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INFORMATION PROCESSING IN THE FROG'S RETINA

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THE OHIO STATE UNIVERSITY RESEARCH FOUNDATION

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FOREWORD

This study was sponsored by the Biophysics Laboratory, Aerospace Medical Research Laboratories, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio. The research was conducted by the Institute for Research in Vision of The Ohio State University, Columbus, Ohio, under Contract No. AF 33(657)7578 of The Ohio State University Research Foundation. Dr. Leo E. Lipetz, Associate Professor of the Institute for Research in Vision, was project supervisor. Lt Colonel Jack E. Steele served as contract monitor for the Aerospace Medical Research Laboratories.

The work was performed between 1 September 1957 and 29 February 1964.

The electron micrography was done by Mr. Thomas M. Richardson, and was interpreted by Dr. Lipetz and Mr. Richardson.

This technical report has been reviewed and is approved.

J. W. HEIM, PhD
Technical Director
Biophysics Laboratory

ABSTRACT

The information handling properties of the frog's retina were studied by three techniques. (a) An attempt was made to investigate the mutual interaction of excitatory and inhibitory connections to bipolar cells by studying the averaged electroretinogram response to stimulation with various patterns of light. It was found that present techniques of electroretinography and photometry did not permit the required stability of measurement. (b) Light and electron microscope studies were made of the cell types and connections of the frog's retina. Illustrative micrographs are included in the report. The new findings were made that the Landolt club of the small bipolar cell is a mitochondria-packed process and that it terminates as a cilium in the aqueous space between the visual cells. It is suggested that the function of these structures is to detect the presence of bleached, unregenerated visual pigment molecules in the surrounding visual cells. (c) A technique was developed which seems to result in selective staining of the active bipolar-ganglion cell synapses. With it functional and anatomical studies were correlated to identify the four ganglion cell anatomic types which transmit information each as to the presence in its receptive field of (1) an edge, (2) a small dark image, (3) a changing light distribution, and (4) a dimming of light intensity. The large bipolar cells were found most likely to be carriers of "light adaptation" information, the small bipolars of "edge" information.

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I. STUDY OF RECEPTIVE FIELDS OF BIPOLAR CELLS

This portion of the work was an attempt to investigate the mutual interaction of excitatory and inhibitory connections to bipolar cells by studying the averaged electroretinogram (ERG) response of the frog's eye to stimulation with various patterns of light.

A. Accuracy required

As a start, the simplifying assumptions were made that: (a) Those visual receptors that directly synapse with a bipolar cell provide excitation to it, (b) A surrounding annulus of receptors connect to the bipolar via intermediate cells and provide inhibition to it, (c) The amplitude of the ERG generated at the receptor-bipolar junction is a linear function of the excess of excitation over inhibition at that bipolar. The effects on the amplitude of the ERG of distributing the illuminated area of retina in differently spaced hexagonal patterns of different size spots was then calculated. It was found that some arrays would stimulate more of the excitatory receptors than inhibitory receptors for each bipolar cell. Other arrays would stimulate less excitatory and more inhibitory receptors. On the basis of the above assumptions, it was calculated that the maximum difference in amplitude of the ERG for those two extremes would be only about 7%.

This meant that a useful quantitative study could be conducted only if the variations in ERG from all other causes were held to less than 1/2%. Further, if the receptor-bipolar excitatory and inhibitory connections were not arranged in the simple pattern assumed above, then even greater reliability of measurement would be needed. Therefore, attention was concentrated on technique to find out if sufficient reliability could be obtained.

B. Contact lens electrode

To lead the ERG potentials from the frog's eye, a special contact lens electrode was developed. This required, first, that casts be made of the frog's eye. A special procedure was developed for this. See the Appendix for details. The casts were sent to a commercial manufacturer of contact lenses and an experimental contact lens electrode fabricated. Upon trying the electrode it was found that the eyes of the bullfrogs used varied varied greatly in size, and the contact lens electrode fitted well to only a very small percent of the frogs. When well fitted, the lens would stay in place for periods up to 1-1/2 hours.

C. Stimulus patterns

For this study the frog's eye was required to view a hexagonal array of illuminated disks against a dark background. This was provided by transilluminating with an electronic flashlamp a photographic transparency which

was opaque except for the pattern of transparent disks. At first such transparencies were prepared by photographing ink patterns drawn by a draftsman. It was found that the human visual system could easily detect small irregularities in the drawn pattern. Since the response of the frog's eye might also be affected by the irregularities, this technique was modified. Sheets of metal were obtained from a machine shop, each sheet containing an exact hexagonal array of circular holes of a given, constant size. These metal sheets were transilluminated and photographed to prepare the transparencies.

