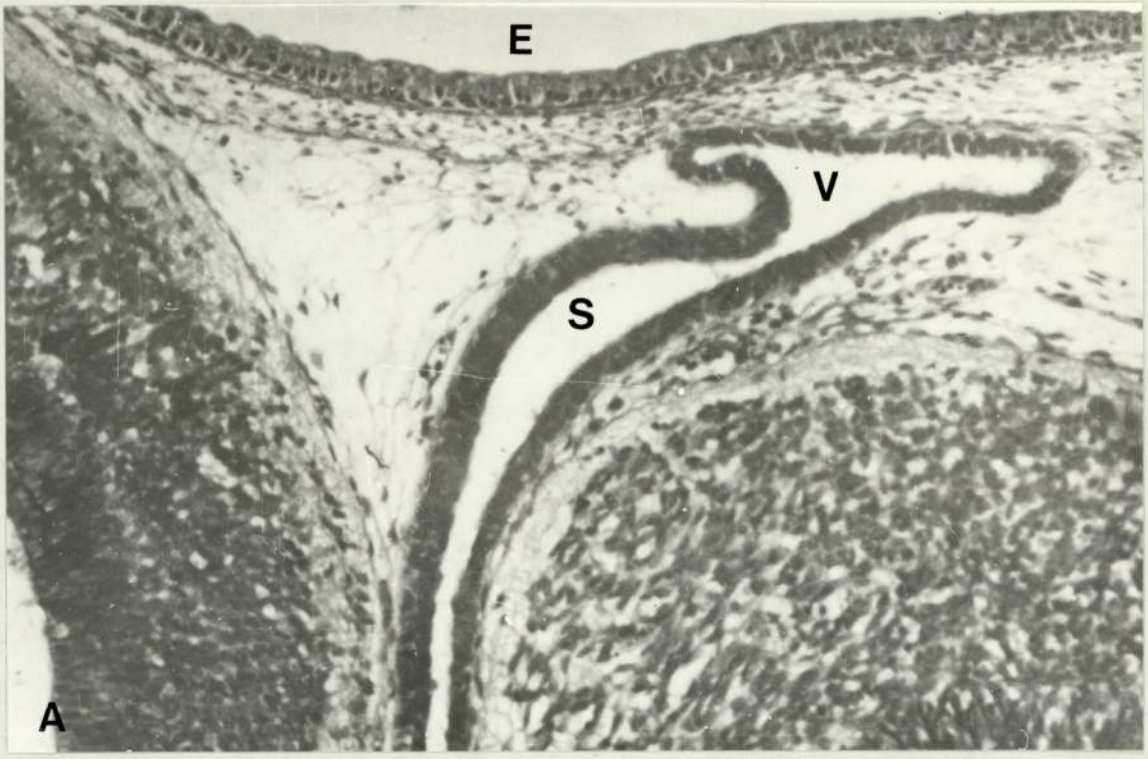
The pineal vesicle can be seen to enter the braincase (B). The three layers of the pineal are clearly visible, 1- nuclear layer; 2 - middle layer; 3 - meninx primitiva. The enlarged vesicular lumen (L) is seen to be flattened in transverse section. Fusion of the meninx primitiva and braincase is evident. (arrow). x 140.


B
L
1
2
3

Figs. 14a, b. Sagittal sections through the pineal of S.canicula (30 mm. specimen), formalin fixed paraffin sections stained by Haematoxylin and Eosin.

a. The pineal stalk (S) which may have a wide lumen enters the vesicle (V) from immediately below the epidermis (E). x 210.

b. The pineal vesicle (V) appears to consist primarily of nucleated cells at this stage of development. x 340.


E
V
S
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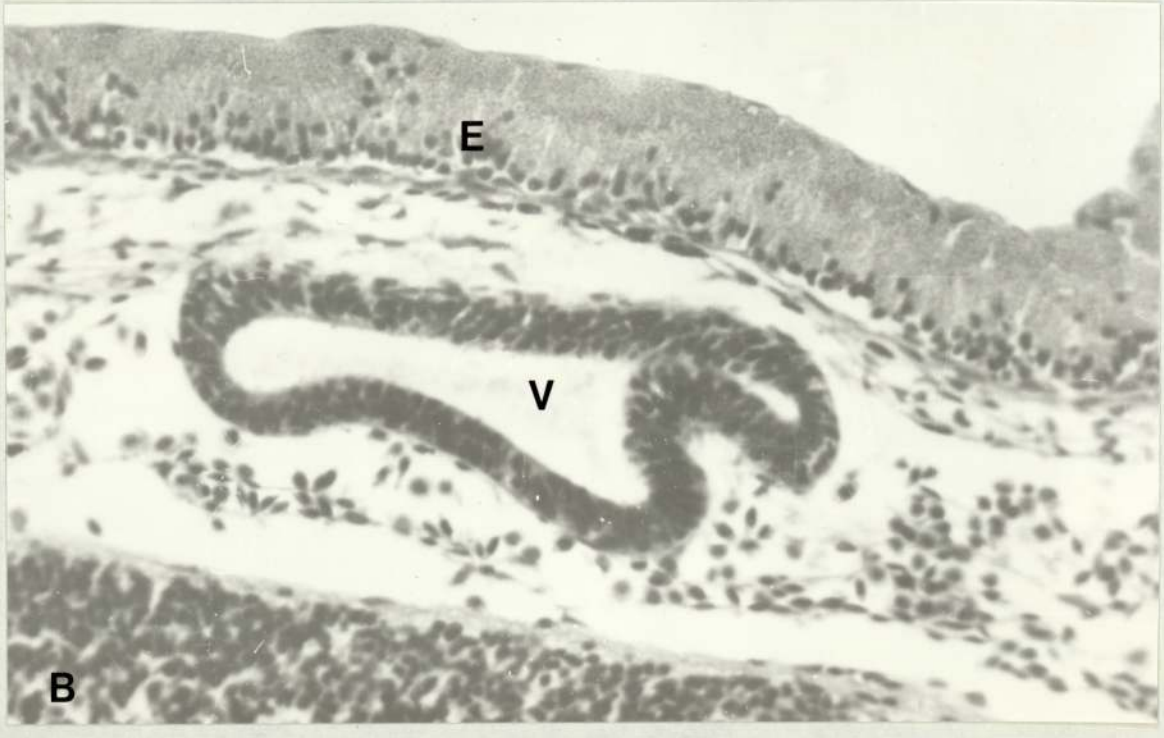

E
V
B

Fig. 15 Sagittal section through the pineal of S.canicula, formalin fixed paraffin sections stained by Haematoxylin and Eosin.

In sagittal section, the shape of the adult pineal differs from that of the juvenile specimen. The large lumen (L) is lined by the nucleated layer (1) which is bounded by a fibrous layer (2) surrounded by a connective tissue capsule (3) containing a large capillary (C). x 120.

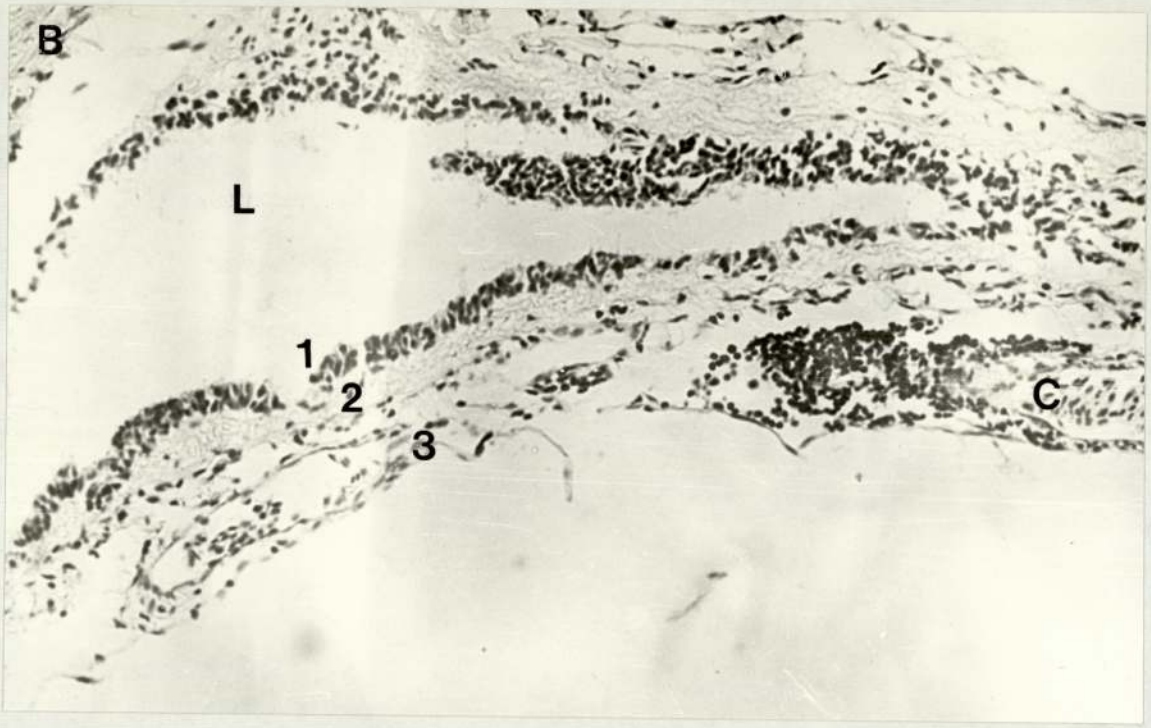
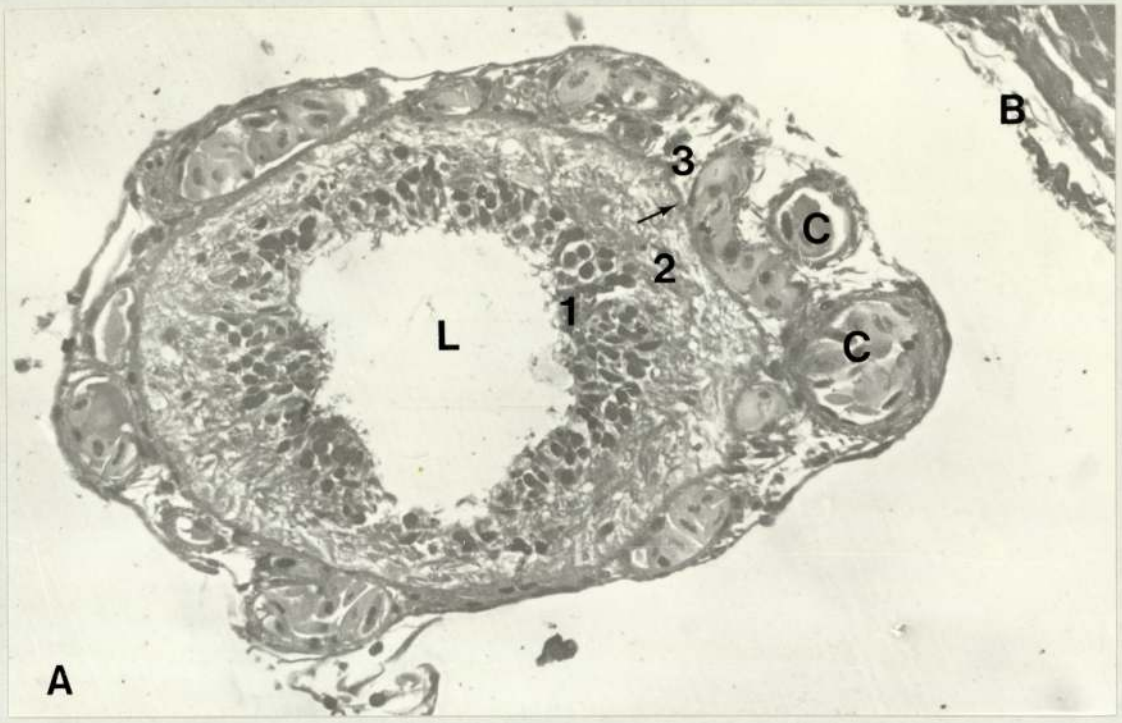

B
L
1
2
3
C

Fig. 16a. b. Median sections through the posterior region of the pineal vesicle of S.canicula, formalin fixed methacrylate sections stained by Solochrome cyanin.

a. The rounded lumen (L) of the vesicle at this level is bounded by the nuclear layer (1) which itself is in contact with the fibrous layer (2), surrounded by a capillary (C) - rich connective tissue (3) capsule. A distinct boundary (arrows) separates layers 2 and 3. x 300.

b. As a, illustrating a very loose connective tissue layer. x 320.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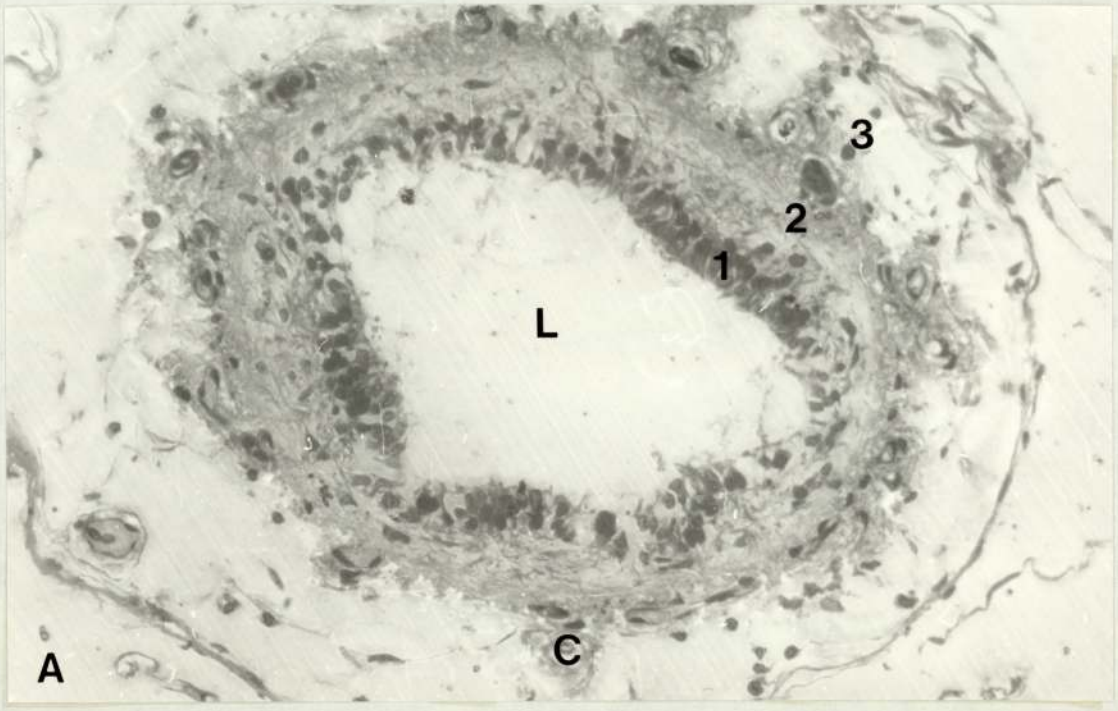
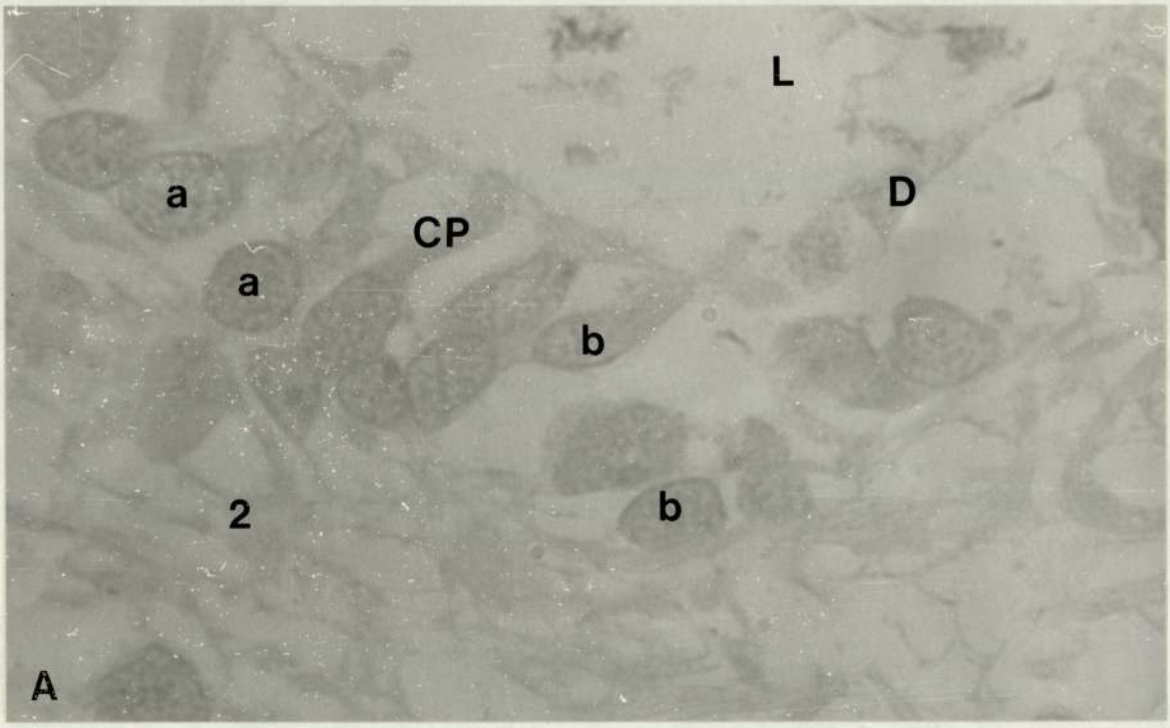

3
2
1
L
A
C

Figs. 17a, b. Median sections through the pineal of S.canicula formalin fixed methacrylate sections stained by modified Dominichi method (a) and Haematoxylin-PAS (b).

a. The nuclear layer and part of the fibrous layer (2) of the pineal vesicle. The nucleated cells consist of two types, those with oval nuclei (a), and those with irregular nuclei (b).
Come of the cells possess cytoplasmic processes (CP) which project into the lumen (L). The latter contains some cellular debris (D) x 1200.

b. Distinct glycogen granules (g) are visible in the lumenal (L) extremities of the cells of the nuclear layer (1). x 1000.


