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CYTOLOGICAL AND CYTOCHEMICAL STUDIES ON THE FRONTAL AND PINEAL ORGANS OF THE TREEFROG, *HYLA REGILLA**

By

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With 16 Figures in the Text

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This study on the amphibian frontal organ (stirnorgan) and pineal organ (epiphysis) is similar to that made recently by us (EAKIN, QUAY and WESTFALL 1961) on the parietal eye of the lizard, *Sceloporus occidentalis*. The information here presented on the fine structure, glycogen and indole content of these diencephalic derivatives will supplement the cytological studies with light microscopy made by workers in the early part of this century (cf. BARGMANN 1943) and by the recent resurgence of research on the pineal complex of vertebrates. A brief review of the relevant literature may be found in a paper by KELLY and VAN DE KAMER (1960).

Material and Methods

Tadpoles reared from eggs of the Pacific Treefrog, *Hyla regilla*, collected near Berkeley, were used in this investigation when they reached stage I of development (TAYLOR and KOLLROS 1946). In experiments designed to test the sensitivity of the frontal and pineal organs to light, two lots, each of ten animals of uniform size, were placed in dishes filled with pond water. One (experimental) was exposed to continuous illumination by a water-filtered beam of light (approximately 1000 foot candles) from a 6.5 volt miniature lamp; the other (control) was enclosed in a light-proof maze of black paper boxes, arranged to permit circulation of air. Both preparations were placed for 48 hours in a constant temperature cabinet set at 20° C. At the end of this time the animals were fixed in 10% neutral (sodium phosphate) buffered formalin for 24 hours or in 1% OsO_4, buffered to pH 7.3 with veronal acetate, at 0° C for 3 hours. Light-adapted tadpoles were fixed in bright light, the dark-adapted specimens in a room faintly illuminated with a deep red light. For details on procedures used for electron microscopy see EAKIN and WESTFALL (1961) and WESTFALL and HEALY (1962).

Forty experimental and 40 control animals fixed in formalin were embedded in paraffin, sectioned at 8 μ, and stained by the periodic acid Schiff (PAS) technique (LILLIE 1954). The tissues were oxidized for 10 minutes followed by a 15-minute treatment with Schiff reagent. In a few instances the sections of a frontal or pineal organ were mounted on two slides, one of which was incubated $1^1/_2$ to 4 hours at 37° C in 0.1% alpha amylase in 0.01 M NaCl, and the other one in 0.01 M NaCl alone, before both were collodionized, oxidized and treated with leucofuchsin. Slides of control and experimental organs were stained simultaneously.

For better fixation with OsO_4 some animals were first anesthetized by adding a few drops of dilute ether to the operating dish, after which the skin was excised from the dorsum of the head thereby exposing the frontal organ and the meninges above the epiphysis. The tadpoles were then dropped into the fixative. The frontal organ and the diencephalic roof were dissected out of the specimens while in 70% ethanol, and from the latter piece the epiphysis was isolated while in Epon.

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To test for the presence of certain indole compounds in the frontal and pineal organs of larvae of *Hyla regilla*, tadpoles in various stages of metamorphosis were frozen with dry ice; the skin was removed from the dorsum of the heads as the animals thawed; and the stirnorgan and epiphysis were extirpated along with a small piece of attached meninges. From 10—30 organs of each kind were collected at any given time, placed separately in vials each containing 0.3 ml of 0.2 N HCl and 0.5% ascorbic acid, and stored at —80° C between samplings. Several batches of 50 to 100 frontal organs and one of 70 pineal organs were tested for indoles and 5-hydroxy + 5-methoxy indoles with an Aminco-Bowman spectrophotofluorometer. After homogenization and centrifugation, aliquots of 250 λ volume were placed in quartz cuvettes and fluorescence and excitation spectra were recorded for both

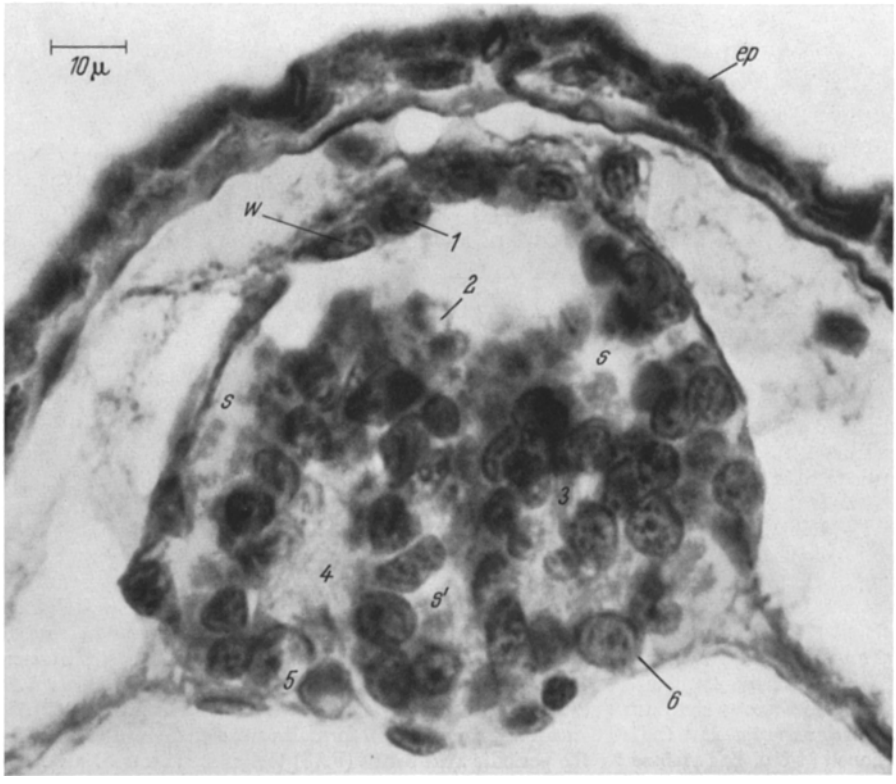

Fig. 1. Cross section of the stirnorgan of a young tadpole (stage I of TAYLOR and KOLLROS 1946). *ep* epidermis; *s* lumen of organ; *s'* part of median arm of lumen; *w* outer wall; *1*—*6* positions of electron micrographs (Figs. 2—7).

the original solutions (slightly diluted, = 0.1 N HCl for indoles) and after the addition of 75 λ concentrated HCl (= 3.0 N HCl for 5-hydroxy and 5-methoxy indoles).

Observations

Frontal Organ

In the young tadpole of *Hyla regilla* the stirnorgan is clearly visible through the transparent integument as a spherical body resting upon the melanophore-rich meninges above the telencephalon (EAKIN and WESTFALL 1961). In cross section one observes that the cavity of the frontal organ is roughly M-shaped. Here and there, however, the walls of the organ are so closely apposed that the

lumen is occluded. In Fig. 1 the arm of the cavity (*s*) to the reader's left is completely open, but that to the right is closed ventrally; the dorsal part of the median arm is obliterated whereas the ventral part appears as a pocket (*s'*). The outer wall (*w*) of the organ is a single layer of cells; the inner masses of cells, one on

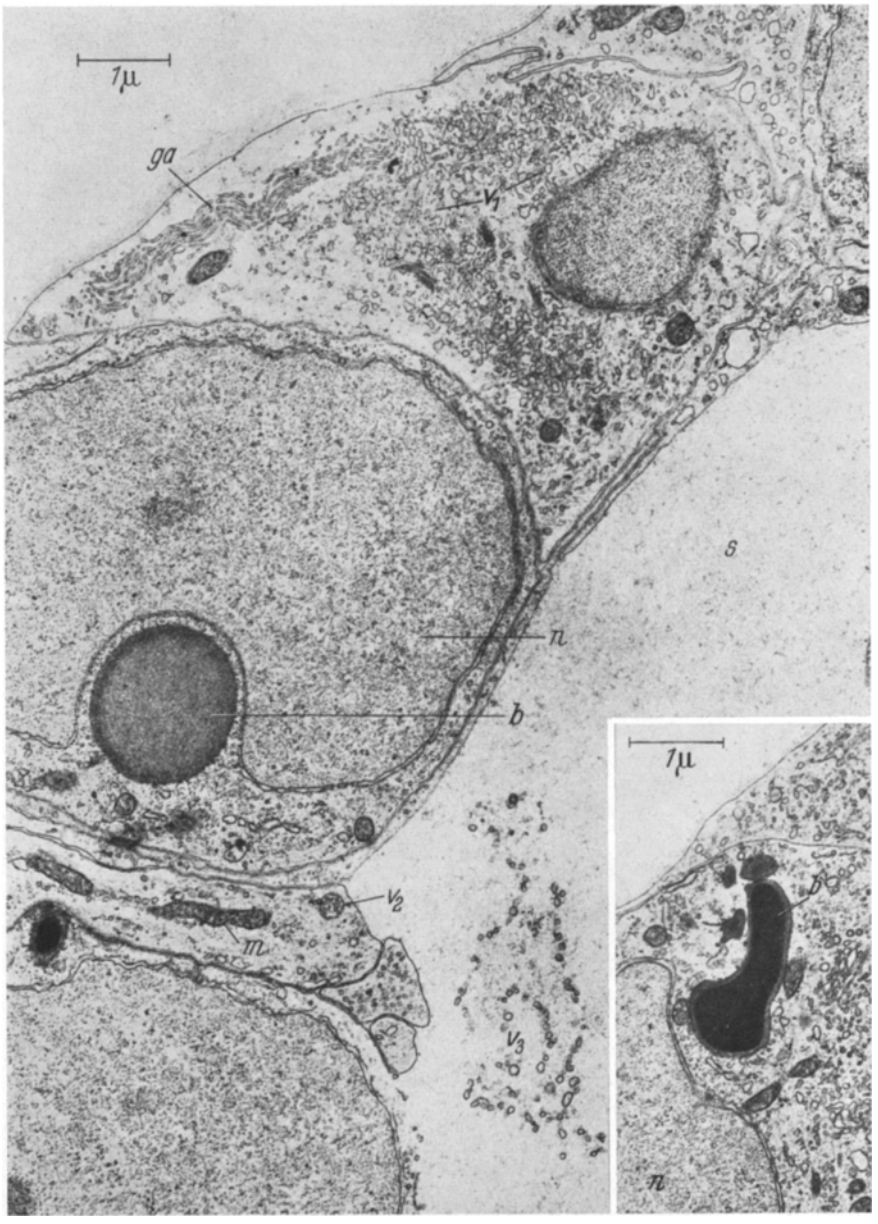

Fig. 2 and insert. Parts of several cells from dorso-lateral wall of light-adapted stirnorgan (position 1, Fig. 1). *b* electron opaque body; *ga* smooth endoplasmic reticulum (Golgi apparatus ?); *m* mitochondria; *n* nucleus; *s* lumen of organ; v_1 small vesicles; v_2 larger vesicles with vesicular inclusions; v_3 vesicles in lumen