D. Recording of the ERG

The potential difference between the contact lens electrode on the frog's eye and a remote electrode on the frog's head was amplified and recorded with a pen-writing oscillograph. As had been expected, the ERG produced on flash illumination of the smaller patterns was often so small as to be below the noise level. This difficulty was overcome by adding on a computer of average transients (CAT) the ERGs produced by a series of flash illuminations. The noise, being random with respect to the flash, canceled out; the ERGs summed. Preliminary experiments showed that as many as 1000 ERGs would have to be summed to produce a reliably measureable ERG under some of the stimulus conditions. It was also found that the frog's eye showed cumulative light adaptation unless a period of about one second was allowed between flashes.

This meant that some stimulus conditions would require 1000 seconds for a single measurement.

E. Results

Tests with a constant stimulus flash showed that whenever the contact lens electrode shifted on the eye, or came off and was replaced, or whenever the remote electrode shifted, there was a change in the amplitude of the recorded ERG of more than 5%. Clearly, this change was as large as the amplitude differences being sought in the responses to the different stimuli. Therefore, any ERG measurements which were to be compared would have to be made during a period in which the electrodes remained fixed. Since these periods were a maximum of 1-1/2 hours long, only six of the stimulus conditions which required 1000 seconds of measurement could be compared. A maximum of about 20 to 30 of the more luminous stimulus conditions could be compared in such a period. Thus, it still seemed just barely feasible to make the needed measurements.

The final stumbling block was the necessity of having the total light from a number of different stimulus patterns equated with 1/2%. This requires working at the very limits of the photometric art.

The results of all these sources of inaccuracy in measurement was that no measurements were obtained which were reliable enough to permit a test of the hypothesized assumptions regarding the arrangements and relative

extents of the excitatory and the inhibitory connections of visual receptor cells to a bipolar cell.

F. Conclusion

This type of study is too close to the limits of present day technique to be accomplished without the investment of several more man-years of work on improving those techniques.

II. ANATOMICAL STUDY OF RETINAL NETWORKS

The purpose of this study was to increase our knowledge of the types of retinal cells which with their interconnections form the various information processing networks of the frog's retina. The ultimate aim is to correlate these anatomical networks with what is known of the functional net works of the retina.

A. Relation between microscopic appearance and true structure

The retina is less than a half millimeter thick, so to distinguish any features in it we must use a microscope. To be able to examine the cellular arrangements within the thickness of the retina, thin sections must be cut through that thickness and laid flat for microscopic examination. To make the cutting easier, the retina must be stiffened. This could be done by freezing the retina. More commonly it is done by impregnating the retina with a liquid which then hardens. This process is called "embedding".

For a century the customary embedding medium has been a form of paraffin. The water is dissolved out of the tissues with alcohol, the alcohol removed with xylene, and then hot liquid paraffin is substituted. The paraffin is then slowly cooled to hardness. Sections as thin as 5 microns can be cut.

A more recent embedding technique is to use, instead of paraffin, an alcohol solution of the monomer of an epoxy resin to impregnate the cells. A catalyst is added beforehand and causes the epoxy to polymerize and form a three-dimensional lattice that gives rigidity to all parts of the tissue. Sections as thin as 0.02 micron can be cut.

Upon examination of a retinal section with the light microscope only the heavily pigmented (i.e., light-absorbing) structures can be seen. These are the melanin granules of the pigment epithelium cells, the outer segments of the rods, and the oil droplets of the cones. The other retinal structures are transparent and not seen. To make them visible a chemical must be used that reacts with chemicals present in the tissue to produce a colored substance.

This process is called staining. Before the stain can be added to paraffin-embedded sections, the paraffin must be dissolved out. Usually some lipids are removed from the tissue by this process.

The chemical constituents differ from cell to cell and from one part to another in the same cell. Therefore, it is usually possible to find a stain that will react with the appropriate chemicals so as to color only one type of cell or only one substructure of a cell. Since the chemical reactions involved are usually unknown, staining technique is an art rather than a science.

Normally, after a tissue has been removed from an organism the chemical constituents of the cells will react with each other and destroy the structures making up the cells. To prevent this the tissue is exposed as soon as possible to a chemical (a "fixative") which reacts with the chemical constituents of the tissue to form a stable product that undergoes very little further changes. This process is called fixation. The usual fixatives have been formaldehyde, picric acid, and osmic acid. More recently potassium permanganate and glutaraldehyde have been used.

What all this means is that the chemical structures of the tissue are first changed by fixation, then by dehydration in the embedding process, and further changed during the removal of paraffin, if that was used for embedding. Certain of the chemical constituents then present are reacted with another chemical to produce a colored product. It is the distribution of this colored product that is viewed with the light microscope. Obviously the structures seen are not the structures present in the living cell; they are only a transformation and selection of certain features of the original structure. This fact is well known to research anatomists, yet it is frequently overlooked by physiologists. As a result, physiologists often make incorrect interpretations of the functioning of a tissue on the basis of the microscopic appearance of its structure.