L
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D
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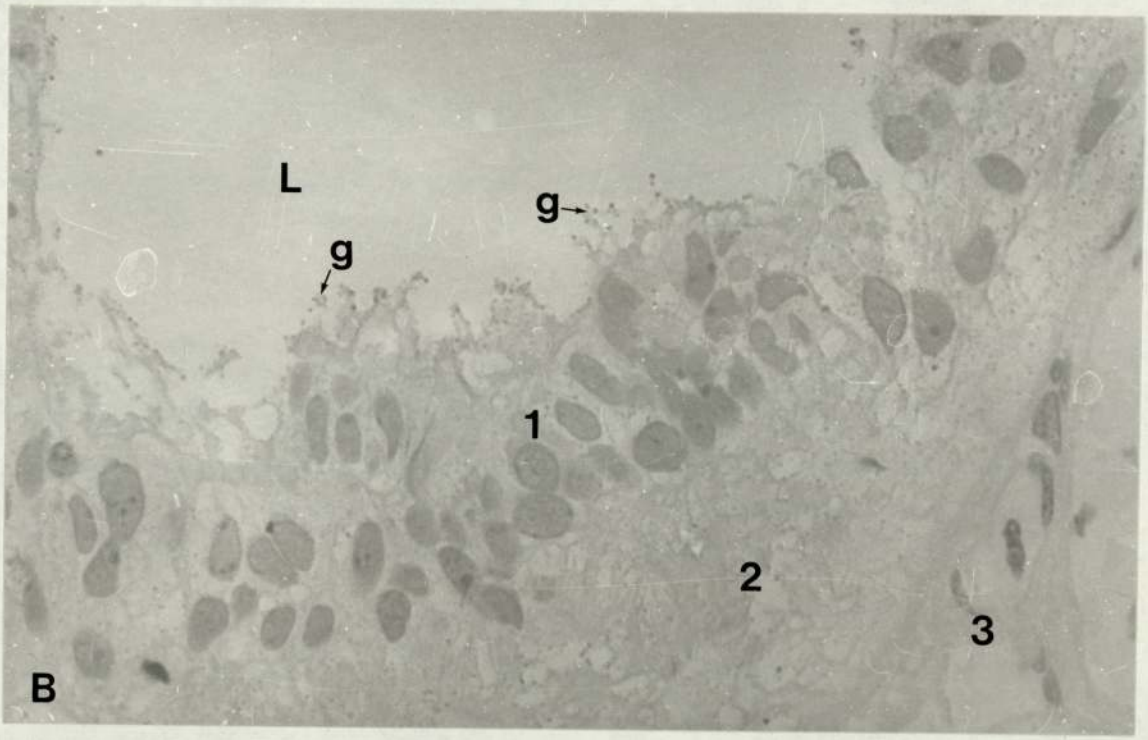

L
g
g
1
2
3
B

2. Electron Microscopy.

The exact nature of the three layers observed in optical sections of the pineal of S.canicula is revealed by the electron microscope.

The outer layer (layer 3 in fig. 18) as indicated by optical staining reactions is composed of collagen (fibres 200-400A) with a relatively small number of fibroblasts. The fibres of this layer are arranged (mainly) in blocks, which themselves are arranged haphazardly. Within the collagen mass, capillaries are present which have typical, flattened endothelial linings. The endothelial cells have irregular nuclei, few mitochondria, an ill-defined endoplasmic reticulum and are sometimes seen to contain electron-dense, homogenous granules. (400-600 nm diameter). In addition, pineocytic vesicles occasionally be seen. (Fig. 20a).

There is often considerable interdigitation between the connective tissue layer and the middle layer. However, there is no fusion of the two layers since a distinct basal lamina is always present. (Figs. 19a, b). Neural junctions may be seen in the region of the basal lamina, but even in this situation, the latter is always complete. (Fig. 19c).

The middle layer, which has a fibrous appearance in optical sections, proves to be composed of neural elements when studied with the electron microscope.

Transverse sections of pineal vesicle and stalk yield a similar pattern of transversely-cut nerve fibres and a wealth of supporting material. These fibres, (diameter 160-670 nm), which are generally non-myelinated, are grouped in large numbers and possess cytoplasm of low density, containing numerous neurofilaments and a few mitochondria. The fibres apparently run up and/or down the stalk to and from the pineal vesicle, although in transverse section a number are seen to run in a circular manner around this vesicle.

A small number of myelinated fibres (diameter approximately 2500 nm), were observed in the pineal vesicle, whose origin has not been traced, but it

is assumed that they follow the main nervous pathway down the stalk. (Figs. 22a, b).

In spite of the presence of such large masses of neural elements, no ganglion cells, or indeed any cell bodies that could be identified as distinctly nervous in origin were observed in any of the sections obtained from vesicle or stalk. In constrast to Rudeberg's (1969) observations, no distinct synapses were observed between the nuclear and nervous layers.

Although typical synaptic structures seem to be missing from the ultrastructural organisation of the dogfish pineal, other nerve endings are present which may serve a similar purpose. These structures occupy a position immediately below the nuclear layer in the region of the basal processes of those cells. They contain dense-core granules (1900-1400A) characterised by a central election-dense mass separated from the encapsulating membrane by a lighter region. (Figs. 23a, b). These granules are very similar to those found in the synapses of some Invertebrates and resemble the neurosecretory granules found in the cells of some of the higher Vertebrates. (Fawcett, 1966). In addition, lighter vesicular structures are also present, together with neurofilaments. In view of the absence of typical synaptic structures, it is suggested that these structures might represent a connection between the nerve layer and the cells of the nuclear layer. This view is strengthened by the fact that these structures are only observed in the area immediately below the nuclear layer.

The nuclear layer itself exhibits considerably complexity. Optical techniques reveal that it is, in the vesicle at least, several cells deep, and using the electron microscope, two basic cell types are evident. These cell types are classified as photoreceptors and supporting cells.

The photoreceptors are characterized by an outer segment which projects into the lumen of the pineal body. This outer segment may be squat or long and tapering and, as far as can be seen, contains at least seventy

sacs. (160 A apart). The latter are, for the most part, open at their marginal ends, conferring a cone-like morphology on the photoreceptors. (Fig. 25b). The outer segments are seldom seen in an intact state and very often detached or broken portions may be observed in the lumen. (Fig. 25c). Even in an intact body the lamellate structure is often degenerate, creating a system of vesicles. These observations may be due to a fixation artefact, but since occasionally perfect (or near-perfect) outer segments are visible this seems unlikely. More probably there is a degenerative cycle of activity in these structures, as for example in the Amphibian retina, (Young and Droz, 1968), when basal growth and distal breakdown of the outer segments occurs.

The lamellate portion of the outer segment arises from a connecting piece which contains a number of fibrils arranged in the conventional 9 + 0 system. These fibrils penetrate the inner segment cytoplasm and are anchored into a basal body. The latter, however, does not appear to possess any striated rootlets. A second centrosomal cylinder is present in the cytoplasm adjacent to the basal body of the first unit.

It is probable that not all photoreceptors have outer segments, since the number of outer segments does not necessarily correspond to the number of inner segments observed in any group of sections.

The photoreceptor inner segment appears to divide into two zones. The lumenal portion, which contains the basal body of the outer segment in a relatively clear area of cytoplasm, is rounded and joined to the nuclear area by a 'waist'. This outer region is extremely rich in large mitochondria which contain sparse tubular cristae. In addition, some cells contain large, electron-dense cytosomes (450-900 nm) bounded by a single unit membrane. (Fig. 24b). The cytoplasm contains large numbers of faint microtubules (220 A diameter) endowing it with a dense appearance. The endoplasmic reticulum appears to be poorly developed in this region. Minute granules are also present throughout the cytoplasm. Rudeberg (1969)

hypothesized that they might be glycogen.

Below the constriction, the cell enlarges to accommodate the nucleus. The latter is usally rounded or slightly ellipsoid with few, shallow indentations, and in the adult appears to possess little visible internal structure. In the immature dogfish, however, the nucleus exhibits a distinct internal arrangement of chromatin - see below. Around the nucleus, the cytoplasm possesses a poorly developed endoplasmic reticulum and Golgi body, and few mitochondria.

At the base of the cell, below the nucleus, the cytoplasm tapers down forming a fine strand which intertwines with those of the other cellular elements. This strand was, on no occasion, seen to contain either synaptic vesicles or rootlets.

The supporting cells, which are present in far greater numbers, seem to consist of two types. These types are recognised by the density of cytoplasm; many have pale abundant cytoplasm, while others have sparse, dense cytoplasm. (Figs. 28a, b, c).

The supporting cells surround the photoreceptors, both at the sides and below, since the deeper lying cells are of the supporting type. These cells which line the lumen are often in such intimate contact with the receptor cells that a vertical section reveals the rounded terminal region of a photoreceptor enclosed by supporting cell cytoplasm (See Figs. 24b, 27a). Also, some type of junctional complex is sometimes observed between the different cell species in the region of the photoreceptor 'waist'. This complex is classified as the macula adherens. (Fawcett, 1964).

The translucent supporting cells are characterized by an abundance of pale cytoplasm containing a poorly developed smooth endoplasmic reticulum and a well developed Golgi body. The mitochondria are relatively few in number and contain a small number of tubular cristae. Electron-dense granules with a transparent region immediately below the limiting membrane are occasionally visible. The latter are identical to those observed in the nerve endings described previously. Only small numbers

of microtubules are visible in the cytoplasm.

Perhaps the most obvious characteristic of the supporting element is the presence of microvilli on the free apical surface. The microvilli project irregularly from the cell surface and often form a dense mass reaching far into the lumen. (Fig. 28b). In addition, cilia are present in very small numbers among the microvilli. Each cilium exhibits the familiar 9 + 2 arrangement of fibrils and is anchored into the cytoplasm by a basal body. On one occasion striated rootlets were observed attached to this basal body.

The nucleus of the supporting cell may be irregular, having deep indentations; although often it is seen to be simply rounded or spindle-shaped. Its internal structure is similar to that of the photoreceptor. (See also Section III, 3).

The second type of supporting cell is characterized by extremely dense cytoplasm whose appearance on close examination proves to be due to the presence of large numbers of microtubules. These cells, which we will refer to as opaque cells, possess few mitochondria, a moderately well developed smooth endoplasmic reticulum and a well-developed Golgi body. The nucleus is smooth and spindle-shaped. The cell does not appear to possess microvilli on its free surface in the adult. (see also Section III, 3)

Both species of supporting cell taper at their bases and pass to the nerve layer, but again no synaptic structures are present. The extreme complexity of the nerve layer renders the possibility of tracing the destination of the various nuclear elements very low indeed.

Only a limited number of specimens of Raja clavata were available. Structural analysis is thus necessarily based upon a rather limited amount of information.

The pineal of R.clavata exhibits a basic organisation very similar to that of S.canicula . It consists of an outer capsule composed mainly of collagen containing few fibroblasts and a large number of capillaries. This layer is separated from a poorly-developed nerve layer by a distinct basement membrane, which, like that of S.canicula, remains unbroken.

The nerve layer contains only non-myelinated nerve fibres in a mass of supportive elements and does not appear to have any synaptic contact with the nuclear layer.

The nuclear layer consists of poorly-developed photoreceptors and supporting cells. The outer segments of the photoreceptors are always degenerate, consisting of vesicles rather than lamellae, and at no time were intact photoreceptors observed. However, the possibility that this might be due to a fixation artefact cannot be excluded. The lumen of the pineal body is very small and contains cellular debris.

The pineal body of R.clavata appears to be smaller and less well-developed internally than that of S.canicula. The diameter of the pineal vesicle in a 90 cm specimen was only half that of a 60 cm dogfish.

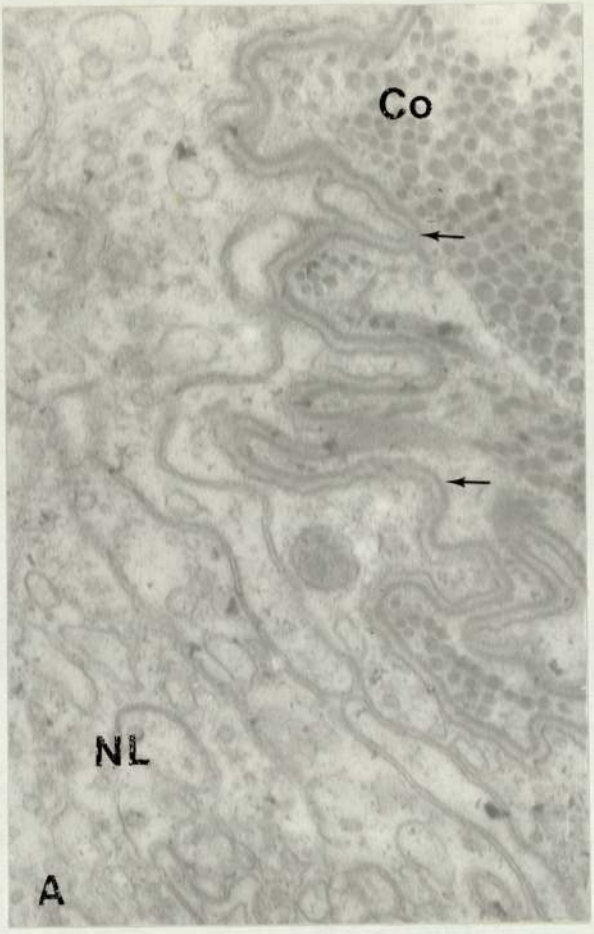
Figs. 19a, b, c, d. Outer region of pineal, S.canicula, median section, gluteraldehyde osmium fixed, stained by uranyl acetate.

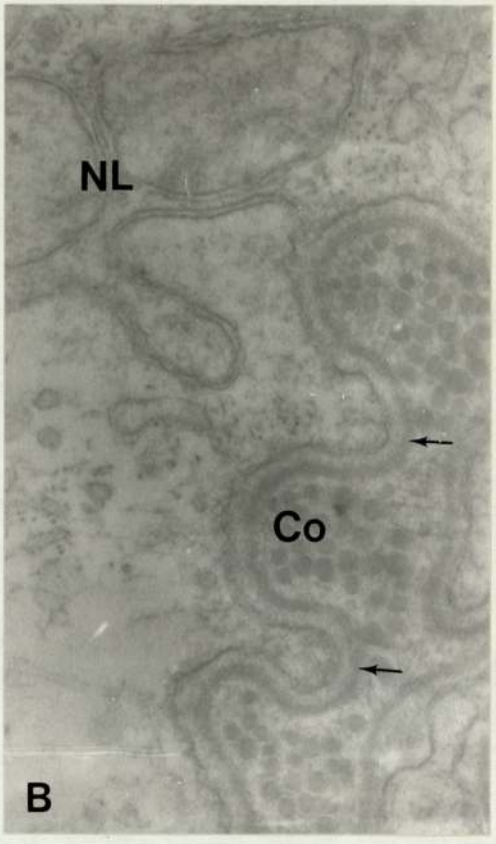
a. Basement membrane between layers 2 and 3 (see Fig.18) arrows - basement membrane; NL - layer 2 - (nerve layer); Co - collagen - layer 3. x 30,000.

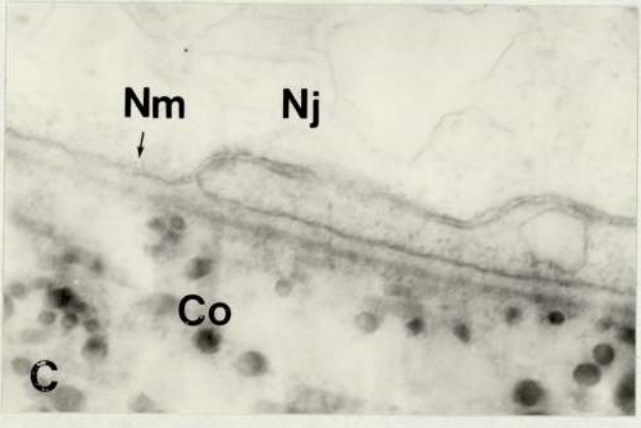
b. x 45,000.

c. Basement membrane, and junction between two nerve elements (Nj), the limiting membrane of the nerve elements can be clearly seen (Nm). x 45,000.

d. Capillary within the connective layer, (Cap.)- containing an erythrocyte (Er). The endothelial cells (End) are visible together with cells (fibroblasts) of the connective layer. (CoC). x 4,300.


Co
NL
A


NL
Co
B


Nm
Nj
Co
C

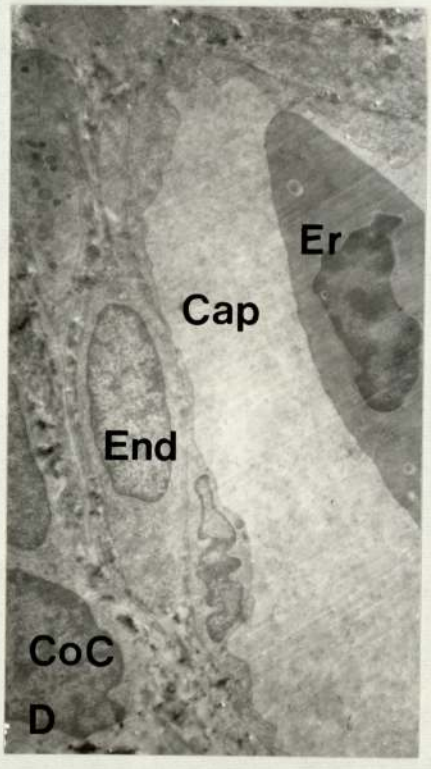
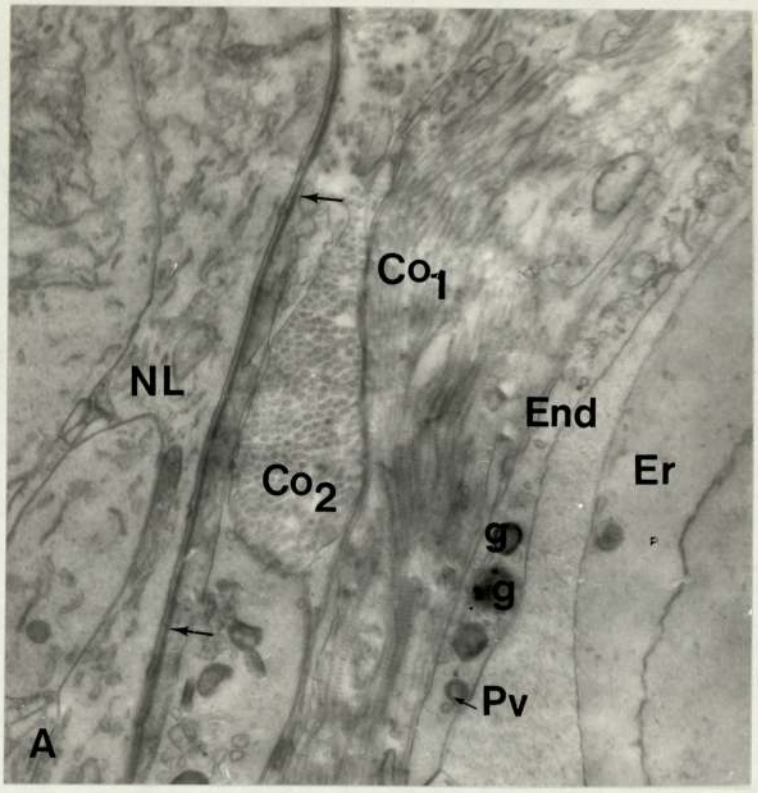

Er
Cap
End
CoC
D

Fig. 20a, b Outer region of pineal of S.canicula, gluteraldehyde-osmium fixed, stained by uranyl acetate .

a. Median section showing capillary (Cap) whose endothelial cells contain dense granules (g) and pinocytic vesicle (Pv). The connective tissue layer contains collagen arranged in blocks; Co. - transverse section through collagen fibre- CO_2 - longitudinal section through collagen fibres. x 10,500.

b. Asa, the endothelial cells contain spherical mitochondria (m). x 6,500.