each side of the median arm of the lumen, are composed of three cell types: receptors, supportive elements, and ganglion cells (HOLMGREN 1918, OKSCHE 1952). Reference will be made to the numbers on this figure in the following description of the fine structure of the frontal organ for the reader's orientation of the electron micrographs.

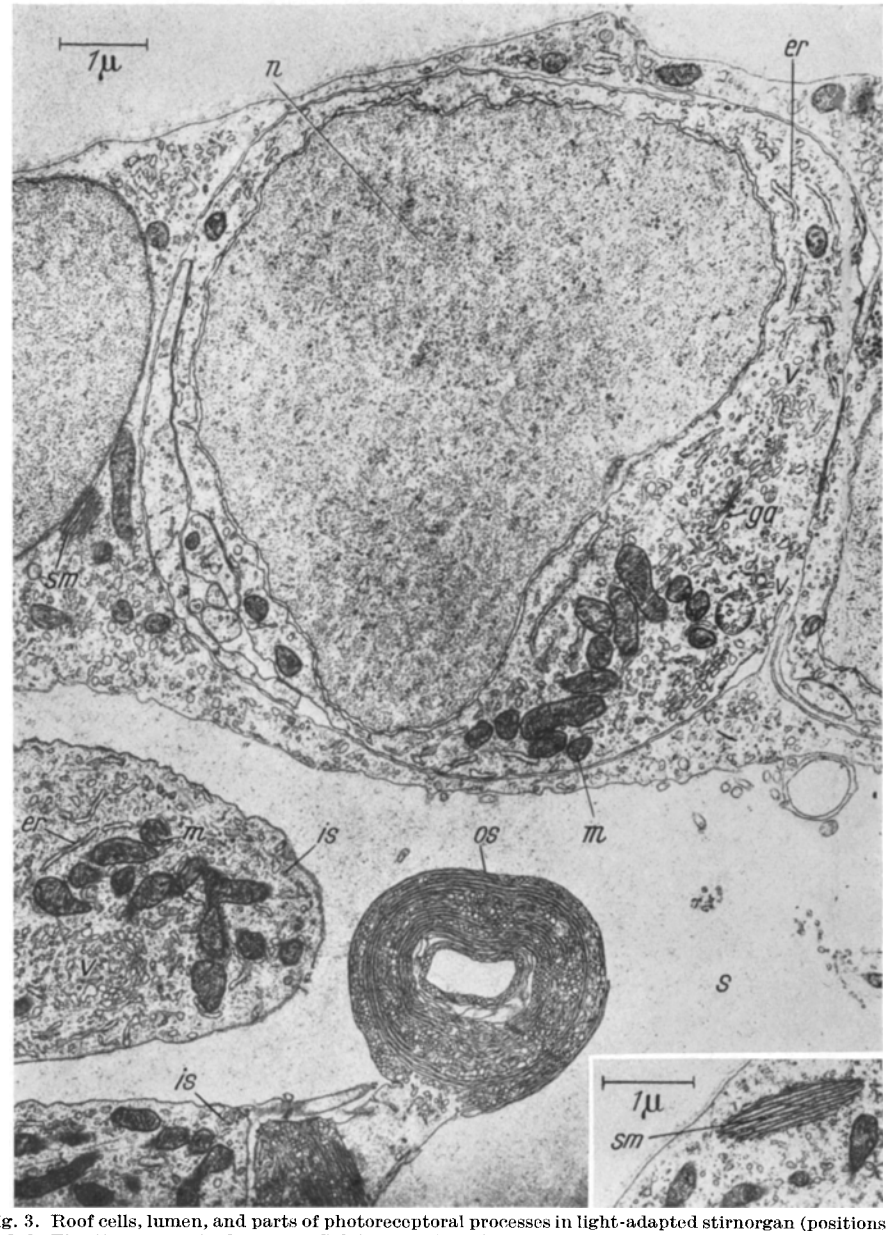

Fig. 3. Roof cells, lumen, and parts of photoreceptoral processes in light-adapted stirnorgan (positions 1 and 2, Fig. 1); *er* ergastoplasm; *ga* Golgi apparatus; *is* inner segment of process; *m* mitochondria; *n* nucleus; *os* outer segment of process; *s* lumen; *sm* ellipsoidal stack of membranes; *v* various kinds of vesicles. Insert: part of roof cell showing stack of membranes

Outer wall. The cells which form the dorsal and lateral walls of the frontal organ are slightly flattened and appear to be knit together by overlapping and interdigitating extensions. The large nuclei are irregularly oval with, occasionally, deep infoldings of the nuclear membrane. Figs. 2 and 3 (position 1, Fig. 1) present typical roof cells showing mitochondria (*m*) which are usually more abundant proximally (i.e. near the luminal surface); granular endoplasmic reticulum or ergastoplasm (*er*); smooth membranes or Golgi apparatus (*ga*) situated near and perhaps related to the numerous small vesicles (v_1); large vesicles (v_2),

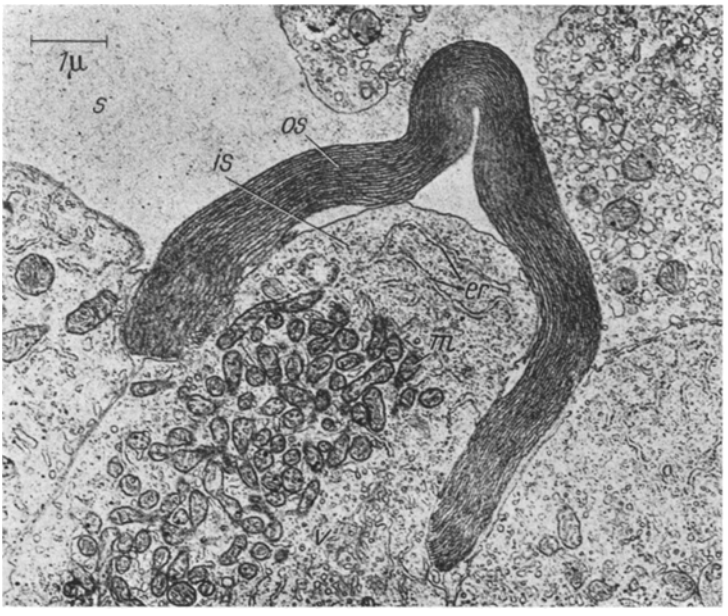

Fig. 4. Photoreceptoral process of dark-adapted stirnorgan (position 2, Fig. 1) showing an apical stack of membranes (outer segment?). *er* ergastoplasm; *is* inner segment; *m* mitochondria; *os* outer segment?; *s* lumen; *v* vesicles

some of which enclose smaller ones; ellipsoidal stacks of membranes (*sm*, insert of Fig. 3) which resemble the myeloid bodies in the pigment epithelium of vertebrate lateral eyes (PORTER and YAMADA 1960, DOWLING and GIBBONS 1962); and opaque bodies (b) lying near the nuclei, often within a nuclear indentation. The latter may be lipoidal in nature, although they are quite different from the typical fat droplets which are numerous in these cells earlier in development (EAKIN and WESTFALL 1961) and which are sometimes observed at this age (not figured). Moreover, these bodies are pleomorphic and many of them have a dense core surrounded by a lighter layer, as the one shown in the insert of Fig. 2. An occasional cloud of small vesicles (v_3) may be seen in the lumen (*s*) of the organ. A few cells contain pigment granules (not shown).