An important example of such a misinterpretation is illustrated by Figure 3. This is a light micrograph of a frog's retina which was fixed with formaldehyde solution, dehydrated, embedded in paraffin, sectioned, the section mounted on a glass slide, then de-paraffined, rehydrated, stained with an aqueous solution of methylene blue, dehydrated, impregnated with a mounting medium and sealed with a glass cover slip. This is a classical procedure on which many textbook illustrations are based. It stains principally the bodies and fibers of neural cells.

Note that at the center of the retina there appears a thick layer made up of many dark ovals. These are the cell bodies of neurons (bipolar cells, amacrine cells, and horizontal cells). The unstained space between the cell bodies has been shown by histologists such as Ramon y Cajal to be partially filled by the Müller cells (glial cells), which could be colored by metallic stains. Nevertheless, it was thought that a large portion of that space simply contained intercellular fluid.

More recently, examinations with the electron microscope have shown

the entire space to be filled by the Müller cells except for a gap between cells that has been variously estimated as 60Å to 200Å wide. What should be emphasized is that there is nothing inherently different about the electron microscope that makes the Müller cells appear to occupy more space. The difference is that the electron microscopic studies were all done with methacrylate- or epoxy-embedded tissues, while paraffin-embedded tissues were used for light microscopy. If the same embedding procedure is used on tissues examined with the light microscope, the Müller cells can be seen to fill completely the spaces between neurons. This is clearly shown by Fig. 1, a light micrograph of epoxy-embedded retina.

The conclusion is that the paraffin-embedding process greatly alters the size and shape of cells, particularly of glial cells. This source of distortion can be avoided by using epoxy-embedding instead, for light as well as electron microscopy.

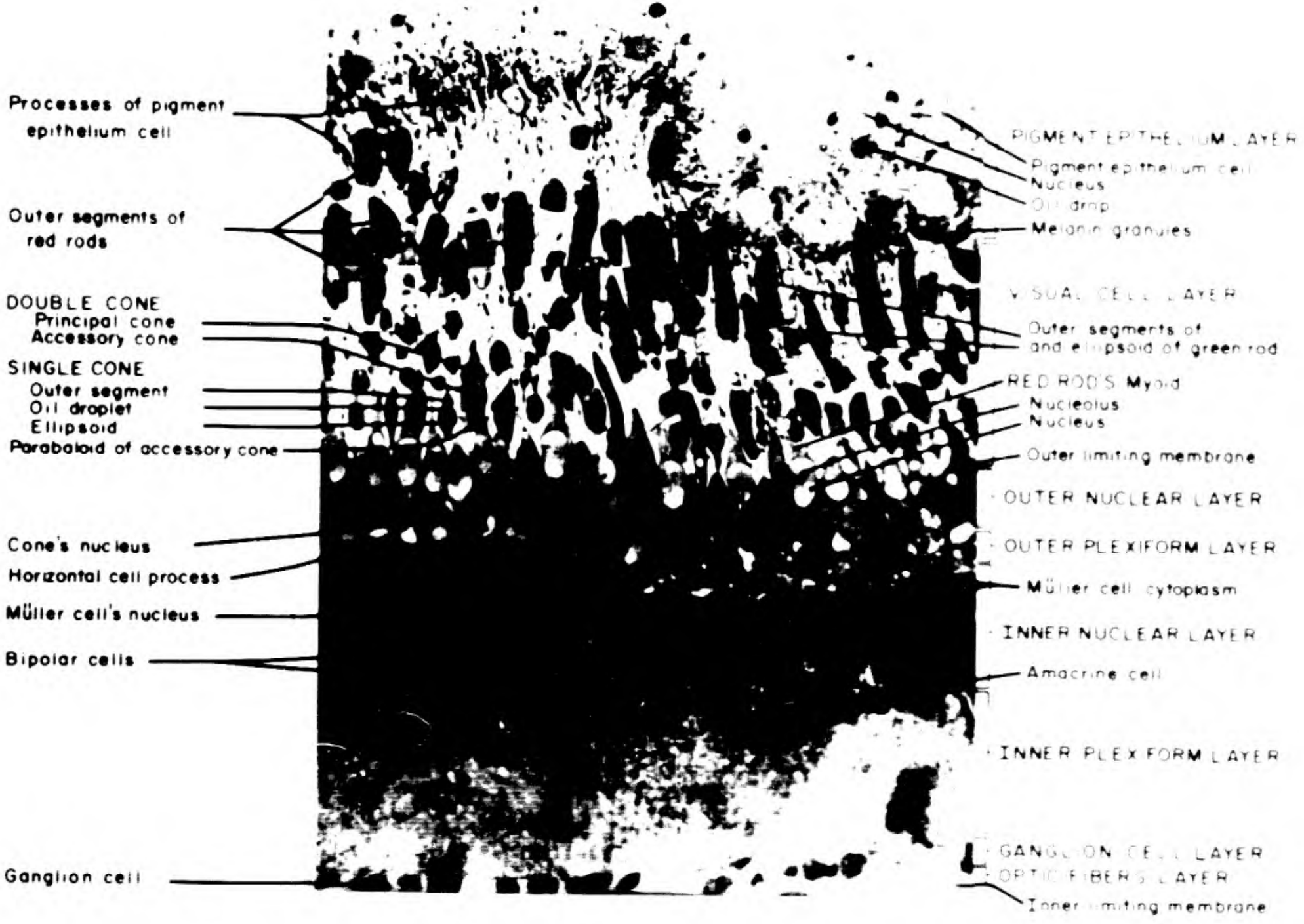
The interpretations of retinal structure presented in the following sections (except for Section II C) are based on light and electron microscope examinations of epoxy-embedded retinas.