In both a and b the basement membrane (arrows) separates the collagen layer (Co) from the nerve layer (NL).


Co1
NL
End
Er
Co2
g
g
Pv
A

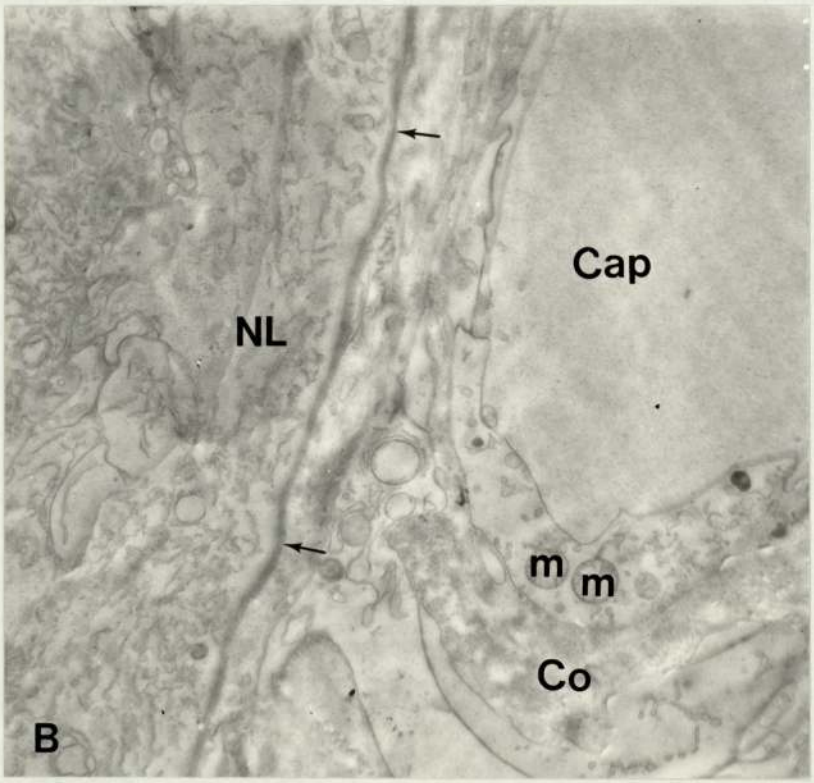
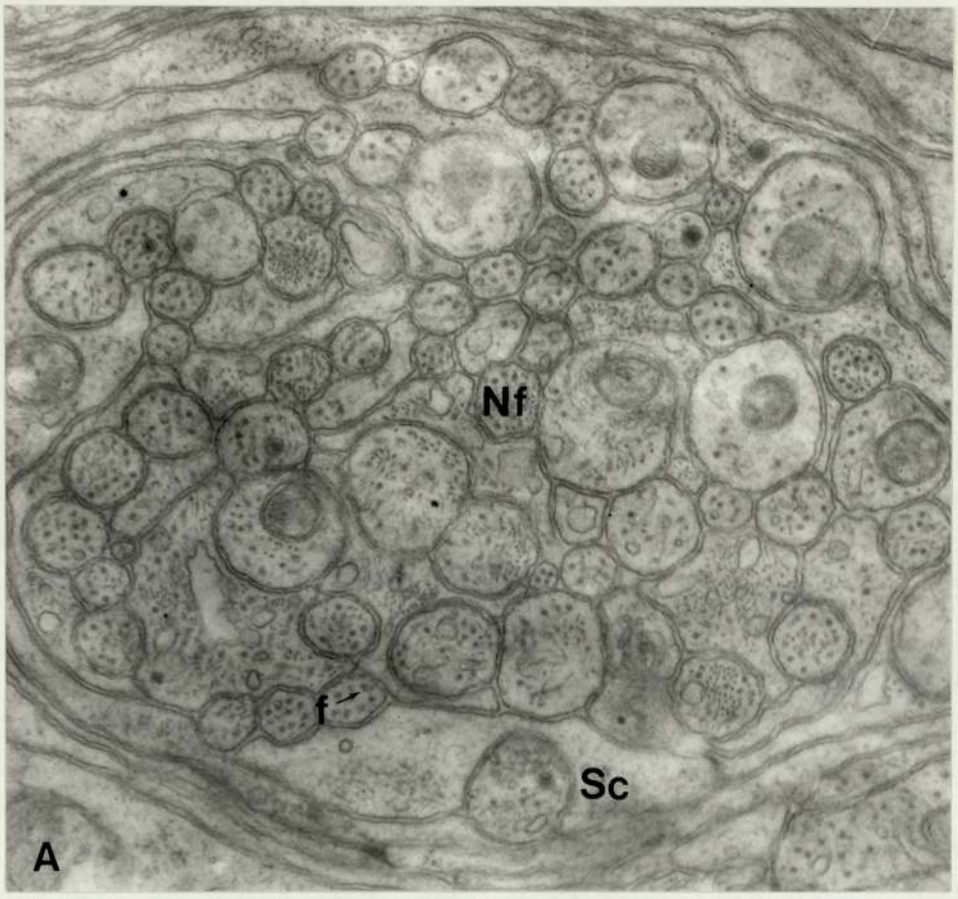

Cap
NL
m
m
Co
B

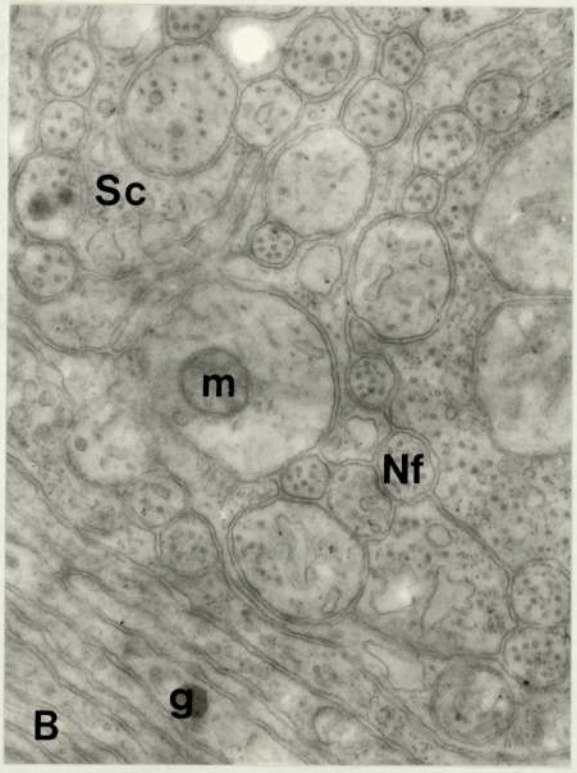
Fig. 21a, b, c. Pineal of S.canicula, layer 2, gluteraldehyde-osmium fixed, stained by uranyl acetate.

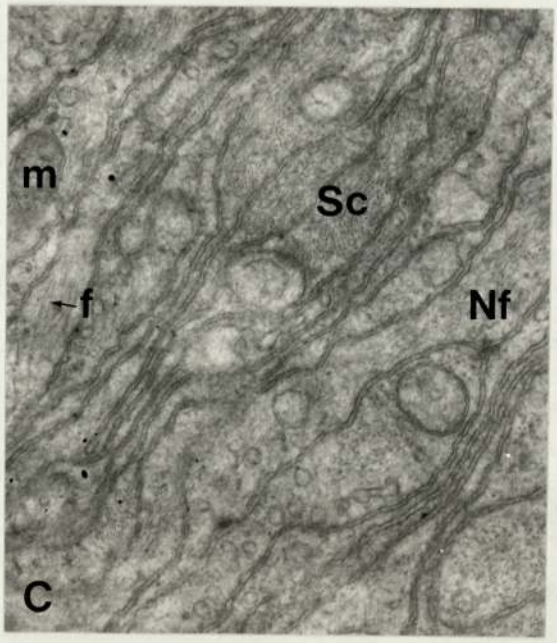
a. Typical median section from layer 2 of the pineal vesicle, showing a large number of non-myelinated nerve fibres (Nf) embedded in supporting cell material (Sc). Neurofilaments (f) are visible within the nerve fibres. x 30,000.

b. Typical section from layer 2 of the stalk; m - mitochondria; g - granule. x 26,000.

c. Median section of pineal vesicle, containing longitudinally sectioned neural elements. Longitudinally-orientated neurofilaments are visible within the fibres. (Nf). x 25,000


Nf
f
Sc
A

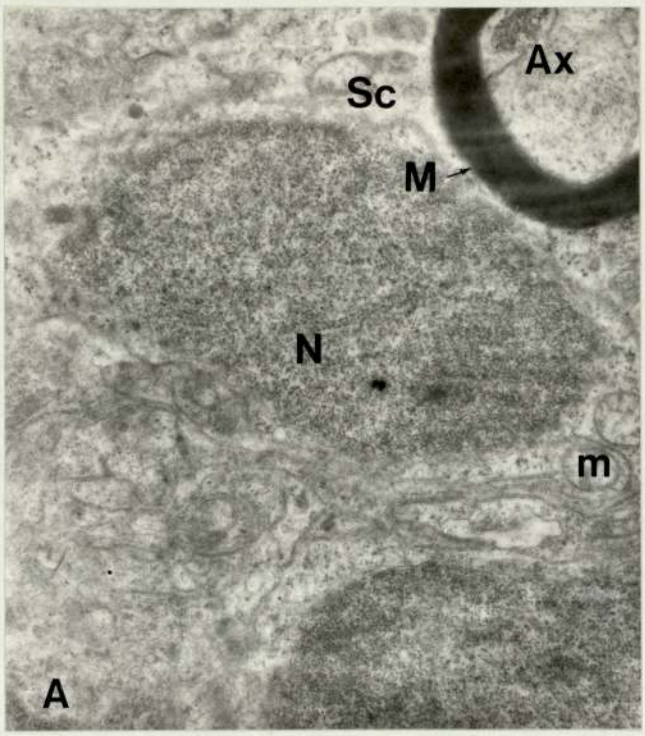

Sc
m
Nf
g
B

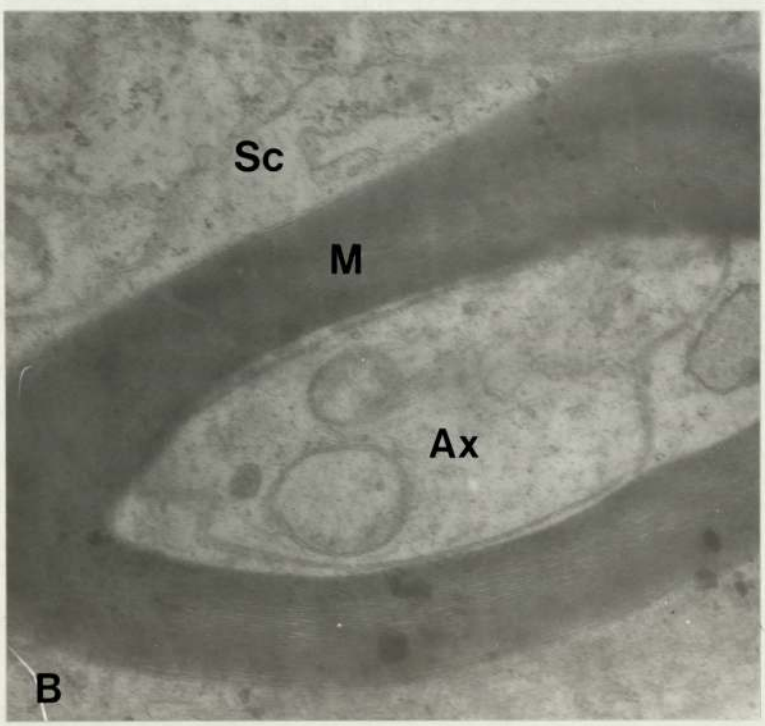

m
Sc
f
Nf
C

Figs. 22a, b. Pineal of S.canicula, median section of vesicle, gluteraldehyde-osmium fixed, stained by uranyl acetate and lead hydroxide.

a. Section of layer 2 to show a myelinated nerve fibre, axon - Ax; myelin - M; supporting cell cytoplasm - Sc; nucleus (possibly of supportive element) - N; mitochondria - m. x 10,000.

b. Axon from layer 2, x 60,000.


Ax
Sc
M
N
m
A

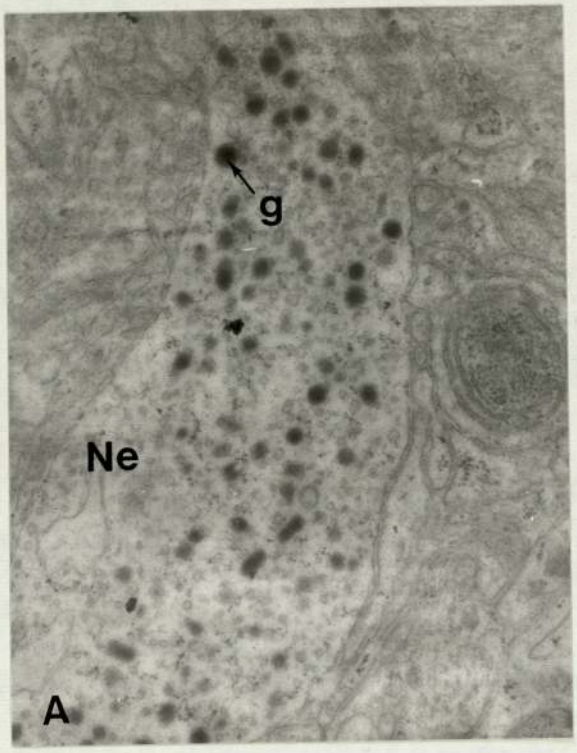

Sc
M
Ax
B

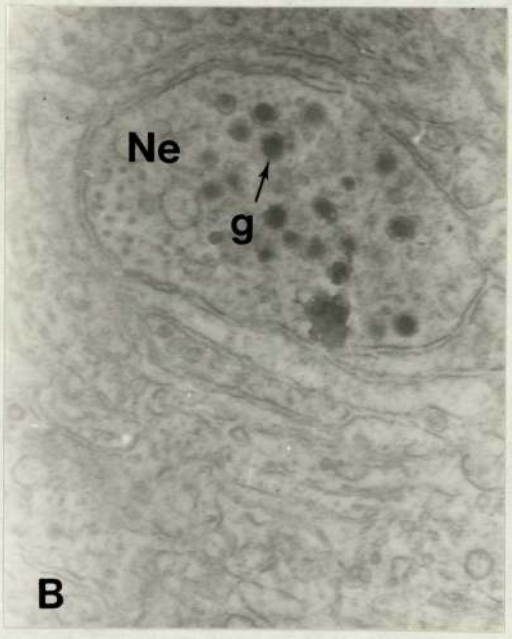
Figs. 23a, b, c. Median section, pineal vesicle of S.canicula, gluteraldehyde-osmium fixed, stained by uranyl acetate and (a and b only) lead tartrate.

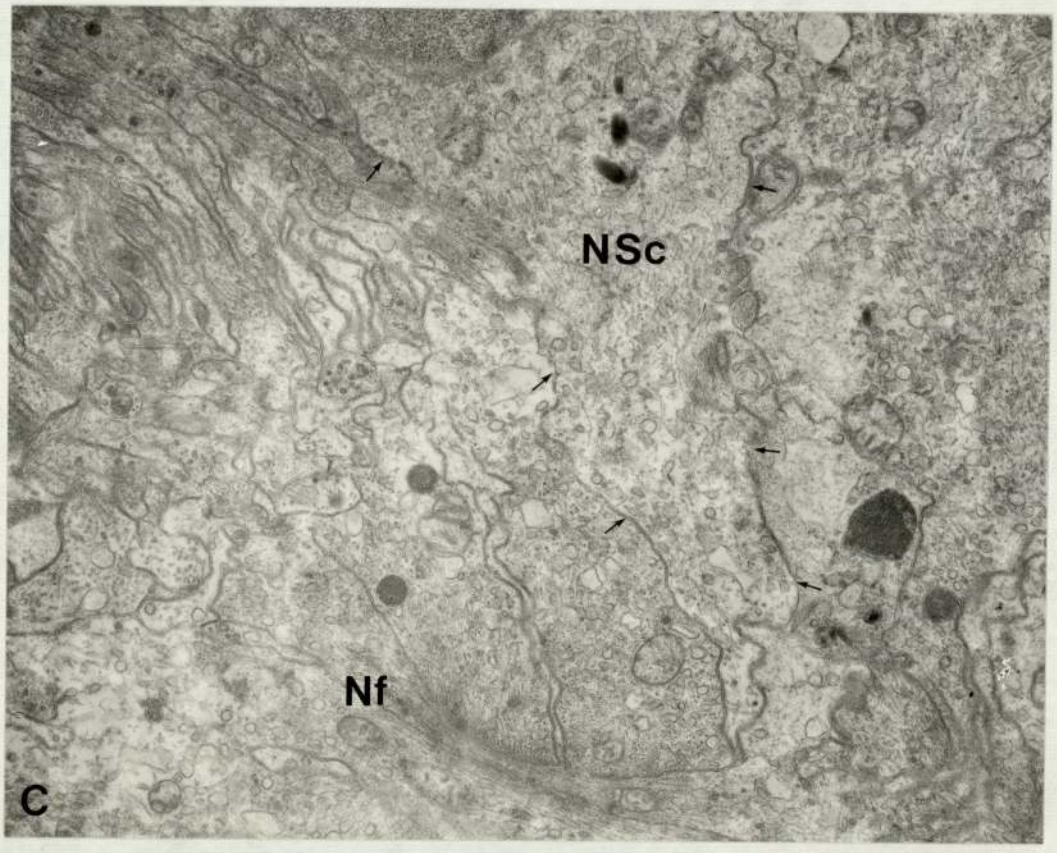
a. Nerve ending (Ne) immediately below the nuclear layer, containing dense-core granules (g) x 23,000.

b. Nerve ending (Ne) containing dense-core granules (g) x 45,000.