Photoreceptors. The photoreceptoral process of the sensory cell in the amphibian frontal organ has been previously described and figured for larvae of *Hyla regilla* (EAKIN 1961a, EAKIN and WESTFALL 1961). OKSCHE (1962) and OKSCHE and VON HARNACK (1962) recently extended these observations to adult frogs

(*Rana temporaria* and *Rana esculenta*). We showed that the photoreceptoral process in the tadpoles of *Hyla regilla* consists of outer and inner segments joined by a short connecting piece containing a nine strand fibrillar apparatus. The outer segment is composed of many double-membrane discs or sacs arranged like those in the cones of the lateral eye and formed by multiple infoldings of the cell mem-

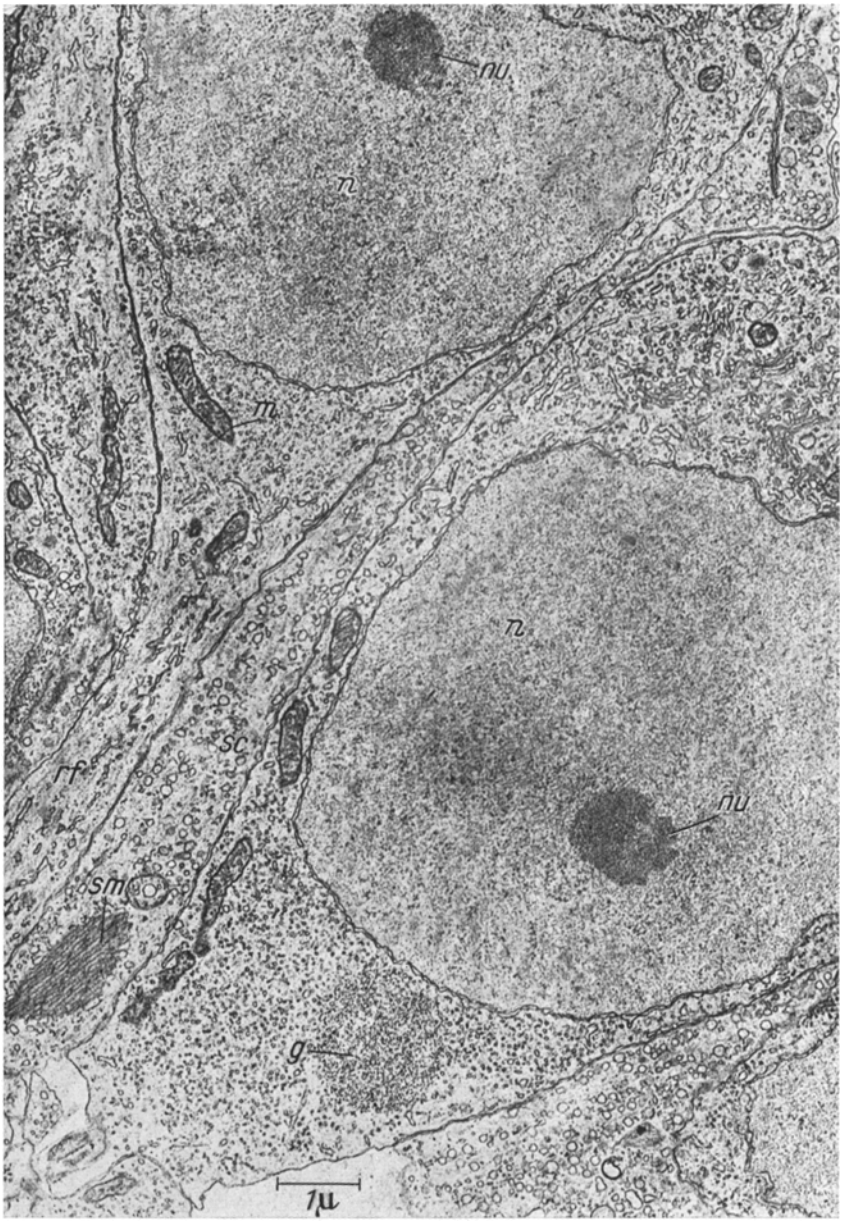

Fig. 5. Basal parts of two sensory cells in dark-adapted stirnorgan (position 3, Fig. 1). *g* cytoplasmic granules (glycogen ?); *m* mitochondria; *n* nucleus; *nu* nucleolus; *rf* receptor fiber; *sc* supporting cell (?); *sm* ellipsoidal stack of membranes

brane. Other processes, not reported earlier, present less regular patterns of organization in their outer segments. In many of them the sacs or tubules appear to be concentrically arranged, often enclosing a clear space at the center (*os*, Fig. 3; position 2, Fig. 1). In other instances the outer segment appears as a short stack of very long discs which are often folded (Fig. 4). In still other examples the outer segment is a highly irregular labyrinth of tubules (not figured).

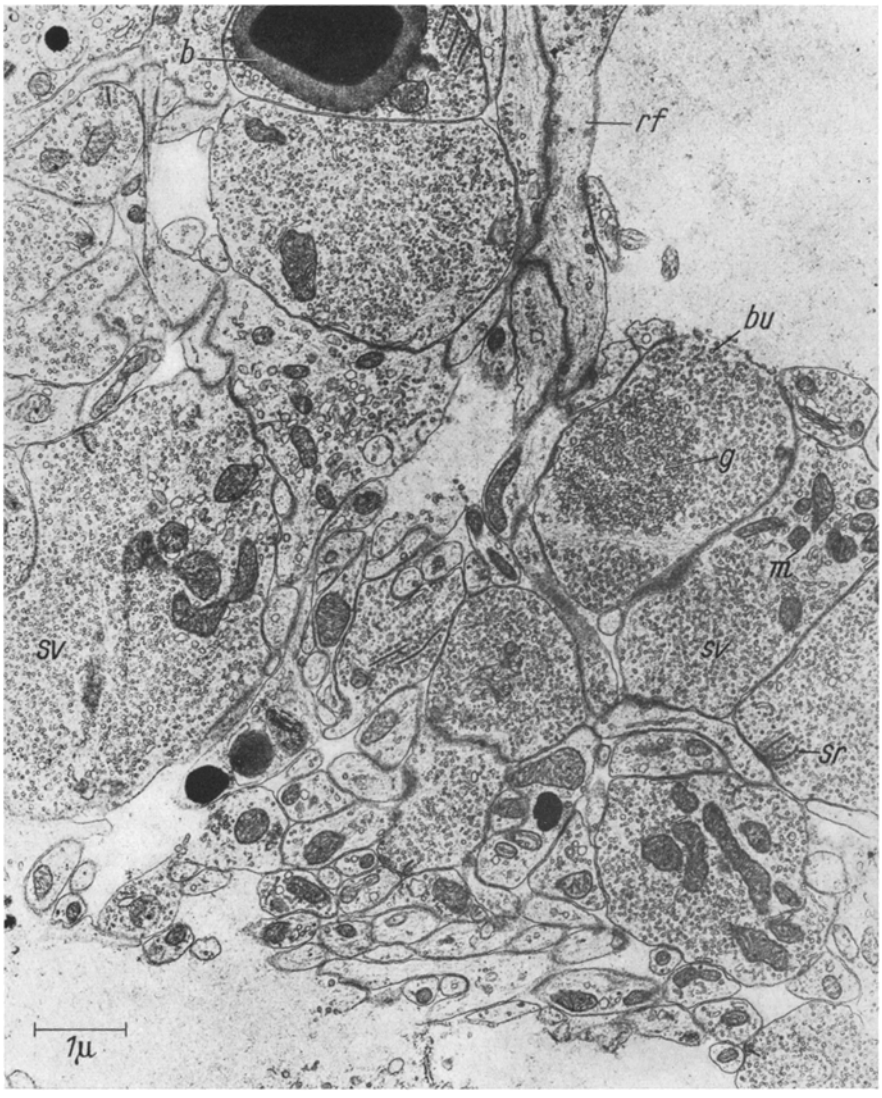

Fig. 6. Neural zone of light-adapted stirnorgan (position 4, Fig. 1). *b* opaque body; *bu* bouton; *g* granules in bouton; *m* mitochondria; *rf* receptor fiber; *sr* synaptic ribbon; *sv* synaptic vesicles

The inner segment (*is*, Figs. 3 and 4), irrespective of the form of the outer segment, is a broad extension of the cell proper into the lumen of the organ. It contains numerous mitochondria (*m*), granular endoplasmic reticulum (*er*),

smooth vesicles (*v*), and in some instances pigment granules, often inside vesicles. The structure of the distal (luminal) end of the cell proper is essentially like that of the inner segment of the process. The large ovoid nucleus of the photoreceptor lies near the apical end of the cell body (*n*, Fig. 5; position 3, Fig. 1). The fine structure of the basal part of the cell differs little from that above the nucleus,

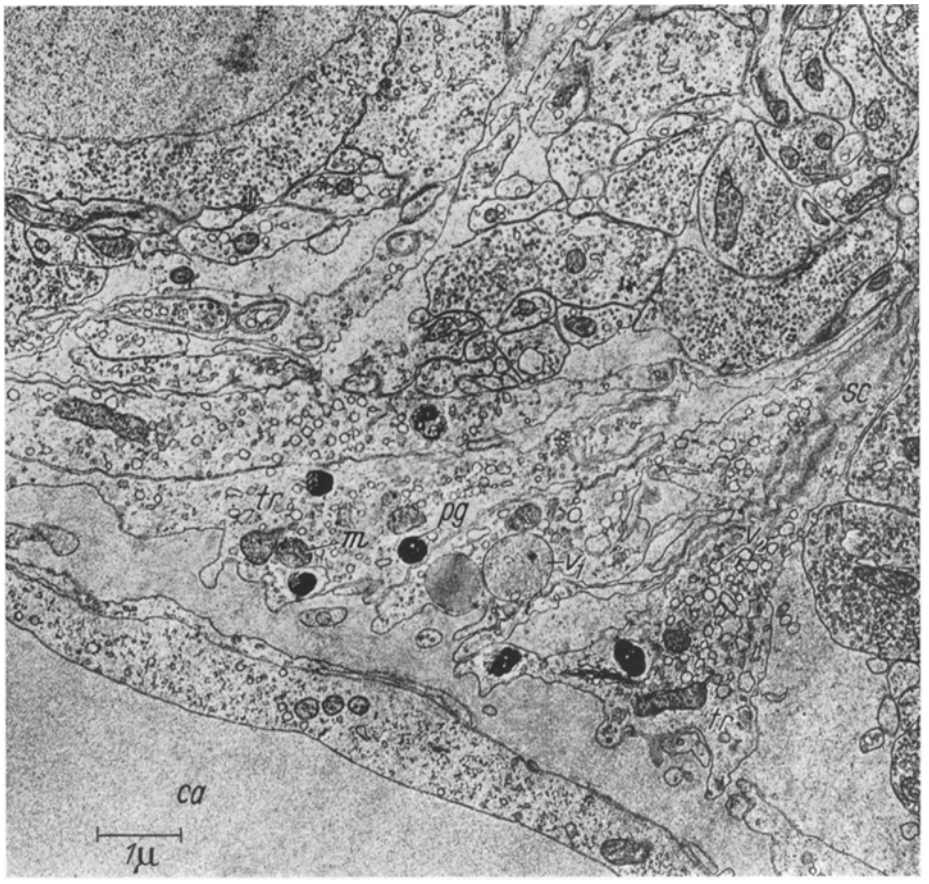

Fig. 7. Trumpet (*tr*) of supporting cell (*sc*) in dark-adapted stirnorgan (position 5, Fig. 1). *ca* capillary; *m* mitochondria; *pg* pigment granules; v_1 large vesicles; v_2 small (pinocytotic?) vesicles