B. Retinal layers and cells

Figure 1 is the composite photomicrograph of a transverse section of a frog's retina (Rana pipiens). The animal was caught in early spring, had been kept in constant temperature and light cycle, and had been fed meal worms three times per week for a few weeks prior to use. It appeared to be in excellent health. The retinas from this animal were exposed to the fixing solution after hemisection with a razor blade of the globe anterior to the ora serrata. The fixative consisted of a 1% solution of osmium tetroxide made isotonic with sucrose and buffered to a pH of 7.4 with sodium veronal acetate. The mixture was cooled to 4°C and the global hemispheres containing the retinas were immersed in the cold fixative for 1 hour at that temperature. The tissue was then rinsed in cold 70% alcohol for 15 minutes (two changes). During this rinse period, while slowly coming to room temperature, the retinas were carefully separated from the scleral tissue and cut into thin strips 1 mm x 4 mm. Dehydration was completed in ascending concentrations of ethyl alcohol; 15 minutes in 95% and 30 minutes in absolute (two changes).

An epoxy resin (Maraglas) was used as the embedding medium. The Maraglas mixture consists of 68% Maraglas, 20% Cardolite NC-513, 10% dibutyl phthalate, and 2% benzyldimethyl amine. The impregnation procedure requires immersion of tissue in propylene oxide for 30 minutes. (Propylene oxide replaces absolute alcohol, which is not miscible with the Maraglas mixture.) The tissue is then placed in a 50/50 mixture of propylene oxide and Maraglas for 1 hour and then into a full strength Maraglas mixture from 15 to 18 hours (or overnight) at 4°C. A freshly-made mixture of Maraglas is used for the final embedding. The tissue is brought to room temperature, and with an applicator stick the strips of retina are placed beneath the surface of the mixture in freshly-filled embedding capsules. A few moments are allowed to

Fig. 1. Composite light micrograph of a transverse section of the retina of leopard frog (Rana pipiens) About X900


Processes of pigment epithelium cell
Outer segments of red rods
DOUBLE CONE
Principal cone
Accessory cone
SINGLE CONE
Outer segment
Oil droplet
Ellipsoid
Parabaloid of accessory cone
Cone's nucleus
Horizontal cell process
Müller cell's nucleus
Bipolar cells
Ganglion cell
PIGMENT EPITHELIUM LAYER
Pigment epithelium cell
Nucleus
Oil drop
Melanin granules
VISUAL CELL LAYER
Outer segments of
and ellipsoid of green rod
RED RODS Myoid
Nucleolus
Nucleus
Outer limiting membrane
OUTER NUCLEAR LAYER
OUTER PLEXIFORM LAYER
Müller cell cytoplasm
INNER NUCLEAR LAYER
Amacrine cell
INNER PLEXIFORM LAYER
GANGLION CELL LAYER
OPTIC FIBERS LAYER
Inner limiting membrane

elapse, while the tissue sinks and the bubbles rise, before the capsules are placed into a pre-heated oven at 60°C for a 72-hour period.

The Maraglas materials and the procedure outlined above yield better gross and ultrastructural preservation than any technique yet tried in our laboratory.

A Porter-Blum ultramicrotome and a Venezuelan diamond knife were used to obtain the thick (1μ) sections used for light microscopy. The thick sections were taken from the knife's edge and floated onto a drop of distilled water on a 1" x 3" glass microscope slide. The slide was dried on a hot plate set for low heat. The sections were stained with a 1% mixture of borax-methylene blue and azure II, by flooding the sections with the staining solution. The slide was then placed on the hot plate for 10 to 15 seconds. After cooling and rinsing gently with a stream of distilled water from a plastic squeeze bottle, the slide was placed back onto the hot plate to dry. (This is necessary to prevent the sections from curling).

The slide can be used at this stage, the sections being still embedded in the Maraglas mixture. However, to obtain a more permanent preparation and to insure more protection for the tissue we routinely dip the slide in xylene and cover with a thin coverglass mounted on Permount or Histoclad mounting medium.