c. Low power micrograph of nerve layer in the region of the inner luclear layer. A nucleated supporting cell is visible (NSc) enclosed in an ill-defined mass of neural elements. A distinct nerve fibe (Nf) can be seen at the bottom. x 10,000


g
Ne
A

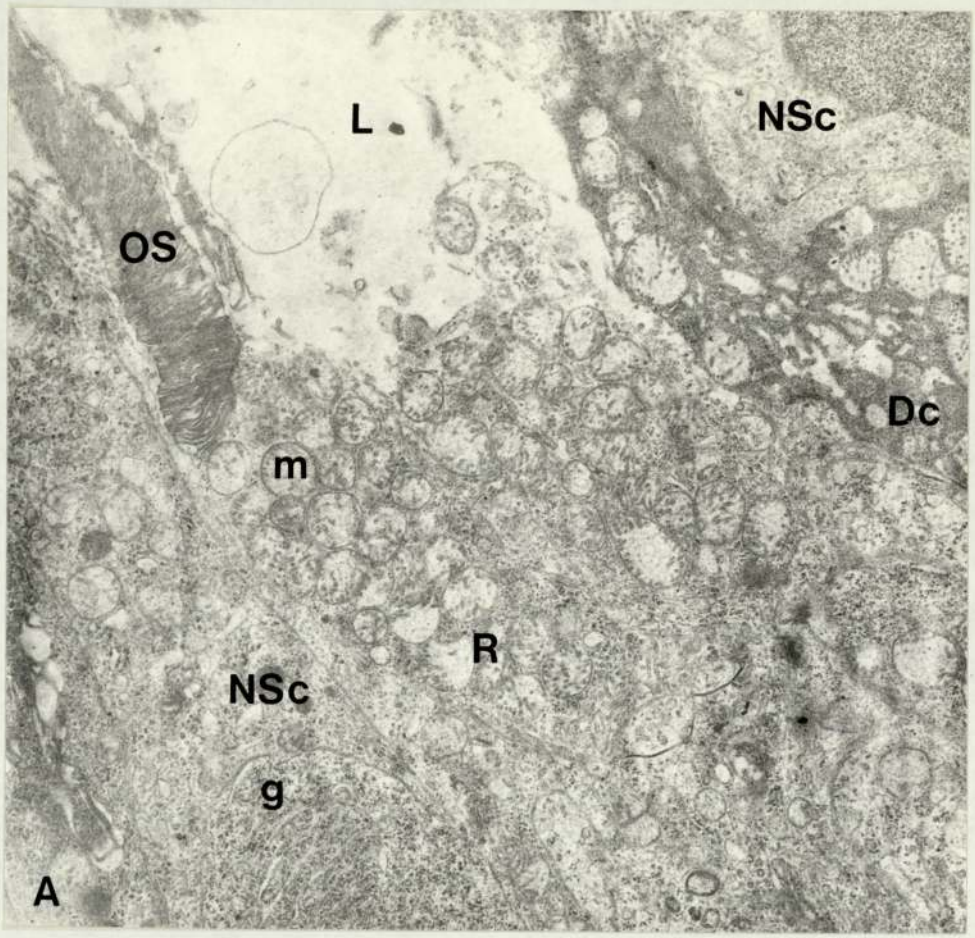

Ne
g
B

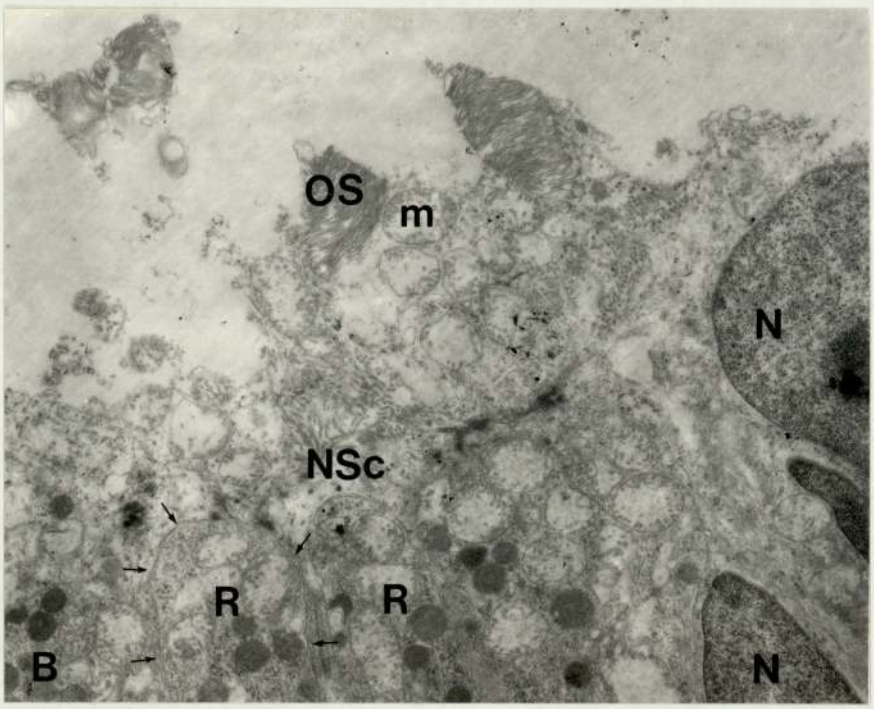

NSc
Nf
C

Figs. 24a, b — Median sections through pineal vesicle of S.canicula; gluteraldehyde-osmium fixed, stained by uranyl acetate.

a. Lumenal portions of a number of nucleated cells. Portions of at least two receptor cells (R) can be seen, each possessing large numbers of mitochondria (m) and one on outer segment (OS) projecting into the lumen (L). In addition, two supporting cells are visible (NSc), one of which encloses a granular area (g). A portion of one cell containing extremely dense cytoplasm (Dc) is also evident. x 8,000.

b. Lumenal portions of a number of nucleated cells, with the rounded apices of two receptor cells (R) actually enclosed by supporting cell (NSc) cytoplasm. Microvilli (mv) on the latter are clearly visible. Cytosomes are evident within the photoreceptors. x 6,600


L
NSc
OS
Dc
m
R
NSc
g
A

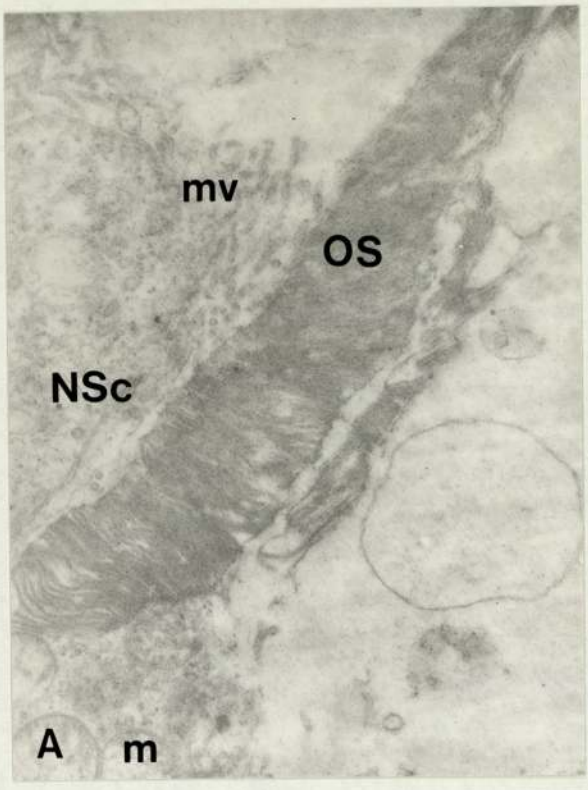

OS
m
N
NSc
R
R
B
N

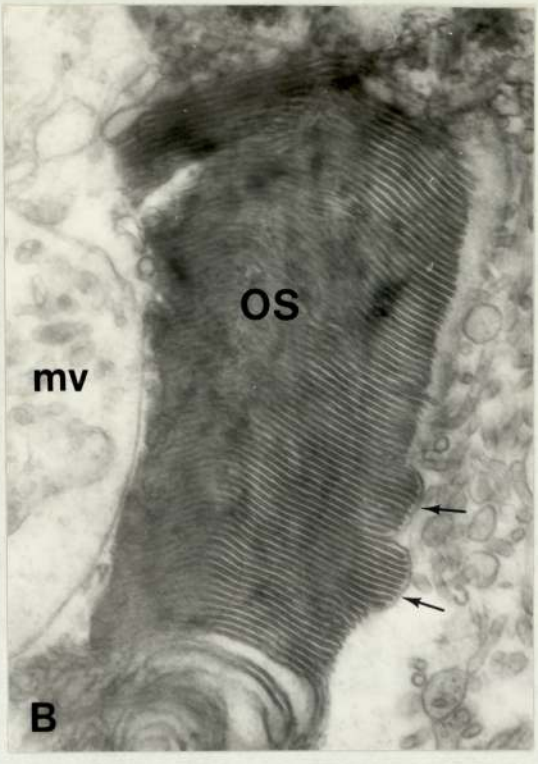
Figs. 25a, b, c. Median section through the pineal vesicle of S.canicula; gluteraldehyde-osmium fixed, stained by uranyl acetate.

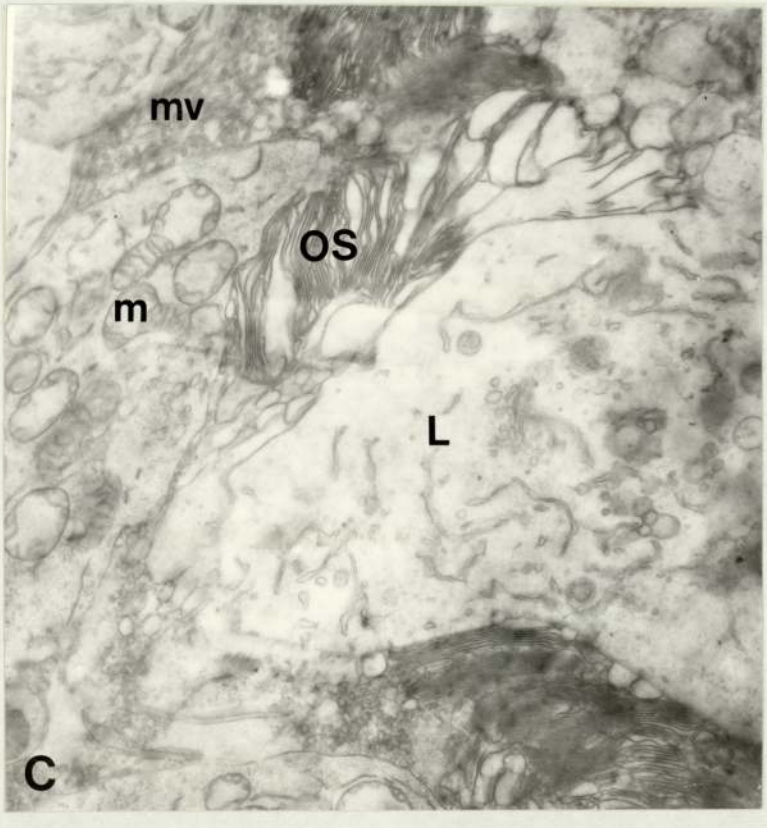
a. Almost complete receptor cell outer segment (OS) with a portion of a supporting cell (NSc) bearing microvilli (mv). x 15,000.

b. Almost complete outer segment (OS) whose lamellae have an occasional outer bounding membrane (arrows) in the basal region. x 28,000.

c. Portion of the lumen (L) to show a number of degenerative outer segments (OS). x 18,000


mv
OS
NSc
A
m


OS
mv
B


mv
OS
m
L
C

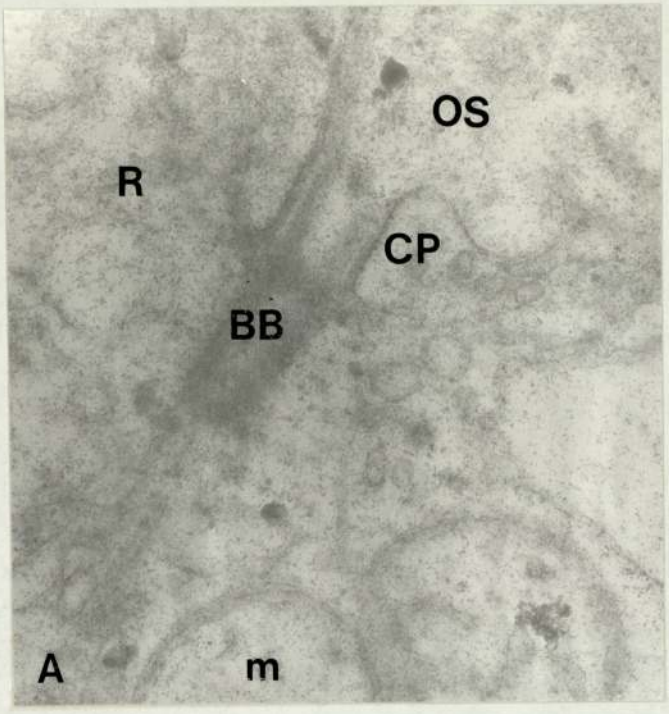
Figs. 26a, b, c, d. Median section through the pineal vesicle of S.canicula; gluteraldehyde-osmium fixed, stained by uranyl acetate and lead tartrate (c only).

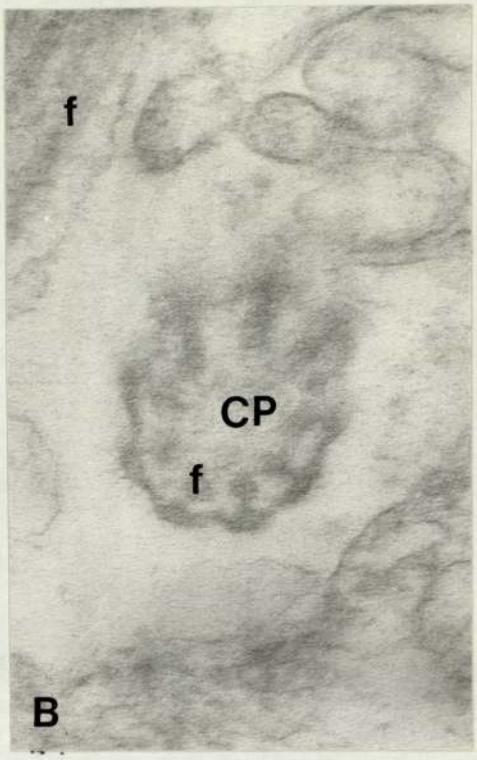
a. Basal body (BB) and connecting piece (CP) of the outer segment (OS) of a single receptor cell (R). x 45,000.

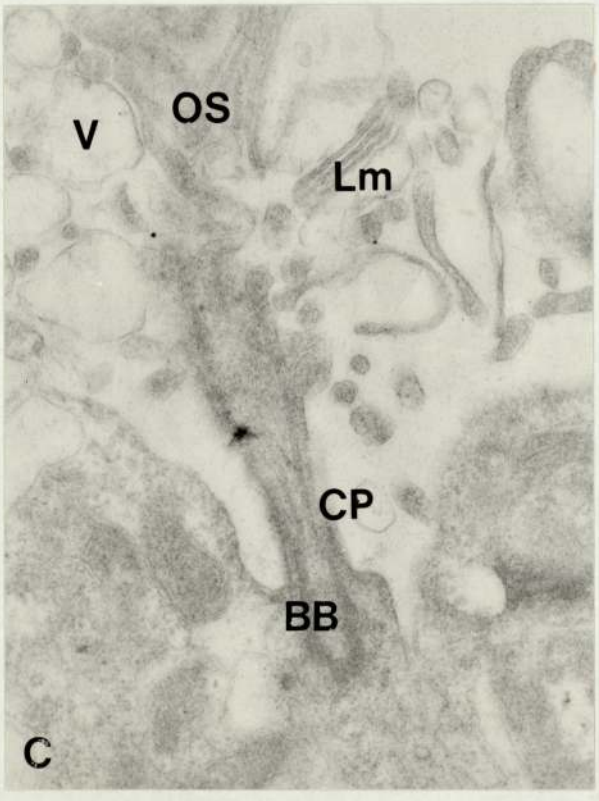
b. Transversely cut connecting piece (CP) and a longitudinally cut piece, both showing a 9 + 0 internal structure of filaments (f). x 60,000.

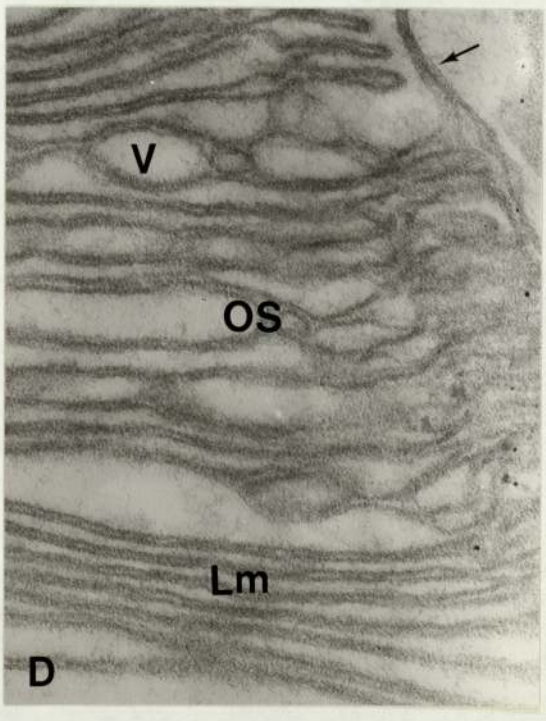
c. Basal body (BB) and connecting piece (CP) of a degenerative outer segment (OS). Many vesicles (v) can be seen, together with a few lamellae (Lm). x 30,000.

d. Outer segment (OS) showing some regular lamellae (Lm) and vesicles (v). An outer limiting membrane may also be seen (arrow). x 60,000.