except that mitochondria are fewer in number and that the cell terminates in a large receptor fiber (*rf*) resembling a nonmyelinated nerve fiber and the analogous fibers of vertebrate lateral eye photoreceptors. Because the fibers have an irregular course one rarely sees more than a short segment of any one fiber. We postulate, however, that each fiber ends in a large bouton, (*bu*, Fig. 6; position 4, Fig. 1) which contains many synaptic vesicles (*sv*). Synaptic ribbons (*sr*) may be seen in some of the boutons. Additionally, one of the boutons has a cloud of dark granules (*g*) which are distinguishable from the synaptic vesicles.

Supportive cells. The identification of the supportive element in our electron micrographs is tentative. These cells exhibit microvilli (not figured) on their

luminal surfaces as do comparable cells in the parietal eye (EAKIN and WESTFALL 1959, 1960), the reptilian homologue of the frontal organ. The supportive cells of the stirnorgan also resemble those in the parietal eye in the possession of foot-

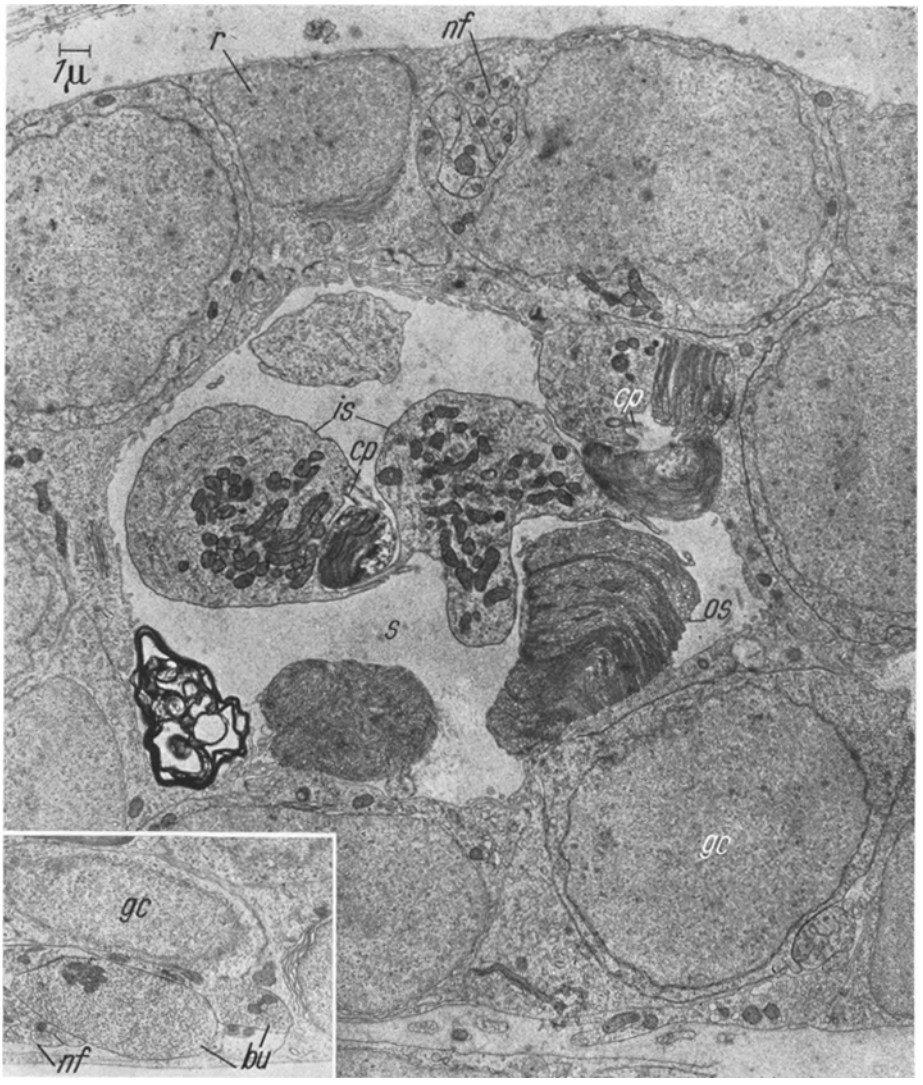

Fig. 8. Central part of dark-adapted epiphysis (cross-sectional view). *cp* connecting piece of photoreceptoral process; *gc* ganglion cell (?); *is* inner segment; *nf* nerve fibers; *os* outer segment; *r* roof; *s* lumen. Insert: part of the floor of another epiphysis showing boutons (*bu*), ganglion cell perhaps (*gc*) and a bundle of nerve fibers (*nf*)

pieces or trumpets. Fig. 7 (position 5, Fig. 1) shows the trumpet (*tr*) of a supportive cell in close relationship to a capillary (*ca*) on the ventral surface of the stirnorgan. The trumpet is very broad and irregularly folded. Within its cytoplasm are mitochondria (*m*), pigment granules (*pg*), large vesicles (v_1) filled with electron opaque material and many small (pinocytotic ?) vesicles (v_2).

Ganglion cells. We are not certain of the identification of ganglion elements in our electron micrographs. According to HOLMGREN (1918) and OKSCHE (1955) these are large methylene blue-staining cells situated in the floor of the frontal

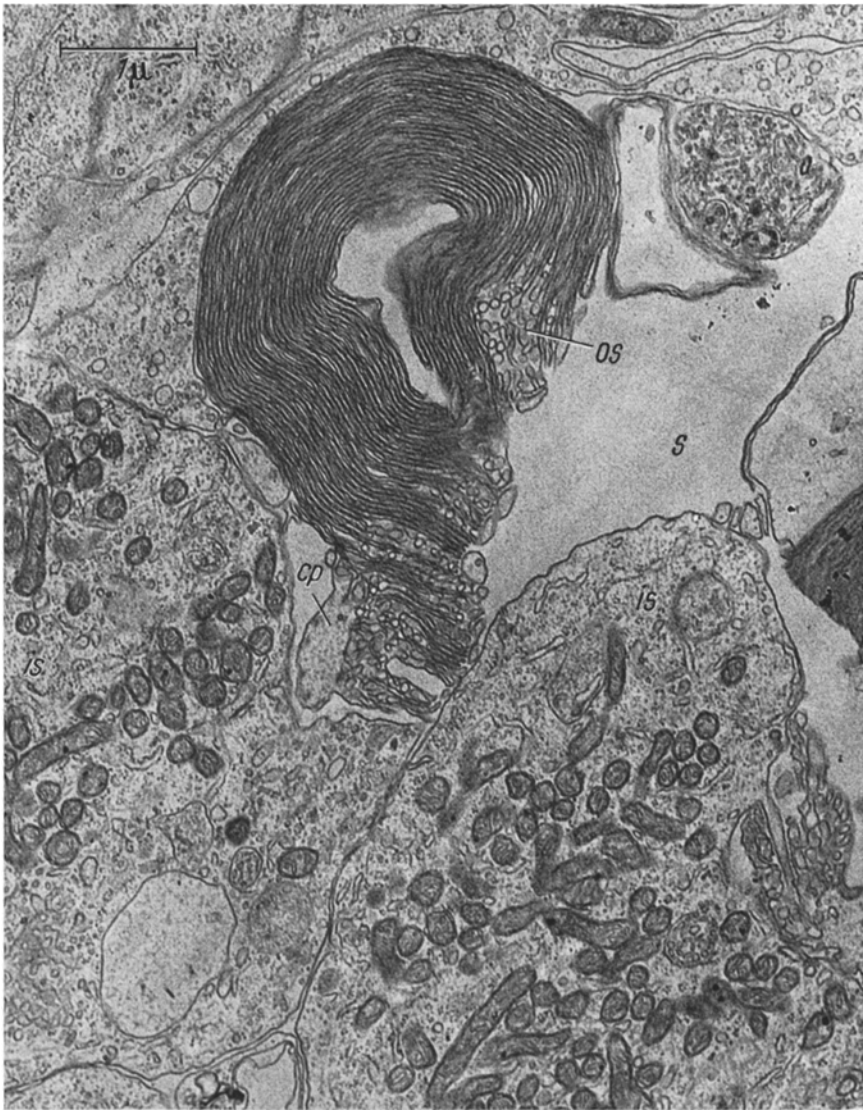

Fig. 9. Parts of photoreceptoral processes of dark-adapted epiphysis. *cp* connecting piece; *is* inner segment; *os* outer segment; *s* lumen

organ (position 6, Fig. 1). Electron micrographs at hand do not clearly demonstrate ganglion cells on the basis of ultrastructural evidence of neurofibrillae or Nissl substance.