The micrographs were made with an AO Spencer Trinocular Microstar microscope and camera using Kodak Plus-X Pan, with a red filter (Wratten No. 29) in the light beam. A 97X oil immersion objective was used. The particular retina photographed is referred to as Trial G-58. Electron micrographs of the same retina are shown in a later section of this report.

C. Selective staining for cellular activity

The discussion of Section II A makes clear that a stain is produced in a tissue section by the reaction of an appropriate chemical with certain chemical constituents of the tissue. Since the chemical constituents of a cell change with its state of activity (e.g., conduction of nerve impulses), in theory it should be possible to find a particular treatment that would stain only those chemical constituents associated with recent activity of the cell. Such a technique would be very useful in identifying the neural paths of the retina which are activated by different types of visual stimuli. It would even show up any parallel paths used in transmission of a given type of visual information; something that cannot be done conveniently by electrophysiological techniques. Therefore, this work has included an exploratory study of selective staining of the retina.

A number of procedures and treatments were tried unsuccessfully. Selective staining was achieved by the following procedure. A group of leopard frogs (Rana pipiens) were held under controlled conditions of

temperature, lighting cycle, and feeding for a month or longer until they appeared to be in a standardized, healthy condition. Next, each frog was dark adapted for 2 hours at 70°F to 76°F. Then, in complete darkness it was held with its right eye in the eye guide of the stimulation apparatus diagramed in Fig. 2. The left eye was covered with a piece of black velvet. The stimulation light was turned on for 40 to 45 seconds, causing an image of the illuminated opal glass to fall on the right retina. Before the end of this period a hypodermic needle was pushed through the nasal cornea of the right eye into the vitreous humor and withdrawn. Then the syringe fastened to the apparatus was advanced by a rack and pinion to a preadjusted stop. This caused its hypodermic needle to penetrate the right temporal corneal limbus, pass under the side of the lens into the vitreous humor, and stop just in front of the central retina. The plunger of the syringe was then slowly depressed to flood the retina with about 0.5 cc of a solution of 10% neutral formalin (pH 6.8). The nasal hole in the eye provided an outlet for aqueous and vitreous humor so that the hydraulic pressure within the eye did not rise to levels damaging to the retina. The injection was finished just at the end of the stimulation period.

Next, under a dim red light, a first hole was made in one side of the left eye by inserting a needle through the black velvet cover over the eye. The needle of another hypodermic syringe was inserted through the velvet into the other side of the eye, and neutral formalin injected over the retinal surface. The frog was then beheaded and pithed. The eyes were then removed, the front halves cut off and the lenses taken out, and the eyes then placed for 1 hour in the dark in 10% neutral formalin. The object of this procedure was to fix the cellular constituents in the state corresponding to the response, or lack of response, of the cell to the light stimulus.

The eyes were then (in the light) trimmed of excess tissue, marked for identification with Turnbull's blue dye, and washed repeatedly with fresh 70% ethanol. They were then put in a solution of two volumes dioxane to one volume of water for 1 hour, transferred to 100% dioxane for 1-3/4 hours, transferred for 1/2 hour to a solution of half dioxane and half paraffin, and then kept overnight in molten 100% paraffin.

The paraffin was solidified and the tissue block trimmed and then 15μ thick sections cut off and mounted with egg albumin on glass slides. The sections were deparaffined by immersion twice for 15 minutes in water-free xylene, 15 minutes in absolute ethanol, and a 1-minute wash with distilled water.

The sections were stained by immersion for 5 minutes in a 0.1% aqueous solution of histologic-quality methylene blue (Loeffler's alkaline methylene blue, Paragon). They were washed in distilled water for 1/2 minute, and then dehydrated by 3 minutes immersion in 95% ethanol, 3 minutes in absolute ethanol, and 5 minutes in water-free xylene. The sections were then impregnated with Canada balsam and sealed with cover slips. In all these procedures the solutions were gently shaken by mounting the container of solution on a turntable which was tilted to 20° from the horizontal and revolved at 2 rpm.