OS
R
CP
BB
A
m


f
CP
f
B

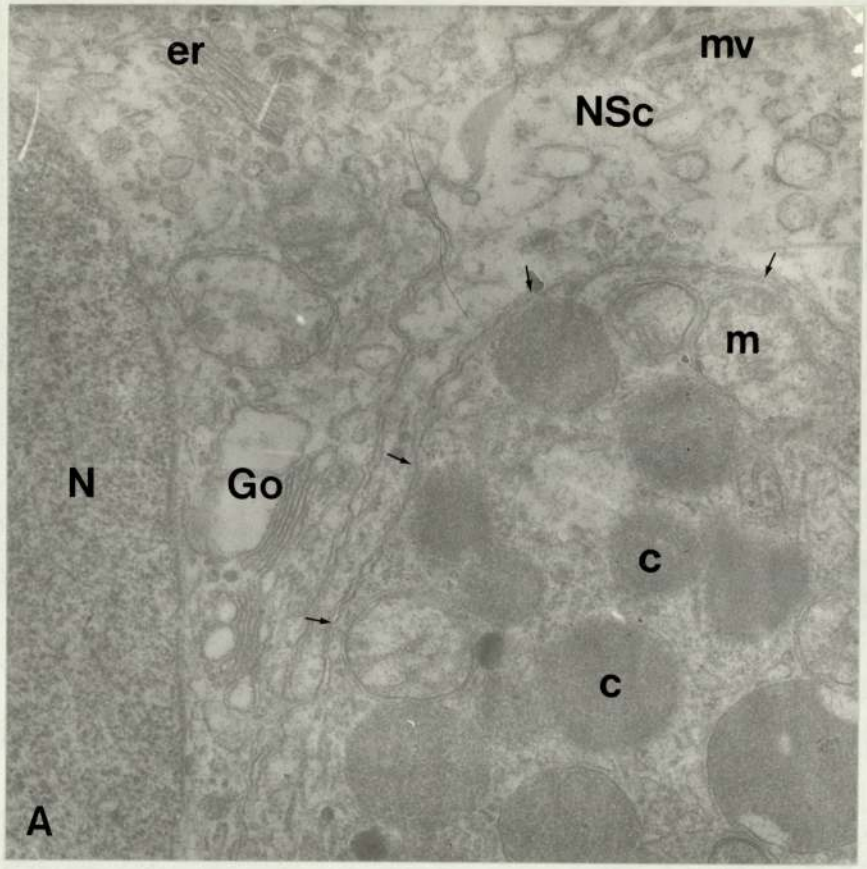

V
OS
Lm
CP
BB
C

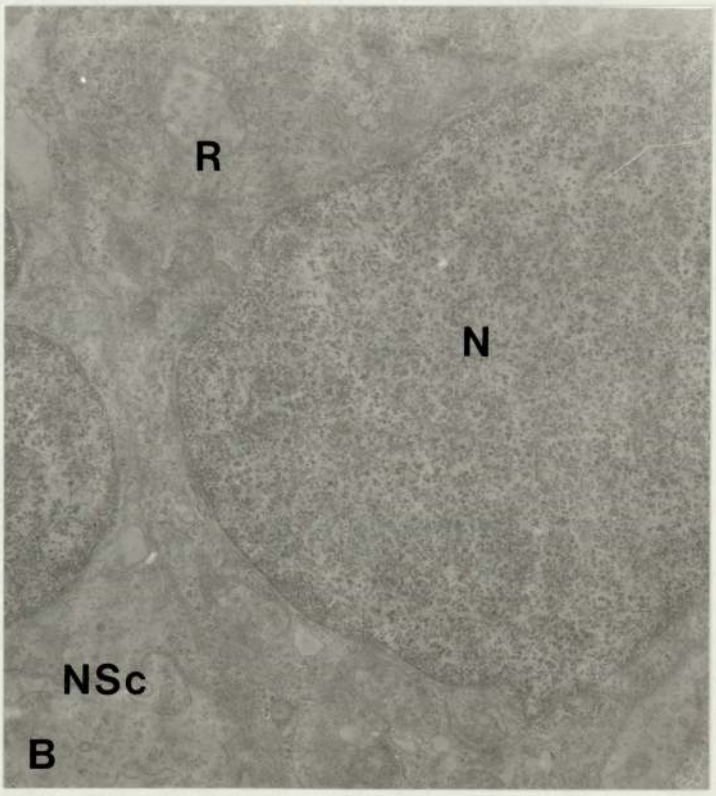

V
OS
Lm
D

Figs. 27a, b. Median section through pineal vesicle of S.canicula; gluteraldehyde-osmium fixed, stained by uranyl acetate.

a. Portion of cytosome (c) and mitochondria (m) - rich receptor cell (delineated by arrows) enclosed by supporting cell cytoplasm (NSc) identified by microvilli (mv) on the upper border. An adjacent supporting cell exhibits a sparse, smooth endoplasmic reticulum (er) and a well-developed Golgi apparatus (Go), together with a nucleus (N) having a well-defined internal structure. x 22,500.

b. Basal region of receptor cell (R) exhibiting a nucleus with no well-defined internal structure. x 11,250.


er
mv
NSc
m
N
Go
c
c
A

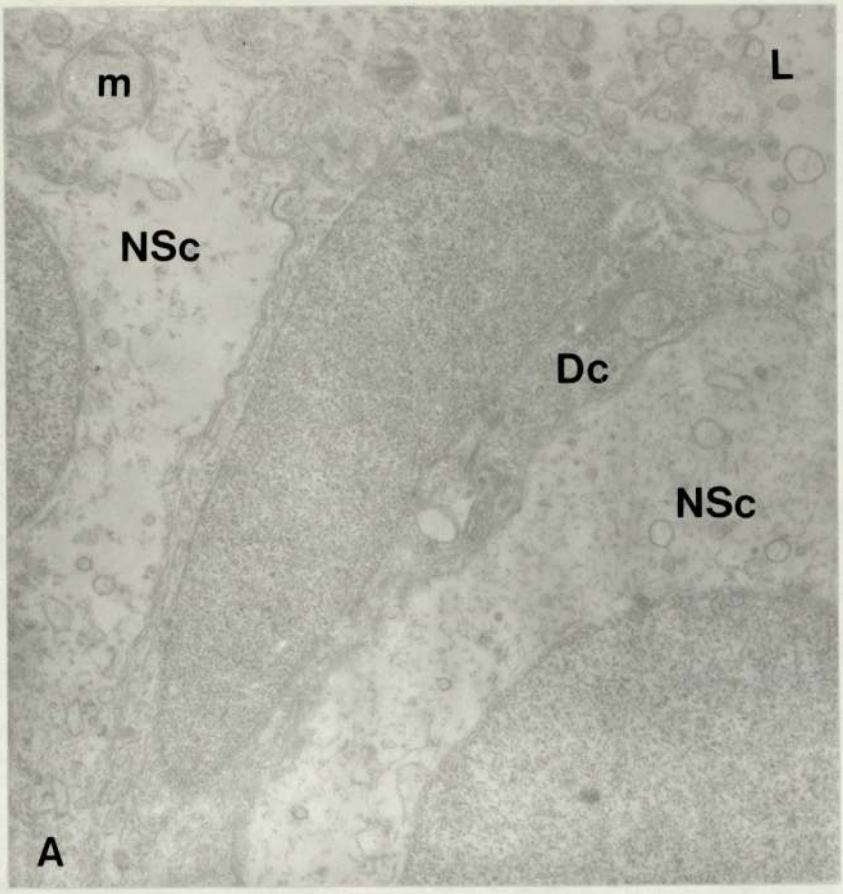

R
N
NSc
B

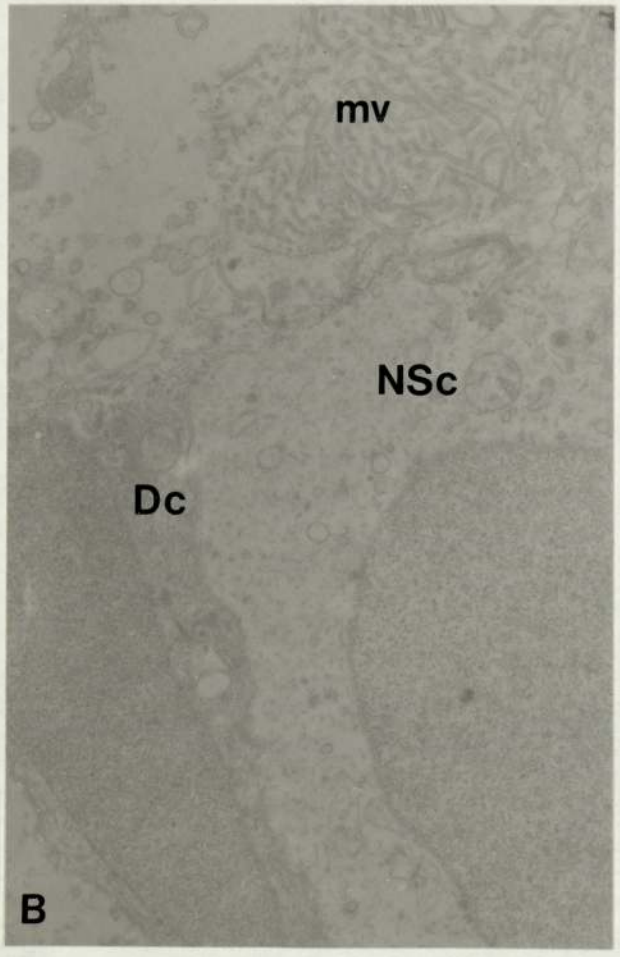
Figs. 28a, b, c. Median section through the pineal vesicle of S.canicula; gluteraldehyde-osmium fixed, stained by uranyl acetate.

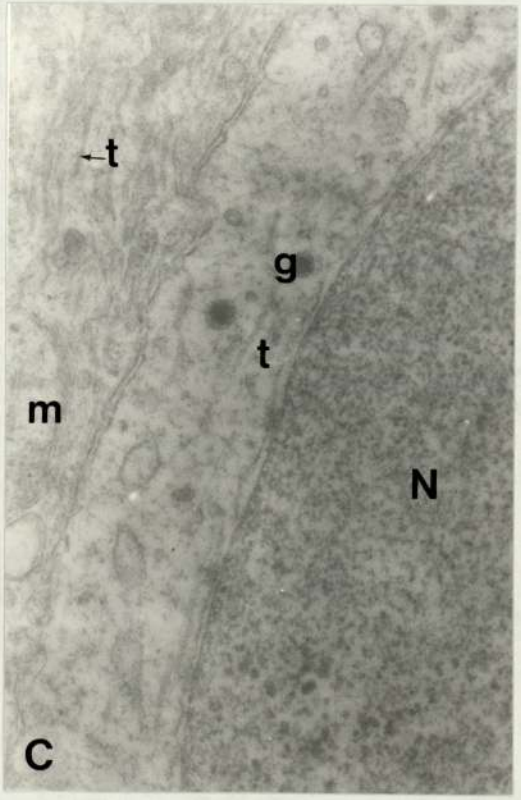
a. Portions of three cells, two of which are supporting cells (NSc) with pale abundant cytoplasm, few mitochondria (m) and extremely sparse endoplasmic reticulum. The third cell has a spindle-shaped nucleus and very dark cytoplasm (Dc), but is thought to be a supporting cell. x 14,000.

b. Similar to a, but showing the microvilli (mv) on the upper surface of one of the supporting cells. x 11,500.

c. Junction between translucent and opaque supporting cells. The primary difference can be seen to be due to the relative numbers of microtubules (t) contained in their cytoplasm. Dense-core granules are also visible in the cytoplasm of the 'light' cell. x 22,500.


m
L
NSc
Dc
NSc
A


mv
NSc
Dc
B


t
g
t
m
N
C

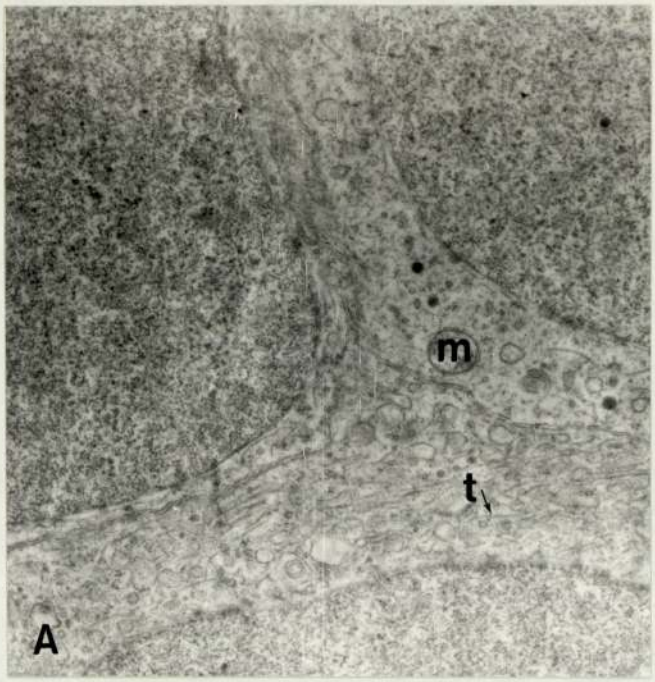
Figs. 29a, b, c, d. Median sections through the pineal vesicle of S.canicula; fixed in gluteraldehyde-osmium tetroxide and stained by uranyl acetate and lead hydroxide (b and c only).

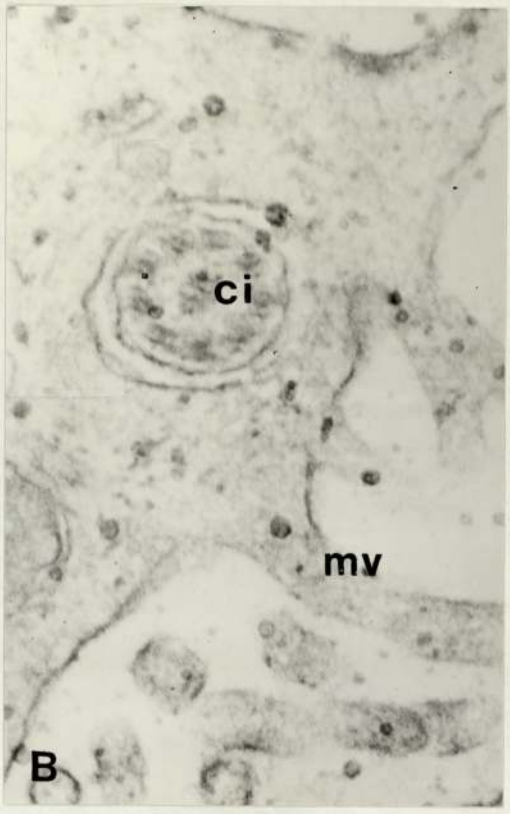
a. Junction between three cells, all of which are almost certainly supporting cells. One of the cells contains a large number of microtubules (t) rendering the cytoplasm denser than that of the other cells. x 12,300.

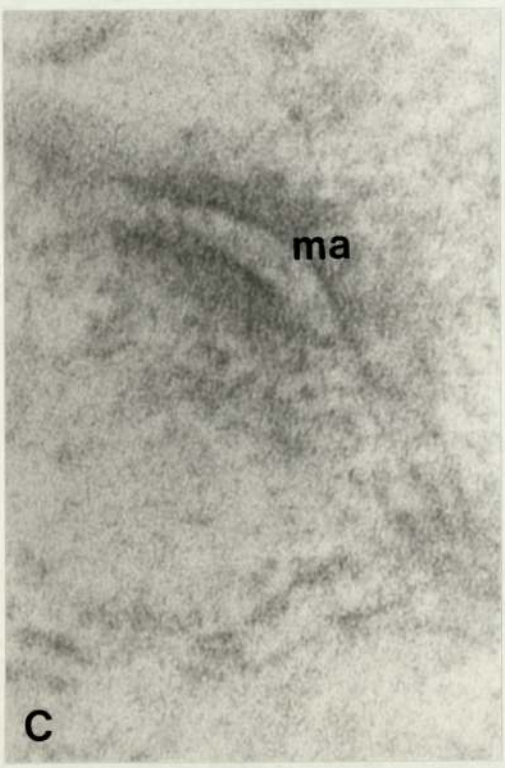
b. Portion of free surface of supporting cell with microville (mv) and cilium (ci) which has the familiar 9 + 2 arrangement of fibrils. x 60,000.

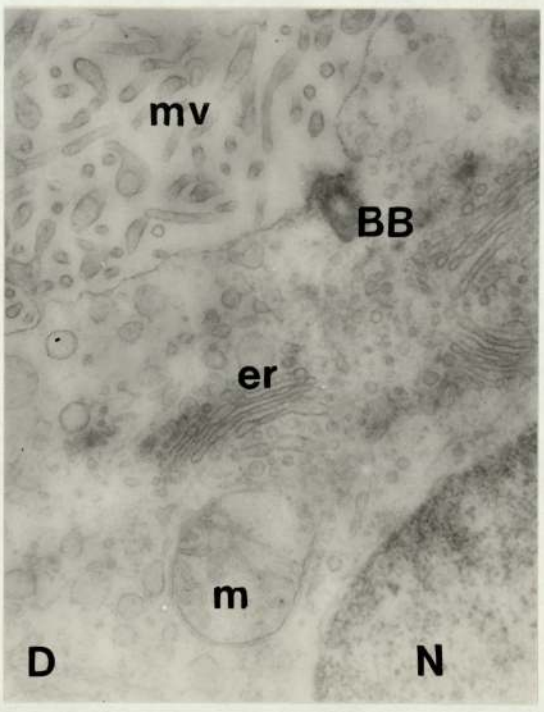
c. Macula adherens between adjacent cells of the nuclear layer. x 170,000.

d. Free surface of supporting cell with smooth endoplasmic reticulum (er) and a mitochondrion (m). The basal body of a single cilium is also visible (BB). x 24,500.


m
t
A


ci
mv
B


ma
C

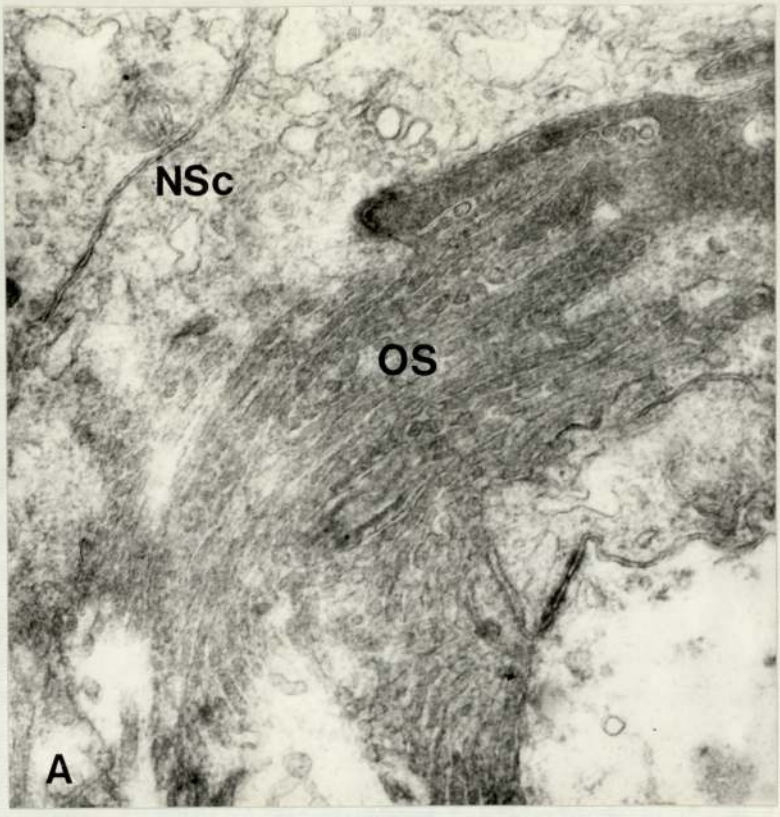

mv
BB
er
m
D
N

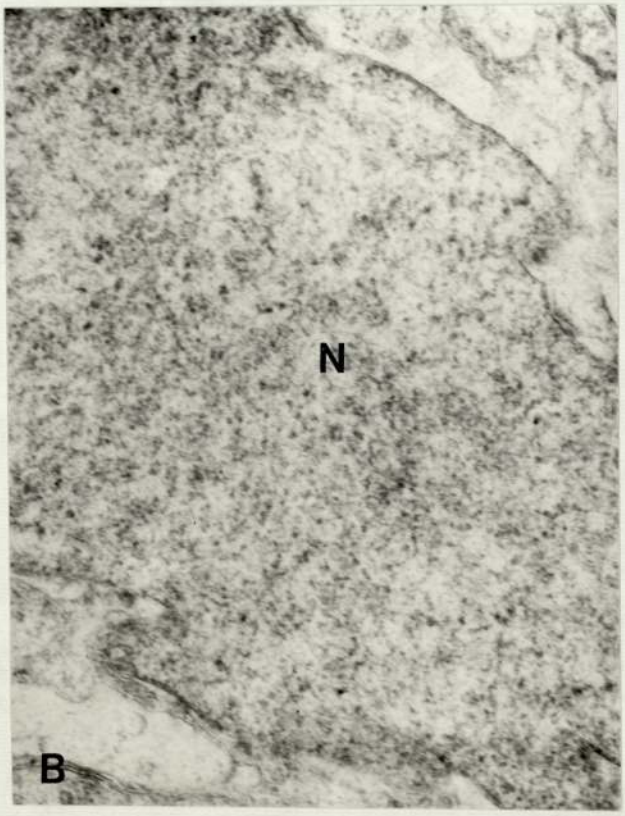
Figs. 30a, b, c. Median section through the pineal vesicle of Raja clavata; gluteraldehyde-osmium fixed, stained by uranyl acetate.