Epiphysis

The morphology and development of the epiphysis or pineal organ of the *Hyla regilla* larva are similar to those of other anurans (STUDNIČKA 1905,

RIECH 1925, VAN DE KAMER 1949). In young tadpoles of this species the epiphysis is a long tubular body lying beneath the meninges and on the roof of the diencephalon. The posterior end of the pineal lumen communicates with the

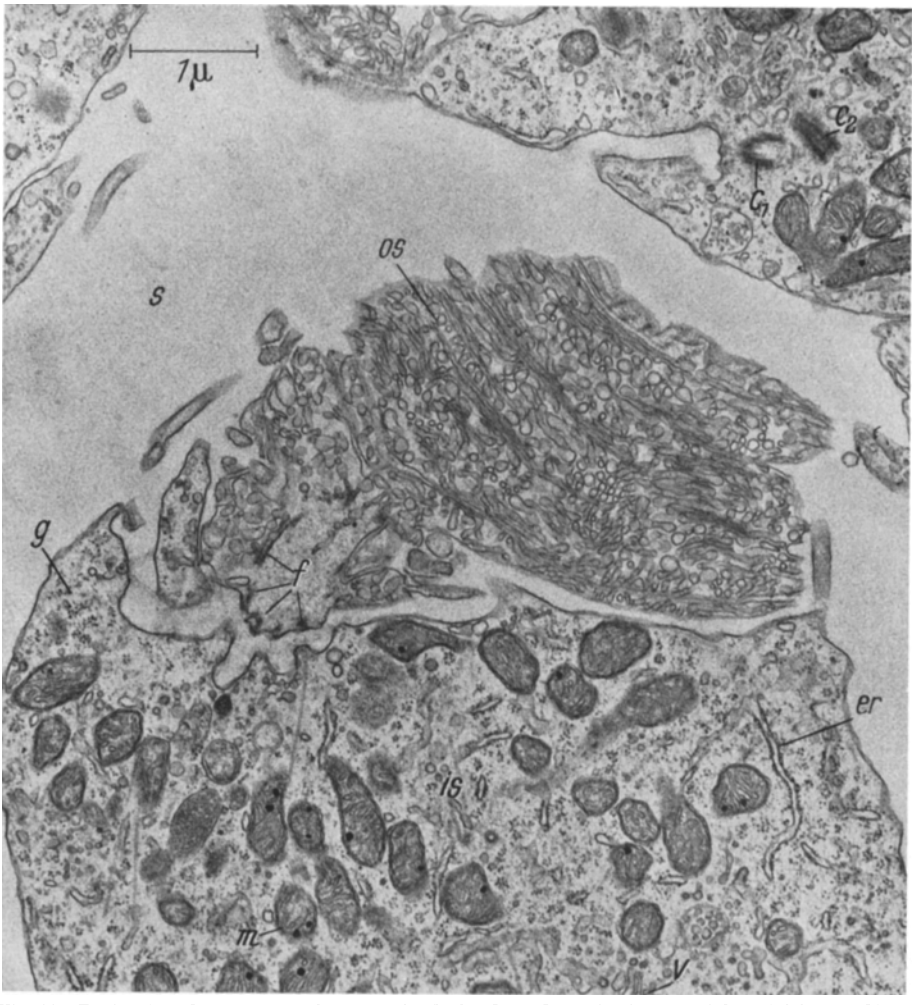

Fig. 10. Parts of a photoreceptoral process in dark-adapted epiphysis. c_1 axial centriole; c_2 oblique centriole; *er* ergastoplasm; *f* fibrils in connecting piece; *g* cytoplasmic granules; *is* inner segment; *m* mitochondria; *os* outer segment; *s* lumen; *v* vesicles

third ventricle by a small pore. The general features of the epiphysis may be seen in Fig. 8 showing the central part of the organ which was sectioned transversely.

Sensory cells. The discovery of ciliary-type photoreceptors in the larval epiphysis of *Hyla regilla* was reported earlier (EAKIN 1961b, 1962), but without details or illustration. Electron microscopy revealed sensory cells similar to those in the frontal organ in that processes, consisting of inner and outer segments joined by connecting pieces, extend into the small cylindrical lumen. The outer segments are highly variable, however, even more than in the stirnorgan, from

examples of a regular arrangement of discs, like those in a typical vertebrate photoreceptor, to whorls of double-membrane discs or sacs (*os*, Fig. 9) or piles of cisternae, like those in Fig. 4, or unorganized masses of tubules (Fig. 10). That the outer segment and connecting piece of the process are derived from a cilium-like outgrowth of an ependymal cell (see EAKIN and WESTFALL 1961) is indicated by the fibrillar apparatus consisting of a ring of nine peripheral fibrils — but no central ones — connected proximally to an axial centriole. Fig. 11 shows the base

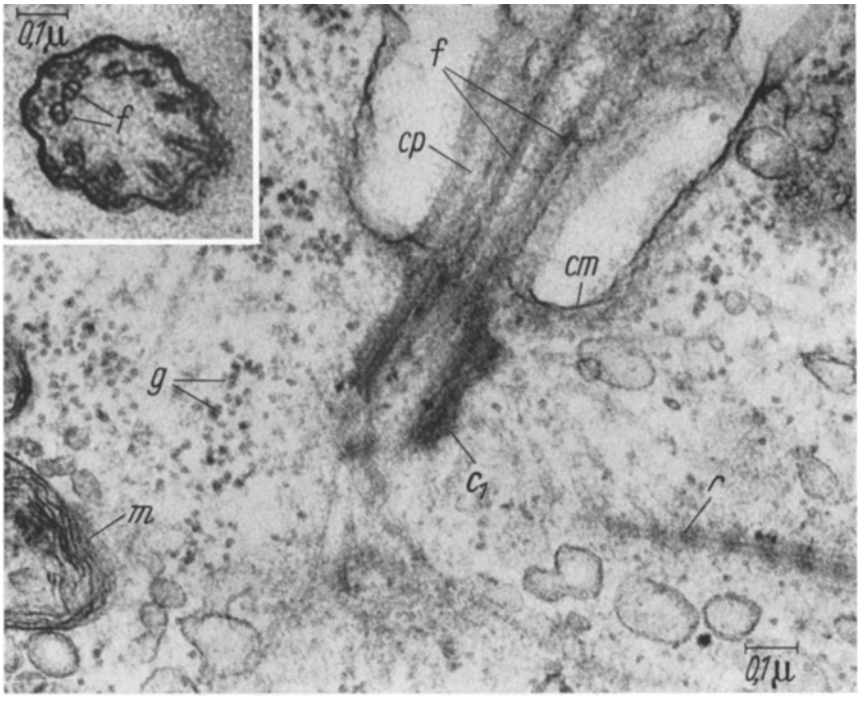

Fig. 11. Base of receptoral process in dark-adapted epiphysis. c_1 axial centriole; *cm* cell membrane; *cp* connecting piece; *f* fibrils; *g* cytoplasmic granules; *m* mitochondria; *r* striated rootlet. Insert: cross-sectional view of connecting piece with two fibrils (*f*) transversely sectioned showing their double nature

of a connecting piece (*cp*) through which pass the fibrils (*f*), the encircling depression of the cell membrane (*cm*), and within the inner segment of the process the following: axial centriole (c_1), granules (*g*), mitochondria (*m*), and a striated rootlet (*r*). A cross section of a connecting piece showing the ring of nine peripheral double fibrils is illustrated in the insert. An oblique section of the process in Fig. 10 presents the base of an unorganized outer segment (*os*), the connecting piece in which several fibrils (*f*) may be identified, and the distal part of the inner segment (*is*) rich in mitochondria, granular endoplasmic reticulum (*er*), smooth vesicles and cisternae (*v*), and cytoplasmic granules (*g*). In the same figure may be seen the centrioles (c_1 and c_2) of another receptor cell.

Other cells. The roof of the epiphysis (*r*, Fig. 8) is a single layer of overlapping cells essentially like those in the outer wall of the frontal organ. Without serial sections it is difficult to distinguish supportive from sensory cells. Moreover, our

electron micrographs did not permit identification of ganglion elements, although certain cells (*gc*, Fig. 8) in the ventral and ventro-lateral walls of the organ possessing large nuclei are possibly neurons. They agree with the descriptions of these cells by HOLMGREN (1918), OKSCHE (1955), and KELLY and VAN DE KAMER (1960).

Bundles of nerve fibers (*nf*, Fig. 8) were observed in the dorsal and dorso-lateral walls of the epiphysis and in the floor of the organ adjacent to ganglion cells. The latter fibers appear in many instances to be the axons of sensory cells, some ending in swollen boutons (*bu*, insert of Fig. 8) filled with synaptic vesicles. Those in the dorsal wall may be fibers in the pineal tract, some of which probably originate in the frontal organ. Without a long series of sections the various neurological relations could not be determined.