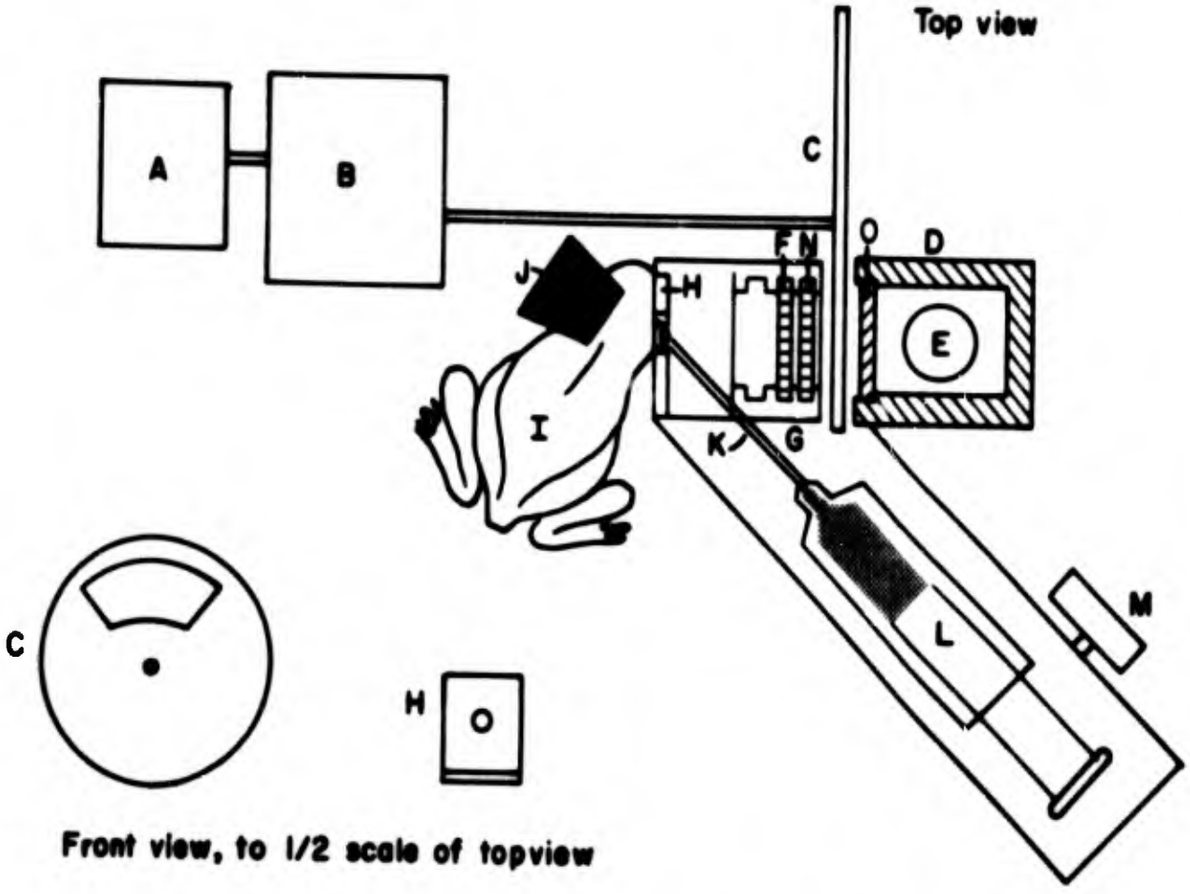


Fig. 2. Apparatus used in the selective staining study to provide controlled stimulation and immediate fixation of the retina

A - Synchronous motor, Bodine 1800 rpm

B - Variable speed drive, reduction to 200 rpm

C - Rotating shutter, of design shown at lower left

D - Light tight box (with lid off in drawing) O - Opalglass

E - Lamp, T12 projection type, 150 watts

F - Neutral density filter

G - Filter holder box

H - Transparent shield for positioning the frog's right eye

I - Frog

J - Black velvet cover over left eye

K - Hypodermic needle

L - Syringe filled with fixative

M - Control of rack and pinion movement which advances needle into eye

N - Heat absorbing filter

Three stimulation conditions were used. The first, continuation of the dark adaptation, was the condition for the left eye of each frog tested and both eyes of a few frogs. The second was the exposure of a right eye for 40 to 45 seconds to a luminance of 1/4 x 26,000 footlamberts (the opal glass window viewed through a 0.60 neutral density filter). The third stimulation condition was exposure of a right eye for 40 to 45 seconds to a luminance of 26,000 footlamberts (the opal glass window viewed through a 0.00 density filter) with the shutter rotating at 3.3 rev/sec and allowing light passage during 1/4 of each revolution. In the final experiment, of the sectioned eyes 2 were in the first stimulation condition, 4 were in the second, and 6 were in the third.

Figure 3 shows the appearance of a section from an eye in the first (dark-adapted) condition. This was typical of what had been found in each of many preliminary experiments. This, and all photomicrographs of the experiment, was made with an AO Spencer Microstar microscope, a 43X objective, Kodak Plus-X Pan film, and transillumination with red light (Wratten No. 29 filter) to increase the contrast of the stain.