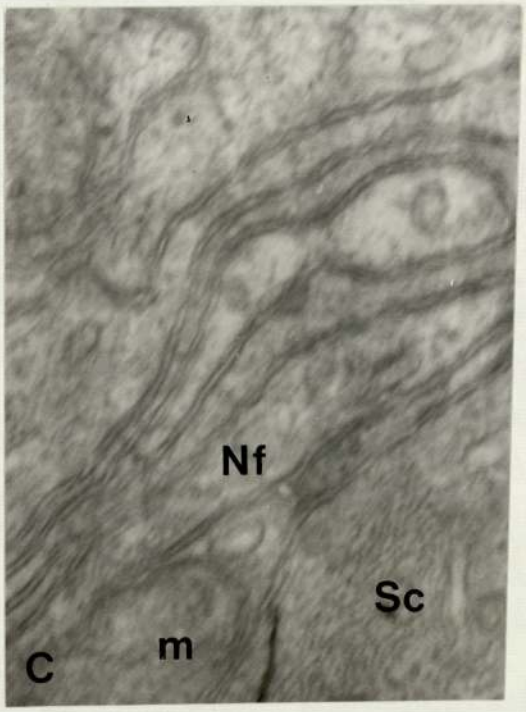
a. Degenerative outer segment (OS) of photoreceptor and a portion of a supporting cell (NSc). x 30,000.

b. Nucleus (N) of a supporting cell, exhibiting poorly defined internal structure and an irregular outline. x 22,500.

c. Section through nerve layer, showing nerve fibres (Nf) and supportive elements (Sc). A mitochondrion is also visible (m). x 60,000.


NSc
OS
A


N
B


Nf
Sc
m
C

3. Examination of Immature Specimens

Optical sections of larval dogfish (see Section III, 1) reveal that the pineal region consists primarily of nucleated cells, with little or no connective tissue capsule or nerve material. This observation is confirmed using the electron microscope. The connective tissue layer has not yet fully formed (in the 40-50 mm specimen) while the nerve layer is only present in rudimentary form.

The nuclear layer exhibits the greatest degree of development and may be several cells deep. Few of these cells are photoreceptors, but many (lumenal) cells bear microvilli and may therefore be classified as supporting cells.

The primary object of this investigation was to examine the effects of light on the developing pineal. This was carried out by simply studying the fish in the presence or absence of light, no other light regimes were attempted. This was primarily because of the difficulty in obtaining suitable specimens. Secondly, it is difficult to create a light/dark cycle which would bear some resemblance to the natural environment. Therefore, light-adapted fish can only be compared directly with dark-adapted fish although, of course, some inferences as to the structure of 'normal' fish might be drawn from the results of studies on the adult. (See Section III, 2).

3. (a) Light adapted fish

Light -adapted specimens exhibit considerable regularity in the structure of the nuclear layer. The layer of cells next to the lumen of the pineal vesicle appears to consist primarily of supporting elements which possess dark cytoplasm. Cells with pale cytoplasm may be interspersed with these and may also form the greater number of those cells which lie deeper down. (Figs. 31a, b).

The opaque supporting cells have irregular nuclei which exhibit a distinct chromatin pattern; being dark at the periphery with dark patches

towards their centres. These nuclei constitute a large part of the cell mass. The cytoplasm contains a well-developed endoplasmic reticulum and Golgi body, while mitochondria are only present in small numbers and contain tubular cristae. As in the adult's opaque cells, the cytoplasm contains large quantities of microtubules. Some cytosomes are also observed similar to those found in the adult receptor cell.

The lumenal cells form a tightly-packed epithelium with microvilli forming an almost continuous surface covering. Between all cells, near to their apices, a junctional complex may be seen. This is presumed to represent macula adherens as in the adult, although since it appears in all sections it may well represent a belt-like structure and be more properly identified as zonula adherens. The translucent supporting cells contain large quantities of pale cytoplasm which has a poorly-developed (smooth) endoplasmic reticulum and a few dense core granules of the type previously described in the adult. A Golgi body was not observed but was presumed to be present since it has been found in the adult; mitochondria were seen only rarely.

Photoreceptors, or rather cells that could be definitely identified as photoreceptors, are present in very small numbers. Those that were seen either had poorly-developed outer segments, or these were lacking entirely. They, like the supporting cells, appear to contain few mitochondria, although some pale vesicular structures were observed in the outer region of the inner segment, an area that is in the adult extremely rich in mitochondria. These vesicles strongly resemble lipid droplets. (Fawcett, 1968).

3. (b) Dark-adapted fish

Dark-adapted specimens lack the regularity of structure exhibited by those which are light-adapted.

The lumenal epithelium again consists primarily of opaque cells although their nuclei are somewhat more regular and lack any detectable internal pattern of chromatin. The cytoplasm contains an endoplasmic

reticulum which has become vesicular, but is dominated by large numbers of large mitochondria (600-1000 nm). These mitochondria contain an electron-transparent matrix of flocculent material and very few tubular cristae, which, when present, are confined to the immediate periphery. The deeper-lying translucent and opaque cells also contain large numbers of mitochondria of a similar type.

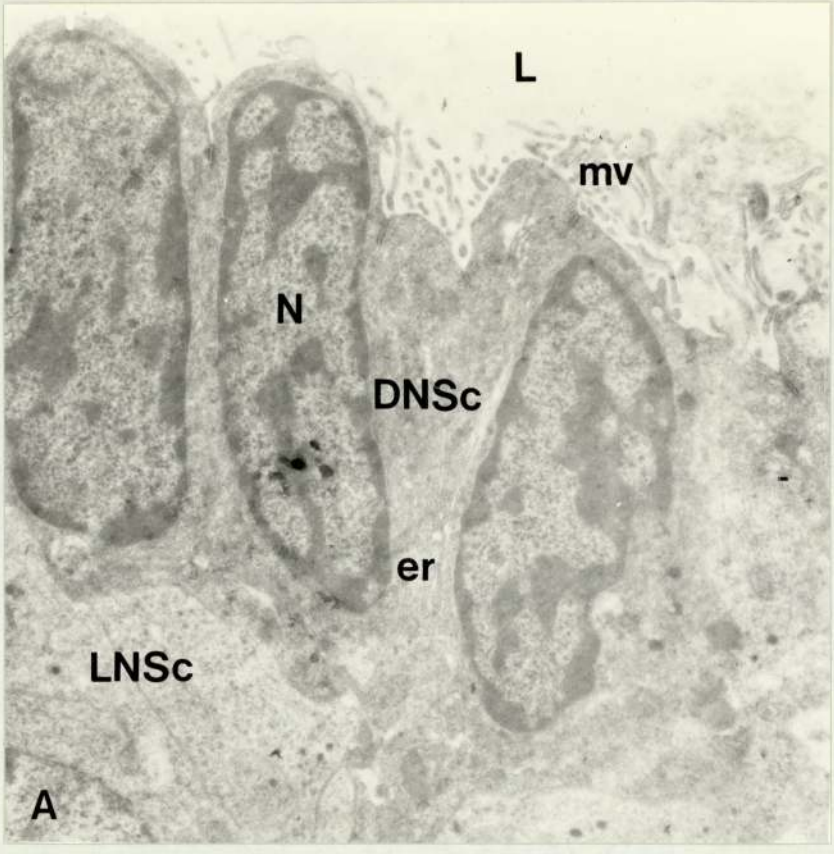
Photoreceptors are again only present in small numbers. They, too, exhibit the increase in numbers of mitochondria already described; however, their outer segments always appear to be poorly developed, containing few lamellae and usually some vesicular material. (fig. 33b).

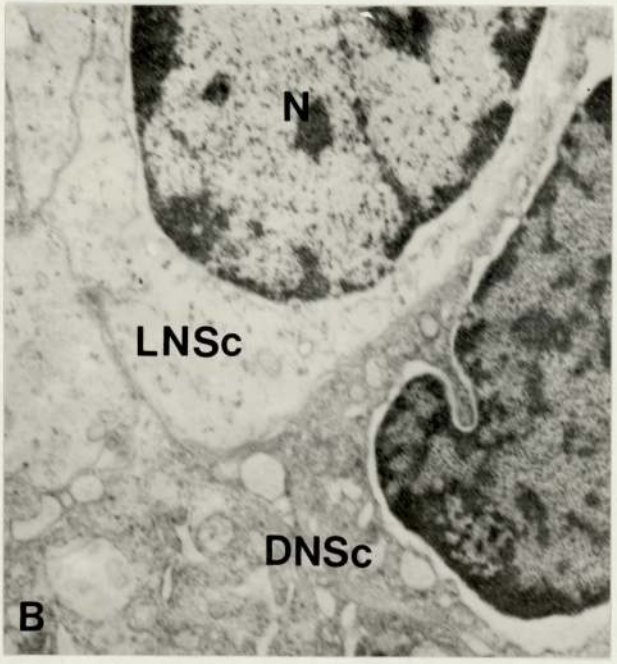
The principle difference between the pineals of light-adapated and dark-adapted juvenile dogfish is in the number and type of mitochondria. Light-adapted pineals resemble most closely those which are present in the adult, while those of dark-adapted fish are atypical. It is interesting that while the number of mitochondria has increased in dark-adapted animals, their internal structure appears somewhat degenerate.

Figs. 31a, b. Sections through pineal vesicle of light-adapted immature (45 mm) S.canicula; gluteraldehyde-osmium fixed, stained by uranyl acetate and lead tartrate. (a only).

a. Low power view of inner nuclear (N) layer. Dark (opaque) nucleated supporting cells (DNSc) bearing microville (mv) at the lumenal (L) surface. A junctional complex (ma) exists between all of the lumenal cells. Below this layer light (translucent) supporting cells are visible (LNSc). x 11,000.

b. Portions of translucent (LNSc) and opaque (DNSc) cells. x11,500.

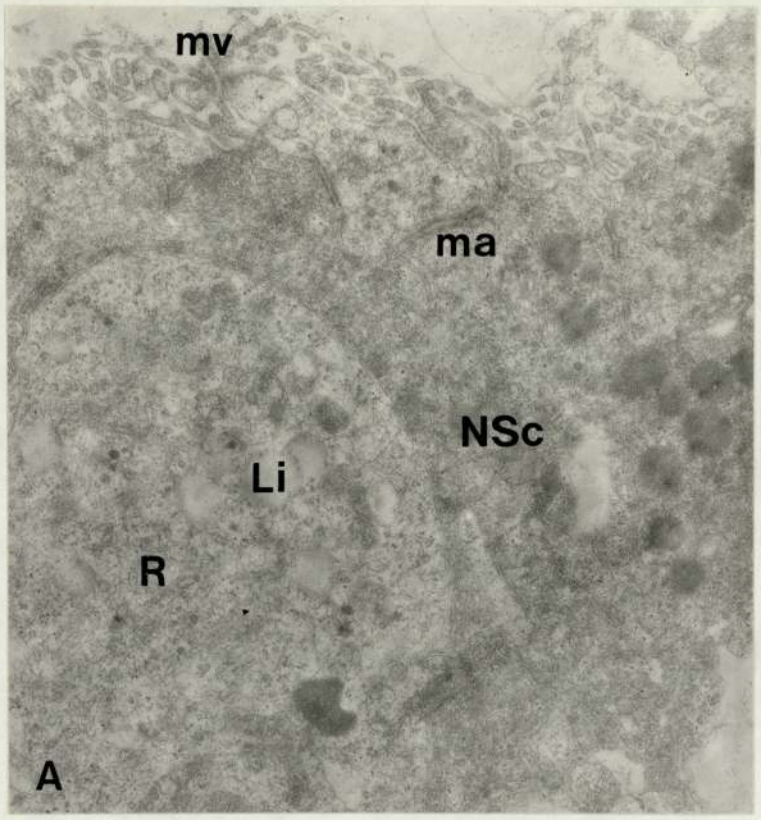

L
mv
N
DNSc
er
LNSc
A

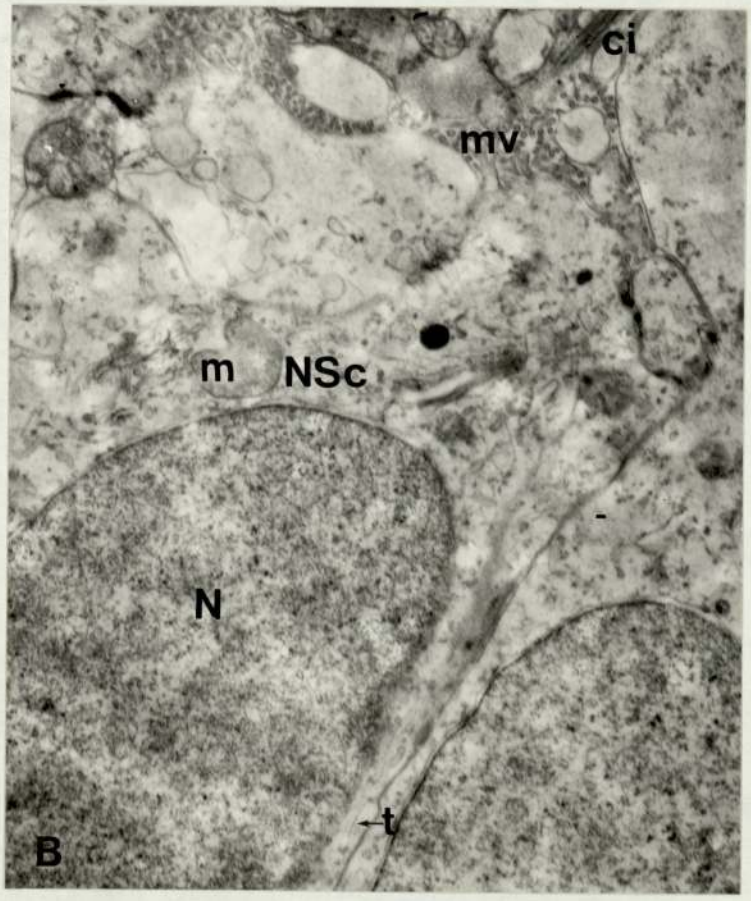

N
LNSc
DNSc
B

Figs. 32a, b. Median sections through the pineal vesicle of light-adapted immature (50 mm) S.canicula; gluteraldehyde-osmium fixed and stained by uranyl acetate.

a. Portion of a receptor cell (R) containing (possibly) lipid droplets (li) surrounded by microvilli (mv) bearing supporting cells (NSc). Junctional complexes (ma) are also visible. x 17,500.

b. Two nucleated (N) supporting cells (NSc) containing mitochondria (m) and microtubules (t) and with microvilli (mv) on their lumenal surfaces. A longitudinally section cilium (ci) is also present. x 11,600.


mv
ma
NSc
Li
R
A

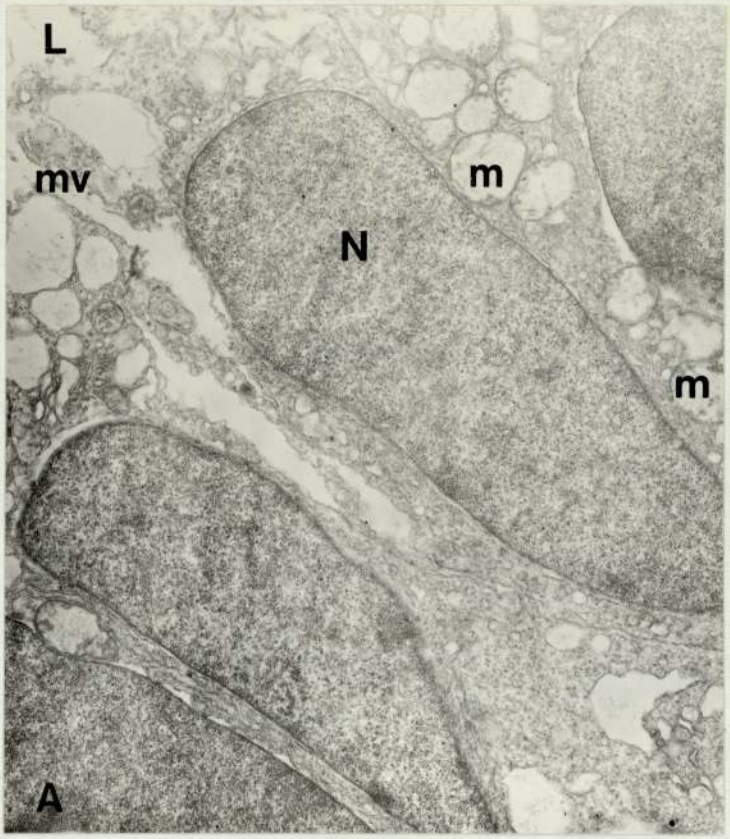

ci
mv
m
NSc
N
t
B

Figs. 33a, b, c. Median sections through pineal vesicle of dark-adapted immature (45 mm) S.canicula; gluteraldehyde-osmium fixed and stained by uranyl acetate.

a. Low power micrograph of a number of supporting cells, whose nuclei have little internal organisation. Numerous enlarged mitochondria (m) are present which lack any internal structure. The lumenal (L) surfaces of these cells bear microvilli (mv). x 11,250.

b. Portion of photoreceptor showing conspicuous structural breakdown, particularly of mitochondria (m). A single degenerative outer segment (OS) is visible, and also a connecting piece. (CP). x 10,000.

c. Portion of supporting cell to show the loss of internal structure in the enlarged mitochondria (m). x 15,000.