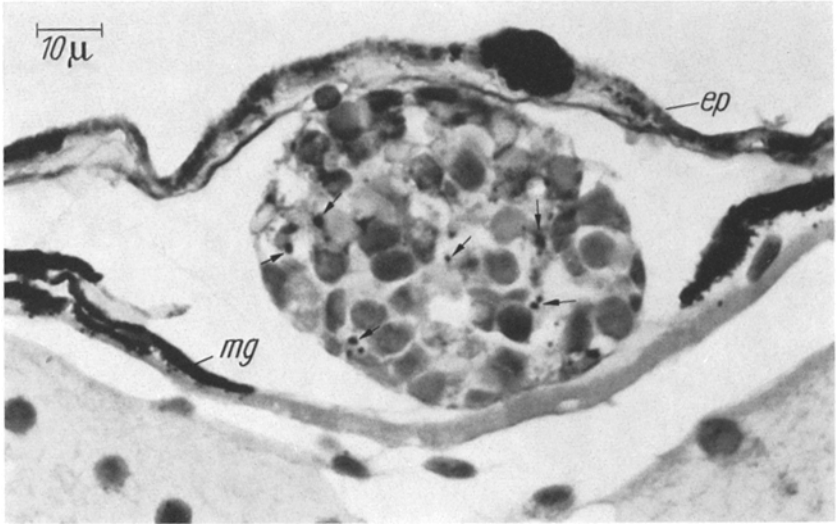

Fig. 12. PAS-positive material (see arrows) in the stirnorgan. *ep* epidermis with melanophores; *mg* meninges with melanophores

Glycogen content

The PAS-positive material in frontal and pineal organs is believed to be glycogen, at least in part, because of the reduction in the amount of this material by treatment with amylase. It is in the form of small, discrete, intensely stained bodies (see arrows, Figs. 12 and 13) which in the stirnorgan are situated on the borders of the lumen; but in the epiphysis they are more frequently in the lateral and ventral walls of the organ. PAS-positive material is particularly abundant in the subcommissural organ. The luminal border of the body is tinged with color which unfortunately does not show well in the photomicrograph (see arrows, Fig. 14). The cytoplasm of some cells immediately adjacent to the ventral margin of the nuclei is strongly stained. Incidentally, the cells contain many brown pigment granules (*pg*), especially abundant dorsally.

The amount of glycogen present in the above organs in the light and dark-adapted tadpoles was rated on a 1 to 5 scale by two of us, working independently

and without knowledge of the identity of the slides. The mean scores of our evaluations for each organ in each animal were treated statistically (Chi-square test). Frequency curves showing the relative amounts of PAS-positive material

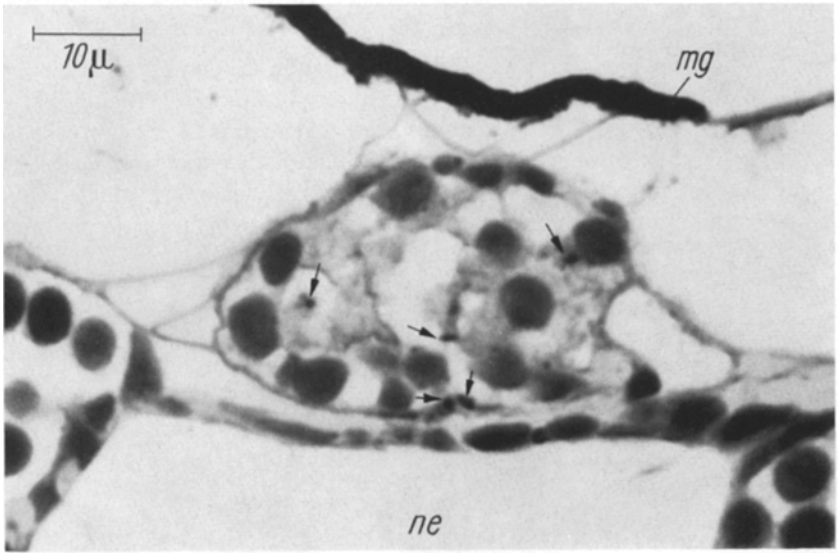

Fig. 13. PAS-positive material (see arrows) in the epiphysis. *mg* meninges; *ne* neurocoel of brain

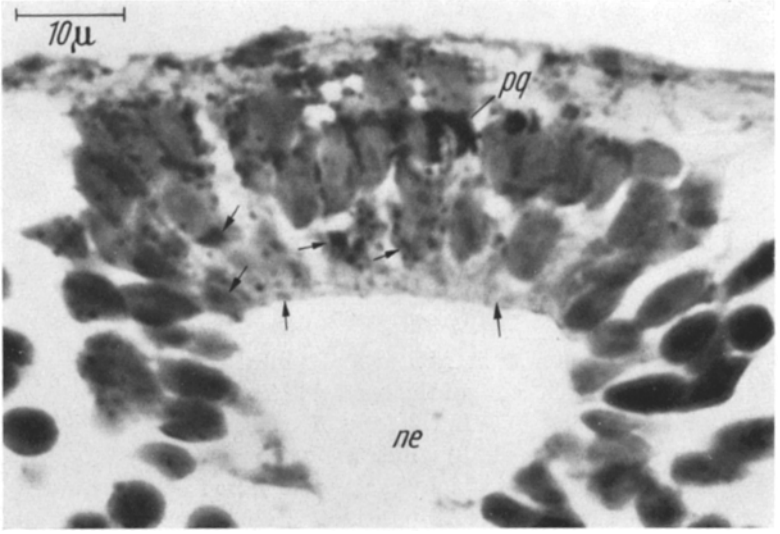

Fig. 14. PAS-positive material (see arrows) in the subcommissural organ. *pq* pigment; *ne* neurocoel of brain

in light- and dark-adapted tadpoles are presented in Fig. 15 for the frontal organ, epiphysis, and subcommissural organ. The frontal organ of dark-adapted animals contained a significantly higher ($P < 0.001$) amount of glycogen than that of animals illuminated for two days. The differences were not significant for the epiphysis and subcommissural organ.

Many fine granules were observed in our electron micrographs of the frontal organ which may be glycogen on the basis of electron microscope observations of other investigators (e.g., REVEL, NAPOLITANO and FAWCETT 1960, KARRER 1961, DROCHMANS 1962) who frequently found the glycogen in the form of clusters or rosettes of granules composed of still finer particles. Great variation in the ultrastructural appearance of glycogen was also reported and attributed to several factors such as basic differences in tissues, metabolic states, and technical procedures used. The granules which we observed in the stirnorgan are especially numerous in the vicinity of the nuclei of the sensory cells (*g*, Fig. 5). Unfortunately, precise correlation between the regions of concentration of the granules in our electron micrographs and the PAS-positive globules in our light micrographs cannot be made. We regard it significant, however, that there appear to be many more granules — indeed clouds of them as shown in Fig. 5 — in dark-adapted tadpoles than in those subjected to intense illumination for two days. This is the only ultrastructural difference we were able to demonstrate between light and dark-treated animals.

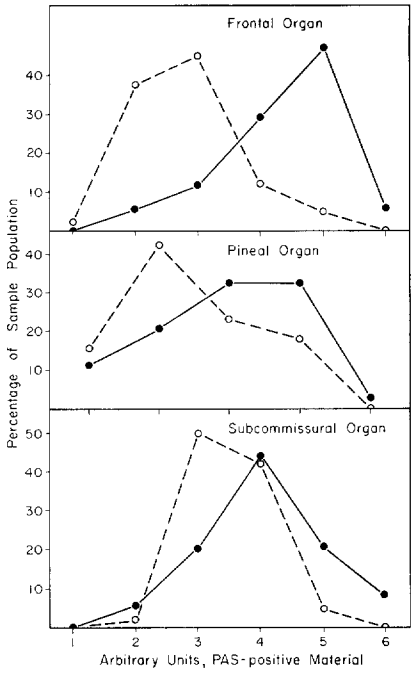

Fig. 15. Frequency curves of units of PAS-positive material in frontal, pineal, and subcommissural organs. Circles and broken line: light-adapted; solid dots and line: dark-adapted

Indole content

Interest in the pineal and stirnorgan indoles stems from the finding of the most potent melanophore-contracting or pigment-concentrating agent, namely, melatonin (a 5-methoxy indole), in mammalian pineals and the demonstration of its hormone-like action on anuran melanophores (LERNER, CASE, TAKAHASHI, LEE and MORI 1958). The melanophore contraction in amphibian larvae subjected to darkness for periods of a few hours has been noted for many years. BAGNARA (1960) has proposed that the mechanism for this lies in the pineal gland which may possibly be stimulated to secrete small amounts of melatonin or a similar substance when sufficient quantities or certain wave lengths of light are lacking. However, the occurrence of either melatonin or related compounds in amphibian pineals or adjacent possibly related organs has yet to be shown.

Histochemical or cytochemical methods for demonstrating presence of melatonin or closely related compounds have not been developed. Chemical and biological methods, however, are available for melatonin assay. Although it is not so sensitive as the bioassay method (amphibian melanophore contraction), the chemical spectrophotofluorometric method is very sensitive and is better known in terms of its chemical specificity than the bioassay method.

Indoles, including tryptophan, serotonin, melatonin, and others, are characterized by maximal fluorescence at 340—350 mμ and a maximal excitation at 290—295 mμ (UDENFRIEND 1962). Indoles with a 5-hydroxy (serotonin etc.) or 5-methoxy (melatonin etc.) group show in addition a stronger fluorescence at 540—550 mμ with excitation at 295 mμ in stronger acid (3 N HCl) (BOGDANSKI, PLETSCHER, BRODIE and UDENFRIEND 1959, QUAY 1963). To test the possible occurrence of these compounds in the stirnorgan of *Hyla regilla* tadpoles, we examined extracts of the organ and of the meninges with a spectrophotofluorometer (see methods). Nearly equally strong fluorescence at 340 to 350 mμ, characteristic of indoles, was obtained from both tissue homogenates (Fig. 16). Added HCl, to bring the concentration to 3 N, did not result in the appearance of even a trace of fluorescence at 540—550 mμ (Fig. 16). Similarly treated homogenates of rat pineal glands had a fluorescence peak at 540—550 mμ which exceeded that at 340 to 350 mμ. These results do not support the hypothesis that the stirnorgan contains melatonin or its precursor 5-hydroxy or 5-methoxy indoles. Nevertheless, the amounts present, even in 100 pooled organs, may be too small for detection by this method. Minimal detectable amounts of 5-hydroxy and 5-methoxy indoles in homogenates such as we used range from about 5 to 20 ng.