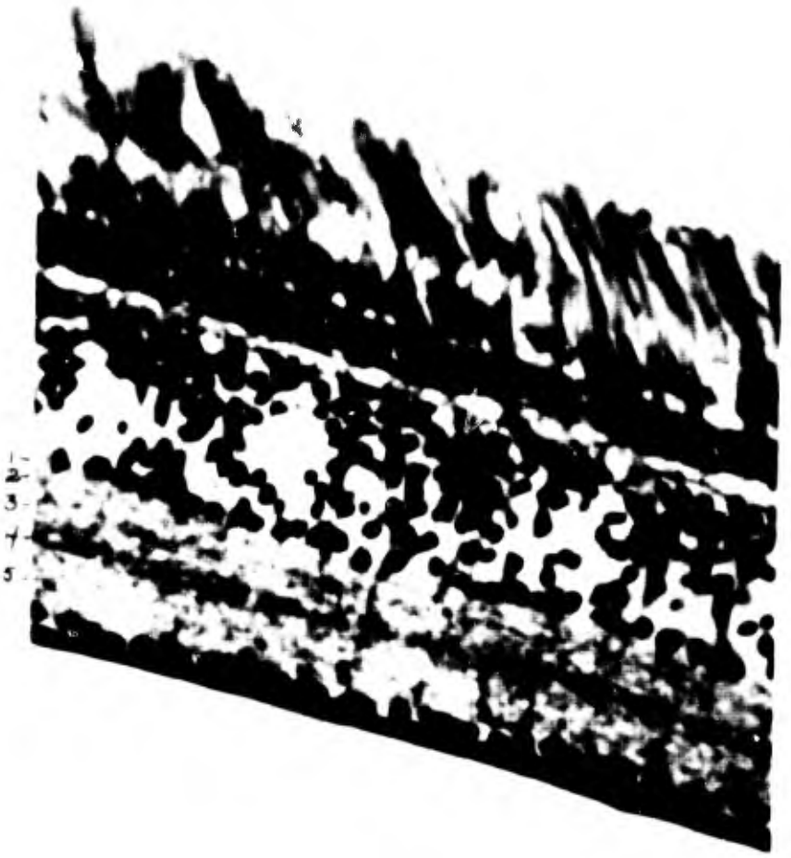
Figures 4 and 5 are of sections from two eyes in the second stimulation condition (steady light). Figures 6 and 7 are of section from two eyes in the third stimulation condition (flickering light).

Microscopic examination of sections of all the eyes studied showed marked differences in staining in the inner plexiform layer (IPL). Examination of serial sections of all the eyes showed that for all three conditions the horizontal levels of synapses in the IPL were stained in fine, long, parallel, horizontal lines only in certain portions of the retina. These portions were almost always central and corresponded roughly to a 40° to 80° retinal field. A few retinas were found to show this staining pattern in a small part of the peripheral retina as well. It may be that these regions corresponded to the retinal portion illuminated by the opal glass window, whose image was a square 36° of visual arc on a side. But that does not explain why the dark-adapted retinas also showed such limited regions. Another possible explanation is that the fixation happened to be more rapid and thorough in those regions --- the hypodermic needle may have injected the fixative just at the retinal region.

Whatever the explanation, those retinal regions were easily recognized and showed a consistency of staining pattern which was not found in other portions of the retina. These regions were photographed in two or more sections of each of the eyes --- a total of 47 photographs. It was noticed that all 8 photographs of dark-adapted retinas showed a light, rather uniform staining of all horizontal synaptic levels in the IPL. Figure 3 is typical of this appearance.

Of the 4 retinas given steady illumination, all the photographs (i.e., 4) of 1 retina looked in the IPL like the dark-adapted retina's staining. All the photographs (i.e., 13) of the other 3 retinas showed either a heavy staining of the middle synaptic level (see Fig. 4), or a heavy staining of that level and nearly as heavy a staining of nearly the entire IPL above it (see Fig. 5).

Fig. 3. Light micrograph (about X400) of dark-adapted retina of leopard frog, fixed in the dark. Methylene blue stain



Figs. 4 & 5. Light micrographs (about X400) of frogs' retinas which had been subjected to high intensity, steady illumination for about 45 seconds before fixing. See the text for details. Methylene blue stain.

Fig. 4

Fig. 5

Fig. 6

Figs. 6 & 7. Light micrographs (about X400) of frogs' retinas which has been subjected to the same total light flux as in the retinas of Figs. 4 & 5, but with the light flickered at 3.3 flashes/sec. See text for details. Methylene blue stain