L
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m
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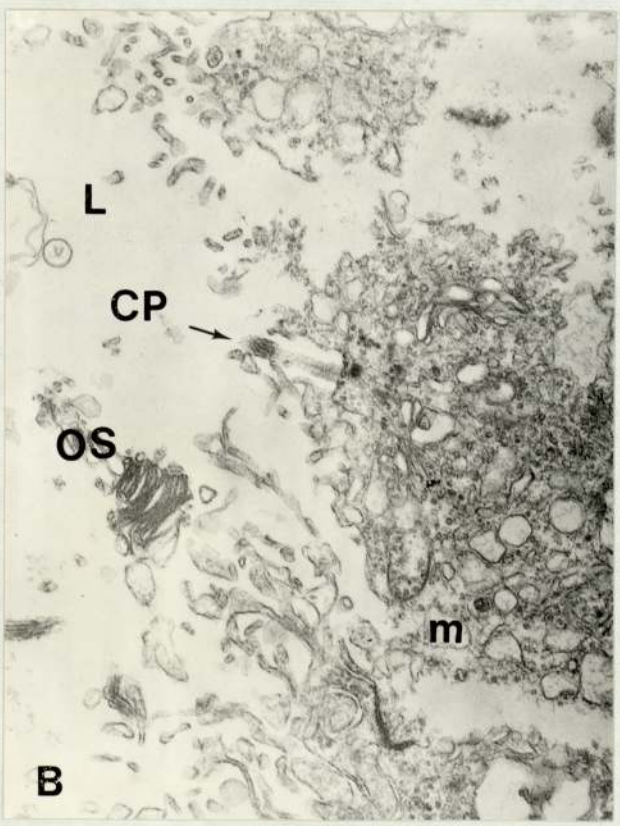
L
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OS
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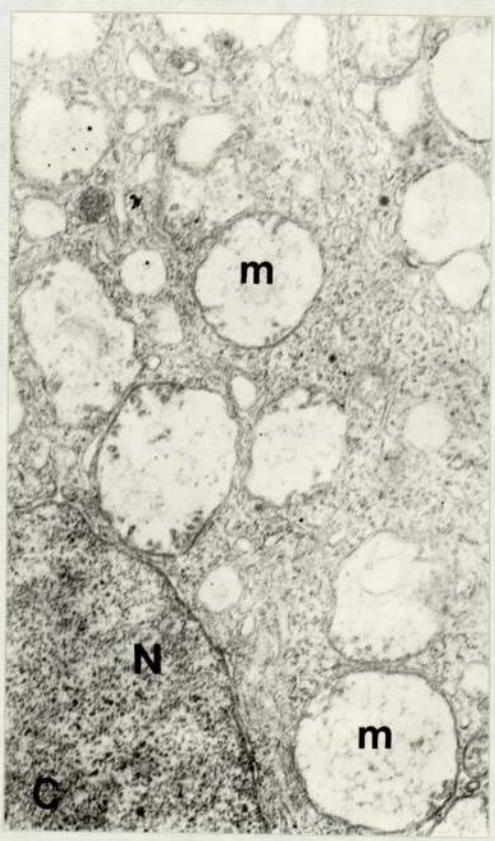

m
N
m
C

4. Assay of Melatonin and Serotonin

4. (a) Chromatographic isolation of Melatonin and Serotonin

Twelve pineals were assayed (in two batches) for melatonin and serotonin. Neither batch yielded a positive reaction, although an extraction check with pure melatonin showed that the procedure was efficient.[1] Also, solutions containing pure serotonin and melatonin were chromatographed and, in the case of melatonin, found to yield Rf values not compatible with those in the literature.

Melatonin: Alkaline system. Rf. 0.90 (0.85)[2]

Acid system. Rf. 0.39 (0.16)[2]

Serotonin: Alkaline system. Rf. 1.0 Acid system. Rf. 0.31

Both solutions gave a reaction typical of indoles, producing dark blue spots when sprayed with Ehlich's reagent, which were intensified by aqua regia. Serotonin yielded a spot darker than that of melatonin, i.e. blue-black as opposed to royal blue.

The solution used in the extraction check yielded a single spot corresponding to Melatonin, the only substance added.

1 - According to Fenwick (1970) the recovery of added melatonin is 75 - 88%.

2 - Rf value for melatonin from Fenwick (1970).

4. (b) Frog skin bioassay of melatonin

A number of tests were run to estimate the efficiency of the method and the apparatus. A field was selected, as described previously (Section II, 4), and a voltage reading taken. The light source was then switched off for one hour, after which time the light source was switched on and a second reading taken. No melatonin was added.

Series I. (No melatonin added).

Initial reading.	Final reading.	Average Difference.
8 - 12 mV.	13 - 17 mV.	5 mV.

It was found that the removal of the light source allowed spontaneous lightening to occur.

The experiment was repeated using selected fields, but again the light source was removed since equal illumination could not be provided over the whole of the five fields chosen.

Series II. (No melatonin added).

Initial reading.	Final reading.	Average Difference.
9 - 13 mV.	13 - 16 mV.	4 mV.

A third series of experiments involved the addition of melatonin (in frog ringer) purely qualitatively at this stage.

Series III. (Melatonin added).

Initial reading.	Final reading.	Average difference.
0 - 2.5mV.	2.5 - 6.5 mV.	3.75 mV.

A final series was carried out, with the addition of melatonin and a constant light source.

Series IV. (Melatonin added - light source continuous).

Initial reading.	Final reading.	Average difference.
9.5 - 11 mV.	11.2 - 12.2 mV.	1.6 mV.

It was decided at this stage, due to the equivocal nature of the results, to abandon this type of assay and replace with a simple Melanocyte Index method.

The darkened melanocytes showed no change when subjected to a constant illumination for a long period of time (up to three hours). It was therefore assumed that any alterations in appearance upon the addition of melatonin would be due to melatonin alone. In a simple quantitative test the addition of trace melatonin (0.001 µg/ml) in Ringer caused complete contraction of the melanocytes. Addition of pineal extract (in Ringer) caused no change in the Melanocyte Index.

It is important to note that the sensitivity of the second bioassay method is such that it will measure at least 0.001 µg/ml of melatonin in a solution. The sensitivity of the TLC method is not known; however, it was employed by Fenwick (1970) to detect melatonin in approximately 1.4 µg quantities. Based upon the results of the most sensitive method, it may be concluded that in the pineal of Scyliorhinus canicula, melatonin was not present in quantities above 0.0001 µg/g of pineal tissue. (Since

the pooled pineals weighed approximately 0.5g). If this is compared to the situation in mammals (0.2 - 0.4 µg/g of tissue) and fish (0.7 µg/g) it seems likely that melatonin is absent from the pineal of S.canicula.

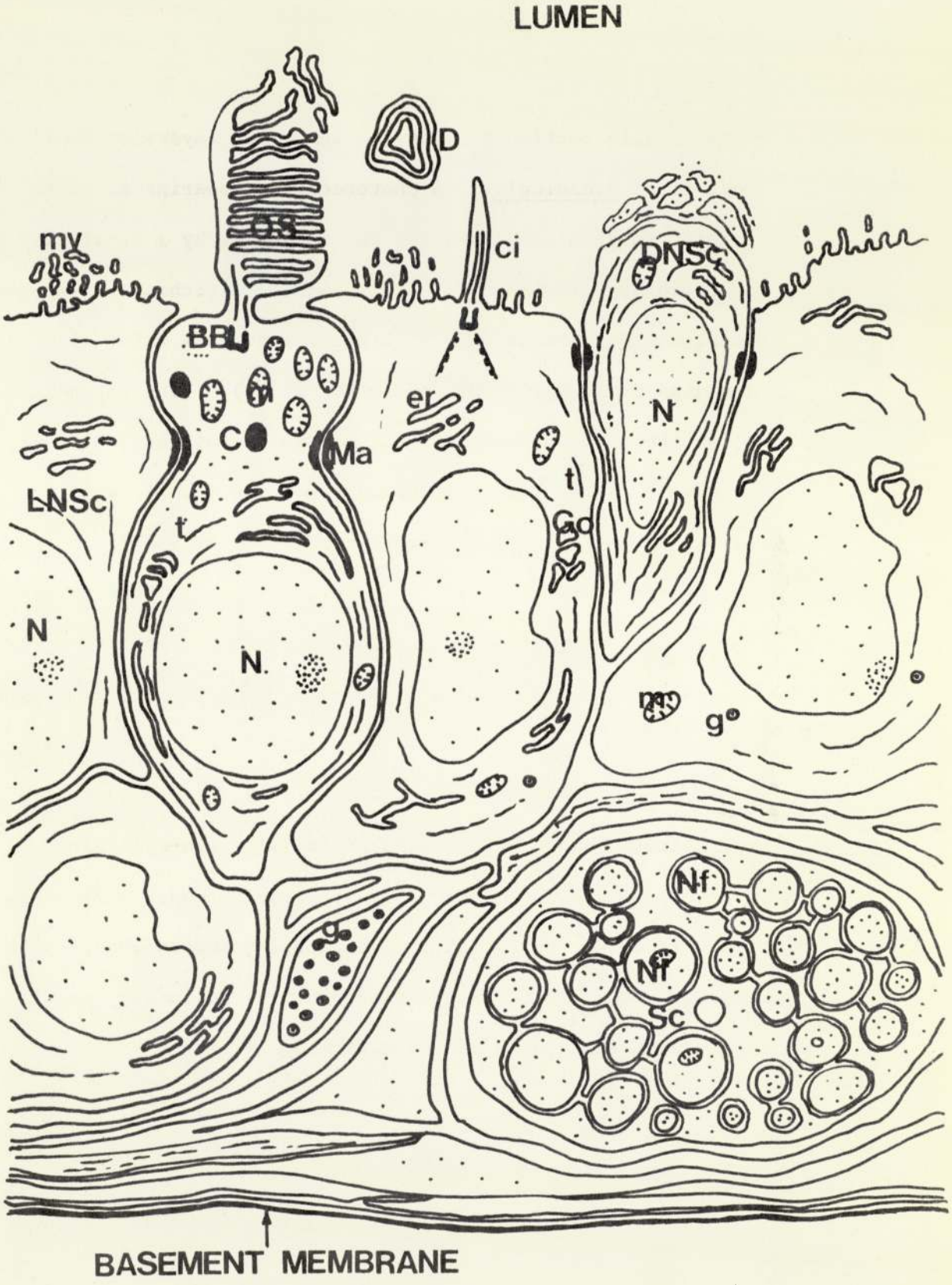
IV. DISCUSSION

The present investigation confirms the observations of such early workers as Balfour (1878) and Sedgewick-Minot (1901) that the epiphysis cerebri of Scyliorhinus canicula arises as a blind ended outgrowth from the roof of the brain. This outgrowth develops into a long stalk with a distinct end-vesicle which maintains a position closely adhered to the underside of the roof of the braincase. This might be considered to represent a position suitable for light reception, although no pigment-free spot exists, as for example, in Xenopus (Van de Kamer et al. 1962) to facilitate this activity. The fact that the pineal lumen is always open and in direct communication with the third ventricle might, however, suggest a different function. It might suggest, as Van de Kamer (1965) proposed, that the pineal is concerned in the secretion of cerebrospinal fluid.

The structure of the pineal of S.canicula, as seen through the optical microscope, does give some support to the last-mentioned theory, since the lumenal cells exhibit processes which pass into the lumen. The latter also contains small quantities of cellular debris. It is interesting that as long ago as 1918, Holmgren hypothesized that these processes might represent the outer segments of photoreceptors. This hypothesis is confirmed by electron microscopy. (See fig. 34).

The presence of photoreceptors in the pineals of both S.canicula and R.clavata is not surprising since they have been discovered in the majority of cold-blooded vertebrates studied so far. These include Lampetra (Collin, 1969), Salmo (Breuker and Horstmann, 1965), Onchorynchus (Hafeez and Ford, 1967), Esox (Owman and Rudeberg, 1970), Mugil, Uranoscopus (Rudeberg, 1966), Thynnus (Murphy, 1971), Phoxinus (Okshe and Kirschstein, 1971), Rana (Kelly and Smith, 1964), Taricha (Hendrickson and Kelly, 1969) Lacerta (Steyn, 1959, 1960; Eakin and Westfall, 1959, 1960), Pseudemys

Fig. 34. Diagrammatic section through the inner two layers of the pineal vesicle of S.canicula. A photoreceptor, bearing an outer segment (OS), attached to the inner segment by a basal body (BB) is visible. Within this cell, numerous mitochondria (m) and cytosomes (C) are present within the cytoplasm which also contains large numbers of microtubules (t). The supporting cells are of two types; most are translucent, having large amounts of pale cytoplasm (LNSc) which contain a poorly developed endoplasmic reticulum (er) and a Golgi body (Go). The second type of supporting cell (DNSc) has little cytoplasm, containing large numbers of microtubules (t). Both types of cell possess microvilli (mv) and cilia (ci) although they are both more easily seen in the first cell type. Between all of the nucleated (N) cells, are found zonula complexes (Ma). The nerve layer consists of a mass of (mainly) non-myelinated nerve fibres (Nf) and glial (supportive) elements (Sc.) Occasionally areas (possibly nerve endings) containing dense-core granules (g) are seen. Similar granules are present in very small numbers in the nucleated supporting cells.


LUMEN
D
mv
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er
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t
LNSc
t
Go
N
N
g
Nf
Nf
Sc
BASEMENT MEMBRANE

fig.34.

(Vivien-Roels, 1969, 1970), Anguis, Iguana (Okshe and Kirschstein, 1968). Photoreceptors have also been shown to exist in the Avian genus, Passer (Okshe and Vaupel-von Harnack, 1965).

It is generally held that the pineal photoreceptors found in all of the aforementioned species may be classified as cone-like (Okshe, 1971); Collin, 1971). In all cases the saccules of the outer segment communicate with the extracellular space and display a distinct lumen in both marginal and central parts unlike rod saccules which have very limited lumeni restricted to the periphery (Cohen, 1968). The photoreceptors of Scyliorhinus exhibit identical characteristics to those of the other species and, therefore, they too have been classified as cone-like.

The outer segments are frequently described in the literature as "well-developed" (Rudeberg, 1969). This does not seem to be true of S.canicula or R.clavata as examined here. In the former, very few outer segments appear to be fully developed, most are broken down and exhibit considerable vesiculation. In the latter, no outer segments appear complete. In spite of the description "well-developed", the majority of published micrographs illustrate outer segments that show at least some degree of disintegration, and it is hypothesized that a fixation artefact might be responsible. (Rudeberg, 1969; Okshe and Kirschstein, 1968). The parietal outer segments of Anguis are highly vesiculated following gluteraldehyde fixation after four to six days in darkness (Petit, 1968). On the other hand, Rohlich and Tar (1968) found that after prolonged dark treatment (one to three weeks) the photoreceptors of planarians exhibited no disintegration when pre-fixed with gluteraldehyde but showed a clear breakdown after treatment with osmium tetroxide alone. This result was thought to be produced by a membrane weakness induced by light deprivation.

It is interesting that a similar pattern of outer segment disintegration in S.canicula was observed in summer and winter specimens (adults)

and light and dark treated larvae. In addition, retinae from the lateral eyes fixed in precisely the same manner exhibited no such artefact. (Smith, 1970).

The nature of the outer segments in S.canicula, R.clavata and the other species quoted must have some physiological significance. It is inconceivable that fixation artefacts are to be found in every experimental procedure carried out on all of the genera mentioned.

One other point is important in this discussion. In all those species which have been studied, although little quantitative data is provided it would seem that these photoreceptors constitute not more than about twenty per cent of the total number of lumenal cells.

It was thought that the number of photoreceptors diminished as the animal grew. For example, Kelly (1965) showed that in Taricha the number of photoreceptors decreased markedly following metamorphosis. This is now known not to be true, Kelly and his co-workers (1971) have shown that, contrary to their original belief, the number of cells remains constant (14 - 18%) in adult life.

Recent evidence has also been put forward to explain the patterns of degeneration seen (at least) in Amphibian pineal receptors. It has been shown that like Amphibian retinal outer segments (Young and Droz, 1968), those of the pineal also exhibit a regeneration cycle, the waste products being removed by macrophages and/or the supportive elements (Kelly, 1971).

It is interesting to consider the place of Selachian and Batoidean photoreceptors in an evolutionary sequence. (Collin (1971) has recently put forward such a phylogenetic sequence. (See fig. 35). He believes that the true photoreceptor cell of the Petromyzontidae, fishes and amphibia has gradually become extinct through the Sauropsida. They are still present in small numbers in lacertilians and chelonians but are absent in birds. In lacertilians, chelonians and birds rudimentary photoreceptors, however, are present. The latter have the basic

Photoreceptor organisation but lack distinct outer segments, instead having a rudimentary outer segment completely lacking cone sacs. Possibly this cell type is also present in ophidians, mammals, amphibia and fishes. Pinealocytes are the predominant elements of ophidian and mammalian pineals but their presence is suspected in chelonians, lacertilians and birds.

It is interesting that the pineal complexes of amphibians and lacertilians which by their position and gross morphology are generally regarded as being well-suited for photoreception should possess rudimentary photoreceptors, thereby indicating a (possible) regression in this function. No rudimentary photoreceptors of the type described by Collin (1971) were observed in Scyliorhinus or Raja but the degenerative appearance of the outer segments might indicate some relationship with the former structures. In some cases quite large vesicles were seen which bore little resemblance to cone-sacs.

It may be, therefore, that the Cyclostomes represent the apex in the evolution of photoreceptive pineals, and that this function is gradually transmuted through the fish, amphibia, reptiles and birds into a purely glandular function in the mammals. If this were true, on strictly anatomical grounds the photoreceptors of fish might be expected to exhibit some degree of degeneration. Even in the Cyclostomes there is never any indication of a pineal retina, like that of the lateral eyes.

The actual structure of the photoreceptor, as presented here, largely confirms the observations of Rudeberg (1969) on S.caniculi. He found that the inner segment consisted of two regions, the outer bulbous portion containing numerous mitochondria but few cytosomes. The evidence presented here indicates that large numbers of cytosomes may be present. The basal body of the outer segment did not possess (according to Rudeberg) any rootlet structures. This is confirmed, although it must be emphasised that striated rootlets belonging to the basal process of a supporting cell cilium were only observed on one occasion. It is therefore not impossible that

Fig. 35. Transformation of the pineal sensory cell line.

	Cell Type	Cell Function	Origin.
(1)	Photoreceptor.	Exclusively photoreceptive.	All Anamniota.
(2)	Rudimentary photoreceptor.	Photoreceptive and secretory.	Chelonia (1^{o}), Lacertilia (1^{o}) Aves (1^{o}), Ophidia? and Mammalia?
(3)	Pinealocyte.	Secretory only.	Mammalia (1^{o}), Ophidia (1^{o}), Chelonia? Lacertilia? Aves?

they exist in the receptor cell. Indeed, they have been described in the receptors of the lizard Anguis (Petit, 1968) and the lamprey, Lampetra (Collin, 1971). Microtubules and granules are also present, although the identification of the latter as glycogen is not certain. Optical techniques reveal that glycogen is present but apparently in the distal extremities of the lumenal cells. (The outer segments?). The electron microscope reveals no special lumenal areas for glycogen storage.

The synaptic vesicles in the basal process of the receptor cell, described by Rudeberg, were not observed. Indeed, it is virtually impossible to trace the route of this basal process when it approaches the nerve layer. Logic would suggest that some synaptic contact between the photoreceptors and the nerve layer must exist. It is very puzzling that this synaptic contact is so difficult to discover. It may be that the synapsing between the photoreceptors and nerve elements is not morphologically distinguishable. This, it must be said, is in contrast to the situation in the Cyclostomes, whose receptor cells possess a distinct synaptic pedicle. The latter ramifies into several branches. These branches contain numerous synaptic vesicles which are (fairly) evenly distributed throughout. There is some accumulation near to synaptic ribbons which themselves are confined to an area very close to the presynaptic membrane. The synaptic pedicle may be in contact with several dendritic processes originating from ganglion cells; alternatively, several pedicles may connect with a single dendritic process. (Collin, 1971).