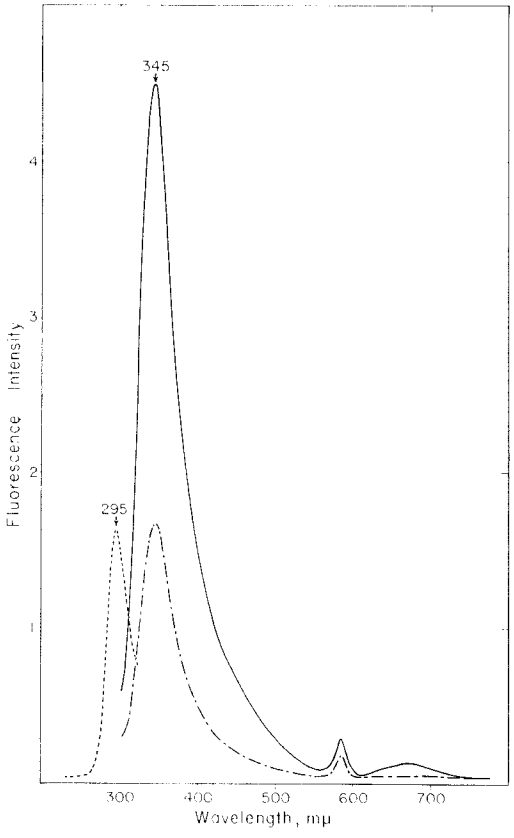

Fig. 16. Fluorescence spectra of frontal organ homogenate in 0.1 N HCl (—) and 3.0 N HCl (— · —) and excitation spectrum (- - - - -) of 345 mμ fluorescence in 3.0 N HCl. Small peaks near 580 mμ are due to Raman scatter

The minimal effective dose of melatonin for producing amphibian melanophore contraction is about 0.1 ng/ml aquarium water (BURGERS and VAN OORDT 1962, BAGNARA personal communication). The minimal effective circulating level within amphibian larva would presumably be less than this.

Discussion

It seems increasingly clear that the amphibian frontal organ is light sensitive. First, it is favorably situated for receiving light: just below a clear spot in the integument (VAN DE KAMER, FEEKES and BURGERS 1962) in the larvae of many if not all anurans and in the adults of certain frogs (STIEDA 1865). Second, there

is a marked similarity in fine structure between the processes of known photoreceptors and those in the stirnorgan, described and figured in this and earlier papers (EAKIN 1961a, EAKIN and WESTFALL 1961). Third, we have shown here that there is a significant reduction in the glycogen content of the stirnorgan after continuous illumination for 48 hours. Fourth, DODT and HEERD (1962) have recorded afferent impulses from the cut pineal tract in adult *Rana temporaria* when light upon the frontal organ is turned on and off. Fifth, the parietal eye, the reptilian homologue of the frontal organ, in the lizard *Sceloporus occidentalis* has been shown to possess cone-like photoreceptors (EAKIN and WESTFALL 1959, 1960). Similar but briefer observations were reported by STEYN (1959, 1960) who studied the third eye of a South African lizard, *Cordylus polyzonus*. Moreover, we demonstrated a reduction in glycogen content in the parietal eye upon light adaptation (EAKIN, QUAY and WESTFALL 1961), and electroretinograms have been obtained recently from the third eye of *Anolis carolinensis* upon photic stimulation (MILLER and WOLBARSHT 1962). Moreover, the pineal and parapineal eyes of larvae of the cyclostome *Petromyzon marinus* have typical vertebrate photoreceptors (EAKIN 1962).

In view of our findings reported here and earlier (EAKIN 1961b, 1962) that in the epiphysis of larvae of *Hyla regilla* there are ciliary-type photoreceptoral processes extending into the lumen, it seems that this organ is probably light sensitive also. KELLY (personal communication) finds similar photoreceptors in the epiphysis of an adult frog (*Rana pipiens*). The reduction in its glycogen content in light-adapted tadpoles might support the above conclusion or the depletion, which is less marked than in the stirnorgan, may result indirectly from the stimulation of the frontal organ. In any event the anatomical position of the pineal organ — beneath several layers of relatively opaque tissue including integumentary and meningeal melanophores — renders it less favorably situated for photoreception than the stirnorgan.

How the frontal and pineal organs use the information obtained by their light-sensitive cells is obscure. Photic excitation of the stirnorgan leads to afferent nervous impulses (DODT and HEERD 1962) which pass back along the pineal tract into the epiphysis where perhaps some terminate but many apparently continue to the posterior commissure (GAUPP 1897) and subcommissural organ (OKSCHE 1955). Nerve fibers originating in the pineal organ are thought to join those enroute from the stirnorgan to the brain. DODT and HEERD (1962) have shown, moreover, that "generally, illumination by white light and stimuli of short wavelengths cause inhibition, whereas darkness and light of medium and long wavelength produce excitation of pineal nerve fibers" (p. 409). These authors suggest the possibility that the sustained inhibition or excitation which they frequently observed could be "due to accumulation of two kinds of secretory material, one released by ultraviolet, the other by orange and red light" (p. 426).

Various investigators have considered the frontal and pineal organs to be secretory (HOLMGREN 1918, OKSCHE 1952, KELLY and VAN DE KAMER 1960). Our electron micrographs, although supporting the photoreceptoral function of these organs, do not negate a secretory role. In fact the appearance of various vesicles, some very large, and numerous granules in the inner segments of the sensory cells suggest secretory activity. Additionally, it is possible that the

disorganized outer segments and isolated whorls of membranes in the lumen of the organs represent a form of cyclical secretion as suggested by HOLMGREN (1918) in his studies with the light microscope. We could not see any ultrastructural difference, however, between light and dark-adapted tadpoles except for a decrease in the former of glycogen granules. Similarly we found the fine structure of the parietal eye to be essentially the same in lizards whether they had been illuminated or kept in the dark (EAKIN, QUAY and WESTFALL 1961). Perhaps the differences in the secretory activities of the pineal organs in these animals, especially considering the treatments we used, are not readily demonstrated in the organelles and inclusions currently studied with the electron microscope.

Various earlier workers have thought that the amphibian pineal organs or complex were involved in the control of pigmentation, especially in anuran larvae (e.g., FUCHS 1914). More recently, BAGNARA (1960) suggested that the epiphysis in larvae of *Xenopus laevis* releases melatonin or a similar substance, when the animals are placed in the dark, and that this mediated the body pallor exhibited briefly by amphibian larvae when placed in the dark. He found that the reaction disappeared if the pineal organ was destroyed by cautery. One of us (EAKIN 1961a) found some evidence that blanching of blinded larvae of *Hyla regilla* was reduced by stirnorganectomy in comparison with blinded controls. He stressed the importance of removing the lateral eyes in experiments designed to test the function of the frontal and pineal organs. BRICK (1962) observed a similar effect of epiphysectomy in *Ambystoma opacum*. KELLY (1962) has recently reported in an unpublished study by KELLY and JOHNSON on the effects of pinealectomy combined with enucleation of the paired eyes in larvae of *A. opacum* and in the newt *Taricha torosa*. Although differences in the reaction of the pigment cells between experimental and sham-operated animals were discernible at first they gradually disappeared. Other workers (e.g., KLEINE 1929, STEBBINS, STEYN and PEERS 1960) were unable to find any measurable effect of stirnorganectomy. These investigators did not remove the lateral eyes, however. KELLY (1962) found wide variation in pigmentary response of host melanophores to pineal organs implanted homoplastically into the tail of larvae of *T. torosa*. Any tendency for punctate pigmentation about the graft was limited to the first four or five days postoperative. Finally, as reported here, we were unable to demonstrate any melatonin or related compounds in extracts of the frontal organ or epiphysis of larvae of *Hyla regilla* with the methods which we used. In conclusion it appears that much more work is needed to clarify the effects, if any, of pineal activity upon the pigmentary system.

The glycogen granules in the retina of the stirnorgan are not readily related on cytological criteria to any of the glycogen-containing organelles of the retinas of lateral eyes of vertebrates. Indeed, the generic variation among vertebrates in lateral eye retinal glycogen both in localization and quantity makes such a relation unlikely as does also the great structural differences between lateral and median eyes. However, in some vertebrate lateral eye retinas glycogen has been found in photoreceptor cells (e.g., CARASSO 1960, YAMADA 1960). Glycogen in these cells can be related in some developing eyes of anurans with the synthesis of outer segment materials (KUROKI 1959), and in at least some lower vertebrates

with normal metabolic activities in the fully formed retina. Reduction of retinal glycogen due to illumination has been demonstrated in a fish, *Carassius auratus* (GOURÉVITCH 1954); the decrease, however, was observed in the layer of bipolar neurones only.

Glycogen depletion has been shown in other organs following acute stimulation, as in the adrenal cortex in cold stress (COHEN 1961). COHEN suggested that such glycogenolysis provides substrate for glucose-6-phosphate dehydrogenase, thus setting into motion the initial steps in the pentose phosphate pathway of carbohydrate metabolism. OKSCHE (1958) found large quantities of glycogen in the ependymal cells of all classes of vertebrates. He studied especially those in the central nervous system of anurans in which he showed that these cells, including those of the epiphysis, were especially rich in glycogen in the winter. He postulated that the ependymal cells supply carbohydrate, the primary energy-yielding substrate for nervous tissue, to adjacent neurones. The significant depletion by light of stirnorgan glycogen in the completely developed frontal organ of *Hyla regilla* may therefore be interpreted as an indication of metabolic stimulation.