The complex structural organisation of synapses observed in the Cyclostomes is shared by many Anamniotic species. Synaptic structures have been observed in amphibians (Kelly, 1965) and teleosts (Okshe and Kirschstein, 1971). Histochemical techniques have revealed acetylcholinesterase activity in the nerve cells of amphibian pineals, but no such activity in the terminals of pineal receptors. (Okshe, 1971).

The present investigation was unable to detect any acetylcholinesterase

activity at all in the pineal vesicle of S.caniculi.

A great deal of work has recently been carried out to investigate the pineal neurons, particularly in Rana (reported by Okshe, 1971). These investigations have been performed using, for example, Golgi-Colonnier stain and intravital methylene blue. There is little doubt that the pineal of R.esculenta contains long stellate neurons which contribute to the formation of the pineal tract. Also there are small bipolar neurons in a chain-like arrangement with the large neurons. There is good correlation between the number of large stellate neurons and the myelinated fibres of the pineal tract (60 - 80). This is an interesting observation, for in the pineal of Scyliorhinus only one or two myelinated fibres were observed. It may be, then, that the number of large (stellate) neurons would correspondingly be very small. Although no quantitative estimation of nerve fibres was attempted in this work, the ratio of non-myelinated to myelinated would almost certainly be of the order of several hundreds to one. The small number of myelinated neurons present is also emphasised by the fact that Rudeberg (1969) was unable to find any such fibres in his preparations. It is interesting to note that Dodt (1971) believes that the electrophysiological activity of the pineal (see below) requires a much greater number of myelinated fibres than was found in the present investigation. Okshe (1971), on the other hand, hypothesises that the ratio of myelinated to unmyelinated fibres is probably not important.

No attempt to determine the destination of the pineal nerve fibres of S.canicula or R.clavata was made during the present investigation. However, it is interesting to note the findings of other workers. Rudeberg (1969) found only one ganglion cell in his preparations, located in the pineal stalk. It would appear, then, that nerve cell bodies do not exist in the pineal vesicle but are confined to the stalk region, probably near to the proximal end. Kappers (1965) has reviewed the literature relevant to the innervation of the pineal in all vertebrates. He points out that

in Squalus bipolar cells are found in the stalk and proximal parts of the organ. Some of the fibres from cell bodies in this region run to the habenular commissure, some may terminate here, while others pass to join the bulk of pineal fubres. In the proximal part of the pineal, the tract divides into two branches which pass in a latero-caudal direction to the mesencephalic ventricular wall. Other fibres, at the caudal border of the proximal region of the epiphysis, branch into two bundles sending one to the left, the other to the right. Some fibres within these bundles later cross over to the opposite side. Many fibres then course into a ventro-caudal direction, while others run to the optic tectum and posterior commissure. Of those fibres which are running ventro-caudally, some go into the dorsal thalamus, while most pass away in the direction of the mesencephalic tegmentum. It is quite probable that a similar route would be followed in other Selachians and therefore in Scyliorhinus. The destination of the nerve tract in Raja would be less certain.

Several electrophysiological investigations have been carried out on the pineals of various vertebrates. These include frogs (Dodt, 1964; Dodt and Heerd, 1962; Hamasaki, 1970), lizards (Hamasaki and Dodt, 1969), teleosts (Dodt, 1963) and more recently the dogfish S.canicula. (Hamasaki, 1970b; Hamasaki and Streck, 1971). The results obtained from the last-mentioned are worthy of close examination.

Hamasaki and Streck (1971) found that the epiphysis of S.canicula is an extremely sensitive light receptor, and that a one-second flash of 4×10^{-4} 1 m/m^2 is sufficient to alter the normal neural activity. This figure for light intensity does not take into account the skin and skull, so that the stimulus on the epiphysis itself is probably of the order 4×10^{-6} 1 m/m^2. They thought that the slow potentials obtained from the epiphysis arose from ganglion cells and represented summated inhibitory post-synaptic potentials spread electronically to the electrode. They suggested that the ganglion cells must have membrane potentials set very

close to the critical level, so that continuous firing would occur. Stimulation of the epiphysis then inhibits this firing.

The spectral sensitivity of both the epiphysis and the lateral eyes exhibits a peak at 500 nm. This is the λmax for rhodopsin. It has already been shown, however, that the photoreceptors have a cone-like morphology. There thus seems to be a conflict between photochemistry and ultrastucture; the first indicating the presence of rods, the second that of cones.

Although the electrophysiological results of Hamasaki and Streck (1971) are interesting they give little insight into the function of the pineal of S.canicula in its normal environment. The results do, however, show that the pineal is very sensitive to light; this might be significant in bottom dwelling fish. (i.e. the sensitivity is 4×10^{-4} 1 m/m^2; full moonlight would, at the surface of the water, give a luminescence of 2×10^{-2} 1 m/m^2). The physiological mechanism proposed by Hamasaki and Streck (1971) depends on the presence of significant numbers of ganglion cells (see below). The presence of such cells has not, however, been detected in the present investigation, certainly in the pineal vesicle although, as was stated earlier, it is possible that ganglion cells occur at the base of the stalk. The apparent lack of distinct synaptic structures is an additional difficulty.

That the electrophysiological results in the various vertebrates are due to the presence of photoreceptors is of little doubt, since these structures seem to be a prerequisite for this type of response. For example, in Passer which has only rudimentary outer segments (Okshe and Vaupel von Harnack, 1965), no such sensitivity is demonstrated. (Ralph and Dawson, 1968). This is also confirmed in frog tadpoles, where the first electroretinographic response to light corresponds in time with the first appearance of retinal outer segments (as revealed by the electron microscope). (Nilsson and Crescitelli, 1970).

Recently, Dodt (1971) has reviewed the literature relating to the

electrophysiology of the pineal body. He points out that it seems necessary to believe that the propagation of impulses in the pineal must differ significantly from that in the lateral eyes. This is self-evident since most of the neural structures of the lateral eye retina have never been found in either the parietal eye or the epiphysis cerebri. In spite of this, the electrical activity in many pineals is very complicated; more so than in Scyliorhinus.

In many experiments, a chromatic and an achromatic response has been obtained from the pineal (e.g. frog by Hamasaki, 1970a). The achromatic response is probably produced by the inhibitory effect of photoreceptors on second-order neurons. (This is the type of response exhibited by S.caniculi). In the chromatic response, two types of sensory cells may be present, one absorbing in the ultraviolet or blue, the other in the green. Hamasaki (1970a) believes that the chromatic response in the frog is due to the two components synapsing with a common ganglion cell. One of the components is excitatory (λmax 515 nm), the other inhibitory (λmax 355nm). The latter has a significantly shorter latent period than the excitatory component, and this is thought to be due to the fact that these photoreceptors synapse directly on to the soma of the ganglion cell. The photoreceptor producing ganglionic excitation would therefore synapse with a dendite. It is unfortunate that only axo-dendritic synapses have been observed. (Okshe, 1971).

Dodt (1971) postulates that achromatic responses may be obtained from myelinated fibres, and chromatic responses only from non-myelinated fibres. This hypothesis, however, does not explain the electrophysiological results from Scyliorhinus, since an achromatic response is produced by an epiphysis containing almost exclusively non-myelinated fibres.

It must be concluded that the ultrastructural organisation of the pineal of S.canicula does not form a basis for clear interpretation of electrophysiological phenomena.

Supporting or ependymal cells have been reported in all species possessing pineal photoreceptors (see above). These cells always bear microvilli, and have a well-developed endoplasmic reticulum and Golgi apparatus. Rudeberg (1969) hypothesised that they are important as a pathway for nutrition and waste products for the photoreceptors, since they always separate the latter from the basement membrane and capillary complex. This hypothesis would seem reasonable based upon the observations presented here. Functionally they may be compared to the pigment cells of the lateral retina.

Two morphologically distinct cells were found in these investigations, both of which were classified as supporting cells. The principle difference between them is the relative abundance and density of cytoplasm; the latter is due to the number of microtubules present. It is interesting that the opaque supporting cell has little cytoplasm, around the nucleus and large numbers of microtubules, like the inner segments of photoreceptors. It is possible that the opaque supporting cell, then, represents a stage in photoreceptor development or degeneration. The presence of cilia in the supporting cells generally might also be taken as evidence that the link between supporting cells and photoreceptors is closer than has previously been held. It is unlikely that the opaque cells represent a fixation artefact since in the larva they are present in large numbers and definitely possess microvilli.

The characteristics of the nuclei of the supporting cells seem to vary. In the adult the nucleus appeared to exhibit a homogenous composition, while in the light adapted larva a distinct pattern of chromatin was observed. This type of pattern was described by Rudeberg (1969) in the adult. No explanation can be offered for this phenomenon. The large, dense core granules (1500-3,000 A) described by Rudeberg were not found in any of the specimens investigated in this work, although much smaller dense core granules were observed on several occasions. (See below).

The possession of microvilli by a cell often indicates that it is involved in either secretion or absorption, (Fawcett, 1966) and it has long been thought that the pineal ependymal cells might be involved in one of these functions. However, the electron microscope reveals no morphological evidence of secretory (or absorptive) activity. Having said that, it seems unlikely that the rôle of the supporting cell is exclusively subservient to the photoreceptors, since in S.caniculi the latter form probably less than 10% of the overall number of nucleated cells.

The cytoplasm of the supporting cell is occasionally seen to contain dense-core granules (900 - 1400 A) morphologically identical to those seen in the nerve layer. Although it is unlikely, it cannot be excluded that those pockets in the nerve layer which contain these granules represent processes of supporting rather than nerve cells. As has been noted previously, it is extremely difficult to trace the basal processes of the nuclear elements. However, in no supporting cell body has a high density of dense-core granules been observed.

The identity of these granules remains a mystery, although several possibilities exist. Collin (1971) describes morphologically identical granules in the photoreceptors of Lampetra planeri; they are present in small numbers in the region of the Golgi Body and the synaptic pedicles. In the pedicles all grades of intermediate form are found between dense granules and clear vesicles. A release mechanism is therefore hypothesised. The nature of these granules is unknown although an indoleamine may be present. Similar granules, thought to contain 5-HT, are found in the rudimentary photoreceptor cells of Lacerta (Collin, 1971). An alternative hypothesis is that these granules contain nor-epinephrine.

Halaris et al. (1967) showed that granules of this type in the pineal organ are reduced by specific agents known to deplete nor-epinephrine. It would be interesting if the ependymal cells were to secrete nor-epinephrine since it would imply that they could pass impulses to other pineal elements.

The presence of granules in nerve endings (for that is where they are most likely situated) implies that efferent fibres must be present. Collin (1971) has proposed that in lizards, efferent autonomic nerve fibres approach the photoreceptors of the pineal, close to their synaptic junctions with the ganglion cells. The presence of efferent fibres in Scyliorhinus, therefore, must not be excluded.

Biochemically, the pineal of S.canicula appears to exhibit little activity. Simple enzymic estimations for acetylcholinesterase and alkaline phosphatase were unable to detect the presence of either of these compounds. More significantly, melatonin and serotonin are also undetectable.

Melatonin has been found in a number of Anamniotic vertebrates, including amphibians (Van der Veerdonk, 1967) and fishes (Fenwick, 1970); serotonin has also been demonstrated in the latter group (Owman and Rudeberg, 1970). HIOMT, the enzyme involved in melatonin formation has been identified in fish, amphibians and reptiles. (Quay, 1965b).

The techniques used for melatonin estimation are noted for their sensitivity, the chromatographic assay having been used by Fenwick (1970) to demonstrate melatonin in the pineal of the salmon Onchorhynchus. The bioassay techniques attempted have been reported in the literature as the most sensitive available (Lerner and Wright, 1960; Mori and Lerner, 1960). However, recent experiments by Hadley and Bagnara (1969) have cast doubts upon the validity of the type of assay which employs electrical measuring devices. This is mainly due to the differing responses of several types of melanophore existing in the frog skin. Secondly, in the experiments carried out in this investigation, caffeine darkened skin was found to lighten spontaneously when the light source was removed. For these reasons a simple Melanocyte Index was used to investigate the presence of melatonin. Since no positive results were obtained (by either method) it must be concluded that melatonin is absent, or present in such minute quantities as to be undetectable in the pineal of S.canicula. (i.e. less

than 0.0001 µg/g).

From the above observations and experiments it appears that the pineal of Scyliorhinus is exclusively a photoreceptor and has no endocrine function. This is rather puzzling since the epiphysis of at least one other fish (Onchorhynchus), which is structurally similar, is known to contain melatonin, while others contain its precursor, serotonin.

The experiments carried out on immature specimens showed that constant darkness produces a spectacular change in the epithelium lining the lumen of the pineal. Primarily there is a large increase in the number of mitochondria; however, these mitochondria are not normal but are very large (0.7 - 1.0 µm) and contain very few tubular cristae together with a matrix of pale flacculent material. This result is precisely that obtained by Halaris (1969) for the effect of light on male rats. In the minnow Gambusia affinis, Chéze and Lahaye (1969) found that twenty days of constant light induced a marked increase in vacuolated cells, in aposecretion and also in a marked melanogenesis. In the characin Astyanax mexicanus, both darkness and light induced variable proliferation of the pineal. The lumen increased in width and the epithelium became more disorganised. (Grunewald-Lœwenstein, 1956).

At first sight, it is difficult to see a common pattern between the results obtained by other workers when compared to the findings presented here. However, a close examination reveals that the results of the only other ultrastructural investigation (Halaris, 1969) can be related to the responses observed in Scyliorhinus.

The mammalian pineal is inervated by a tract which runs from the lateral eyes. The effect of light on the lateral eyes is to set up a stream of impulses in the pineal tract which inhibits the activity of the pineal, in that melatonin output ceases. The selachian pineal is stilulated directly by light and the photoreceptors set up impulses which inhibit the activity of the ganglion cells. (Hamasaki and Streck, 1971).

Thus the pineal epithelium is stimulated by light.

It would seem, then, that the effect of prolonged light on the cells of the mammalian pineal would be identical to the effect of prolonged darkness on the selachian pineal. Thus hypothesis is supported by the fact that in both situations the respective pineals exhibit numerous enlarged mitochondria which lack cristae.

It is, perhaps, unfortunate that the explanation offered by Halaris (1969) will not hold for Scyliorhinus. He supposed that the build-up of serotonin (due to the inactivation of HIOMT) under light conditions was responsible for the effects observed in mammals. Since serotonin appears to be absent in Scyliorhinus, this theory is not tenable, unless it is assumed that serotonin is normally present only in undetectably small quantities, only building up to significant levels under conditions of prolonged darkness. The latter cannot be excluded since the methods used to examine the presence of serotonin in this investigation are generally less sensitive than those used for melatonin (e.g. frog skin assays could not be used). Alternatively, another chemical substance entirely may be responsible for the observed effects.

The pineal of Scyliorhinus (and Raja) is seen as a poorly developed photoreceptive structure, apparently without a secretory rôle. The latter may be linked to the presence of a blood-brain barrier which is particularly well-developed in this region since no interruptions in the basement membrane between the meninx primitiva and nerve layer were ever observed. It possesses only a few well-developed photoreceptor cells which apparently fail to synapse with the numerous non-myelinated and few myelinated fibres which exist in the organ. Indeed, it may be, as Okshe (1971) suggests, that the processes of the photoreceptors actually form a part of the tract. The origin of the nerve fibres remains a mystery, although investigation of the proximal portion of the stalk might prove fruitful in this context.

Phylogenetically, the Chondrichthean pineal seems to represent a level of organisation between one which is purely photoreceptive (Cyclostomes) and one which shows the rudiments of secretory activity (Teleosts, Amphibians, etc.). The organisation of the photoreceptor elements bears this out, for while no rudimentary receptors of the type described by Collin (1971) exist, those cells that are present exhibit significant degeneration. It would seem a valid hypothesis that these cells fit into Collins' cell line theory (see fig. 36) between photoreceptors proper and rudimentary photoreceptors. They are, perhaps, losing photoreceptive ability but have gained no synthetic ability, certainly not with respect to melatonin, nor probably serotonin. The existence of efferent fibres in the organ may be regarded as a corollary to this since they would probably not be present in a purely photoreceptive pineal, but might be expected to be found in one which was changing into a secretory structure.

The rôle of the pineal in the normal life pattern of Scyliorhinus cannot be elucidated using the evidence presented here. It must be noted, however, that a survey of the literature with respect to this and all other genera shows that at no time has a function been firmly ascribed to the pineal. As the number of morphological and biochemical investigations continues to grow, perhaps a functional pattern will begin to emerge.

Fig. 36. Transformation of the pineal sensory cell line.

	Cell Type	Cell Function	Origin
(1)	Photoreceptor.	Exclusively photoreception.	All Anamniota.
(2)	Degenerate photoreceptor.	Exclusively photoreception.	Scyliorhinus (1^{o}) and probably most Anamniota.
(3)	Rudimentary photoreceptor.	Photoreceptive and secretory.	Chelonia (1^{o}), Lacertilia (1^{o}), Aves (1^{o}), Ophidia ? and Mammalia ?
(4)	Pinealocyte.	Secretory only.	Mammalia (1^{o}), Ophidia (1^{o}), Chelonia ? Lacertilia ? Aves ?

(1) → (4) Transfer of stimulation from direct photic to nervous, and therefore a loss of sensory nerve activity. An increase in photoneuroendocrine function as light stimulus is relayed from the lateral eyes.

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APPENDIX I

Key to abbreviations used in the text figures.

1 - Nuclear layer

2 - Fibrous (nerve) layer. (N.L.)

3 - Meninx primitiva. (Co).

3v - Third Ventricle

a - Oval nuclei

Ax - Axon

B - Braincase

BB - Basal body

b - Irregular nuclei

C - Cytosome

C - Capillary - light micrographs only (Cap)

CP - Connecting piece

CP - Cytoplasmic process - light micrographs only

CoC - Fibroblast

ci - Cilium

D - Debris

Dc - Dense cytoplasm

DNSc - Dense nucleated support cell

E - Epidermis

Er - Erythrocyte

End - Endothelium

er - Endoplasmic reticulum

f - Fibril (Fibre)

Go - Golgi body

g - granule

HC - Habenular commissure

L - Lumen

LNSc - Light nucleated support cell

Li - Lipid droplet

Lm - Lamella(e)

Ma - Macula adherens

m - Mitochondrion

mv - Microvilli

N - Nucleus

NSc - Nucleated support cell

Ne - Nerve ending

Nf - Nerve fibre

n - Nucleus

OS - Outer segment

OT - Optic tectum

PC - Posterior commissure

Pv - Pinocytic vesicle

R - Photoreceptor

S - Stalk

Sc - Supporting cell (nerve layer)

t - Microtubule

V - Pineal vesicle

VT - Velum transversum

v - Vesicle

APPENDIX II.

Evolution of the Fishes