Summary

1. The fine structure of photoreceptoral, neural, and supportive elements in the frontal and pineal organs (stirnorgan and epiphysis) of young larvae of the Pacific Treefrog, *Hyla regilla*, is described and figured with electron micrographs. Especially noteworthy is the variation in the outer segments of the photoreceptoral processes in both organs: e.g., linear arrangement of discs like that in rods and cones, whorls of membranes, and unpatterned disposition of tubules and cisternae.

2. The frontal and pineal organs of illuminated and dark-adapted tadpoles were studied for differences in ultrastructure and in PAS-positive material believed to be glycogen at least in part. The dark-adapted stirnorgan had a significantly greater amount of PAS-positive material and more cytoplasmic granules (glycogen?) than the light-adapted frontal organ. Differences in the amount of PAS-positive material in the pineal and subcommissural organs of dark versus light-treated animals were not statistically significant.

3. Assay of 5-hydroxy and 5-methoxy indoles in frontal organs and in meninges (control tissue) of young tadpoles by spectrofluorometry provided no evidence that the stirnorgan contains melatonin or its precursors even in 100 pooled organs.

4. The functional significance of our findings is discussed. In the light of this and other studies we conclude that the stirnorgan is a photoreceptive organ and that the amphibian larval pineal organ is probably light-sensitive. What use the organism makes of the information transmitted by these organs via their neural tracts and possibly by secretory products is not yet evident.

References

BAGNARA, J. T.: Pineal regulation of the body lightening reaction in amphibian larvae. Science **132**, 1481—1483 (1960).

BARGMANN, W.: Die Epiphysis cerebri. In: Handbuch der mikroskopischen Anatomie des Menschen, herausgeg. von W. v. MÖLLENDORFF, Bd. VI/4, Berlin: Springer 1943.

BOGDANSKI, D. F., A. PLETSCHER, B. B. BRODIE and S. UDENFRIEND: Identification and assay of serotonin in brain. J. Pharmacol. **117**, 82—88 (1959).

BRICK, I.: Relationship of the pineal to the pituitary-melanophore effector system in *Ambystoma opacum*. Anat. Rec. **142**, 299 (1962).

BURGERS, A. C. J., and G. J. VAN OORDT: Regulation of pigment migration in the amphibian melanophore. Gen. comp. Endocr., Suppl. **1**, 99—109 (1962).

CARASSO, N.: Rôle de l'ergastoplasme dans l'élaboration du glycogène au cours de la formation du "paraboloïde" des cellules visuelles. C.R. Acad. Sci. (Paris) **250**, 600—602 (1960).

COHEN, R. B.: The histochemical distribution and metabolic significance of glucose-6-phosphate dehydrogenase activity, glycogen and lipid in the stimulated adrenal cortex. Endocrinology **68**, 710—715 (1961).

DODT, E., and E. HEERD: Mode of action of pineal nerve fibers in frogs. J. Neurophysiol. **25**, 405—429 (1962).

DOWLING, J. E., and I. R. GIBBONS: The fine structure of the pigment epithelium in the albino rat. J. Cell Biol. **14**, 459—474 (1962).

DROCHMANS, P.: Morphologie du glycogène. Etude au microscope électronique de colorations negatives glycogène particulaire. J. Ultrastruct. Res. **6**, 141—163 (1962).

EAKIN, R. M.: Photoreceptors in the amphibian frontal organ. Proc. nat. Acad. Sci. (Wash.) **47**, 1084—1088 (1961a).

— Fine structure of some little known photoreceptors. Amer. Zool. **1**, 446 (1961b).

— Lines of evolution of photoreceptors. J. Gen. Physiol. **46**, 359A—360A (1962).

— W. B. QUAY and J. A. WESTFALL: Cytochemical and cytological studies of the parietal eye of the lizard, *Sceloporus occidentalis*. Z. Zellforsch. **53**, 449—470 (1961).

—, and J. A. WESTFALL: Fine structure of the retina in the reptilian third eye. J. biophys. biochem. Cytol. **6**, 133—134 (1959).

— — Further observations on the fine structure of the parietal eye of lizards. J. biophys. biochem. Cytol. 8, 483—499 (1960).

— — The development of photoreceptors in the stirnorgan of the treefrog, *Hyla regilla*. Embryologia (Nagoya) **6**, 84—98 (1961).

FUCHS, R. F.: Der Farbenwechsel und die chromatische Hautfunktion der Tiere. In Handbuch der vergleichenden Physiologie, Bd. **3**, S. 1189—1652. 1914.

GAUPP, E.: Zirbel, Parietalorgan und Paraphysis. Ergebn. Anat. Entwickl.-Gesch. **7**, 208—285 (1897).

GOURÉVITCH, A.: La localisation histologique du glycogène dans la rétine des poissons et sa consommation a la lumière. J. Physiol. (Paris) **46**, 633—641 (1954).

HOLMGREN, N.: Zur Kenntnis der Parietalorgane von *Rana temporaria*. Ark. Zool. (Stockh.) **11**, Nr 24 (1918).

KAMER, J. C. VAN DE: Over de ontwikkeling, de determinatie en de betekenis van de epiphyse en de paraphyse van de amphibïen. Arnhem: van der Wiel 1949.

— C. FEEKES and A. C. J. BURGERS: Histological investigation of the unpigmented meningeal spot on the brain of black background adapted *Xenopus laevis* larvae. Z. Zellforsch. **56**, 359—370 (1962).

KARRER, H. E.: Electron microscope observations on chick embryo liver. J. Ultrastruct. Res. **5**, 116—141 (1961).

KELLY, D. E.: The pineal organ of the newt; a developmental study. Z. Zellforsch. **58**, 693—713 (1963).

—, and J. C. VAN DE KAMER: Cytological and histochemical investigations on the pineal organ of the adult frog (*Rana esculenta*). Z. Zellforsch. **52**, 618—639 (1960).

KLEINE, A.: Über die Parietalorgane bei einheimischen und ausländischen Anuren. Jena. Z. Med. Naturw. **64**, 339—376 (1929).

KUROKI, S.: On the periodic acid Schiff positive substance of Anuran retina and its relation to development. Okajimas Folia anat. jap. **32**, 275—288 (1959).

LERNER, A. B., J. D. CASE, Y. TAKAHASHI, T. H. LEE and W. MORI: Isolation of melatonin, the pineal gland factor that lightens melanocytes. J. Amer. chem. Soc. **80**, 2587 (1958).

LILLIE, R. D.: Histopathologic technic and practical histochemistry. New York: Blakiston Son & Co. 1954.

MILLER, W. H., and M. L. WOLBARSHT: Neural activity in the parietal eye of a lizard. Science **135**, 316—317 (1962).

Oksche, A.: Der Feinbau des Organon frontale bei Rana temporaria und seine funktionelle Bedeutung. Morph. Jb. **92**, 123—167 (1952).
— Untersuchungen über die Nervenzellen und Nervenverbindungen des Stirnorgans, der Epiphyse und des Subkommissuralorgans bei anuren Amphibien. Morph. Jb. **95**, 393—425 (1955).
— Histologische Untersuchungen über die Bedeutung des Ependyms, der Glia und der Plexus chorioidei für den Kohlenhydratstoffwechsel des ZNS. Z. Zellforsch. **48**, 74—129 (1958).
— Histologische, histochemische und experimentelle Studien am Subkommissuralorgan von Anuren (mit Hinweisen auf den Epiphysenkomplex). Z. Zellforsch. **57**, 240—326 (1962).
—, und M. von Harnack: Elektronenmikroskopische Untersuchungen am Stirnorgan (Frontalorgan, Epiphysenendblase) von *Rana temporaria* und *Rana esculenta*. Naturwissenschaften **49**, 429—430 (1962).
Porter, K. R., and E. Yamada: Studies on the endoplasmic reticulum. V. Its form and differentiation in pigment epithelial cells of the frog retina. J. biophys. biochem. Cytol. **8**, 181—205 (1960).
Quay, W. B.: Differential extractions for the spectrophotofluorometric measurement of diverse 5-hydroxy- and 5-methoxyindoles. Analyt. Biochem. **5**, 51—59 (1963).
Revel, J. P., L. Napolitano and D. W. Fawcett: Identification of glycogen in electron micrographs of thin tissue sections. J. biophys. biochem. Cytol. **8**, 575—589 (1960).
Riech, F.: Epiphyse und Paraphyse im Lebenscyclus der Anuren. Z. vergl. Physiol. **2**, 524—570 (1925).
Stebbins, R. C., W. Steyn and C. Peers: Results of stirnorganectomy in tadpoles of the african ranid frog, *Pyxicephalus delalandi*. Herpetol. **16**, 261—275 (1960).
Steyn, W.: Ultrastructure of pineal eye sensory cells. Nature (Lond.) **183**, 764—765 (1959).
— Observations on the ultrastructure of the pineal eye. J. roy. micr. Soc. **79**, 47—58 (1960).
Stieda, L.: Ueber den Bau der Haut des Frosches (*Rana temporaria* L.). Arch. Anat. Physiol. u. wiss. Med. 52—66 (1865).
Studnička, F. K.: Lehrbuch der vergleichenden mikroskopischen Anatomie der Wirbeltiere, ed. A. Oppel, Teil V. Jena: Gustav Fischer 1905.
Taylor, A. C., and J. J. Kollros: Stages in the normal development of Rana pipiens larvae. Anat. Rec. **94**, 7—23 (1946).
Udenfriend, S.: Fluorescence assay in biology and medicine. New York: Academic Press 1962.
Westfall, J. A., and D. L. Healy: A water control device for mounting serial ultrathin sections. Stain Technol. **37**, 118—121 (1962).
Yamada, E.: The fine structure of the paraboloid in the turtle retina as revealed by electron microscopy. Anat. Rec. **137**, 172 (1960).

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