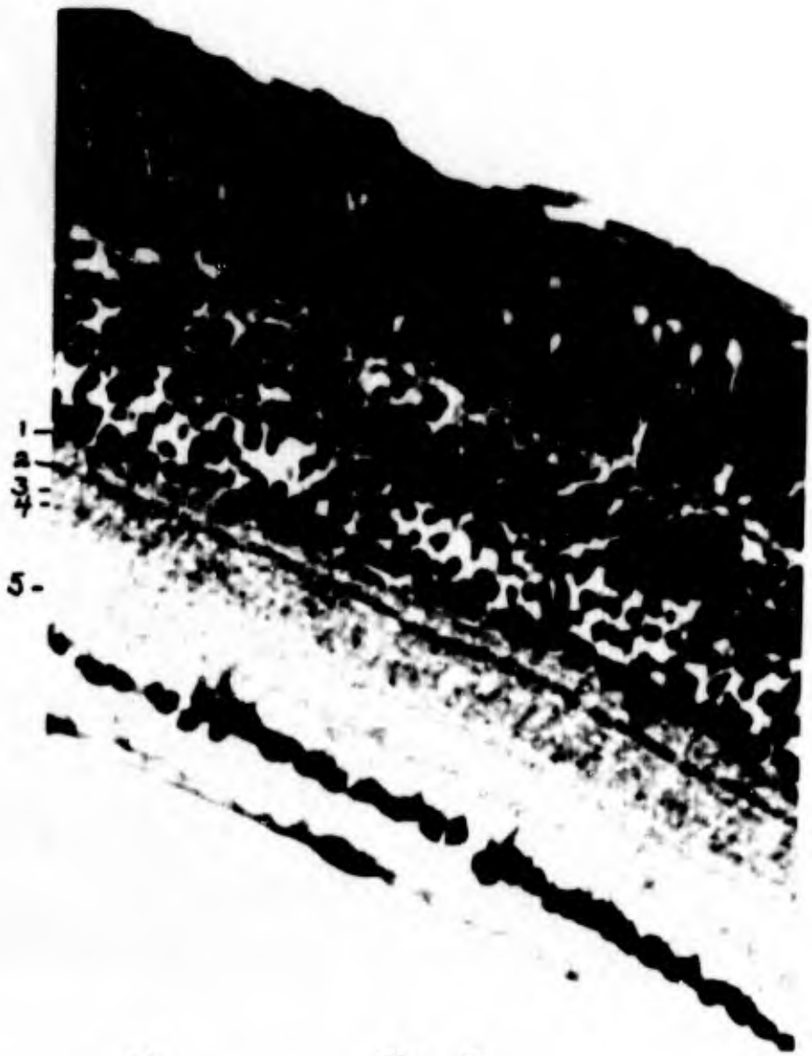


Fig. 7

Of the 6 retinas given steady illumination, both photographs of one retina and 1 of the 3 photographs of a second retina resembled the dark-adapted retinas in staining of the IPL. The remaining 2 photographs of the second retina and all (i.e., 18) photographs of the other four retinas showed either heavy staining of a single synaptic level near the top of the IPL (see Fig. 7), or else heavy staining of both that level and the middle synaptic level (see Fig. 6).

These differences in staining of the IPL correlate well with the stimulation condition. The 8 photographs that did not match these criteria all looked like dark-adapted rather than illuminated retinas, and might in fact be portions of retinas that had not been illuminated by the rather restricted light stimulus (which fell on only 1/25 of the retinal area).

To test whether these criteria were sufficiently objective to be used to determine from the staining appearance which stimulus condition had been experienced, the photographs were shuffled and given to an experimenter to sort according to appearance. In the first sorting 10 of the 46 photographs were categorized differently than had been done above. In the second, 9 out of 46, and in the third, 9 out of 46. In each case 2 to 4 photographs of each stimulus condition were incorrectly categorized. Therefore, in this limited experiment, the criteria chosen gave repeatable identification of the retinal staining pattern in 80% of the cases.

This experiment cannot be regarded as being conclusive or final. It does show that identifiable, repeatable differences in staining pattern can be produced by different stimulation conditions. However, it would be desirable to find whether the patterns could be made identifiable in 100%, rather then 80%, of the cases, by (a) illuminating a much larger portion of the retina, (b) applying the fixative more rapidly and uniformly to the retina. Also, it should be tested whether these staining patterns are repeatable from one batch of frogs to another batch.

Keeping these limitations of this selective staining study in mind, it will be shown below, as a demonstration of the utility of this technique, how the results of this study can be used to analyze retinal function.

Figure 8 summarizes the results of many year's study of the frog's retina by Ramon y Cajal.[1] The drawings indicate the appearance of the cellular structures which were stained by Golgi's technique (osmium fixation, metallic salt stains). He found that the IPL was occupied by teledendrons of the bipolar cells, dendrites of the ganglion cells, processes from the amacrine cells, and processes of the Müller cells. The Müller cell processes (not shown in the drawings) occupy the spaces between the other processes, and could not be the fine, parallel, horizontal fibers stained in the present study; neither could the teledendrons of the small bipolar cells, which have too short a horizontal extension. The teledendrons of the large bipolar cell, the ganglion cell dendrites and the amacrine cell processes could correspond to the stained fibers.

Plate II. Cells of the Retina of the Frog; Stained with Silver Chromate According to the Procedure of "Double Impregnation."

Fig. 1. a Bipolar cells whose descending processes form three overlapping ramifications; b dislocated (deplacirte) bipolar cell; c bipolar cell with a lower branching which spreads out in the fifth sublayer (g); f bipolar cells with large upper tufts; e brush shaped horizontal cells; d bipolar cell which forms downward directed ramifications in the first and second sublayers.

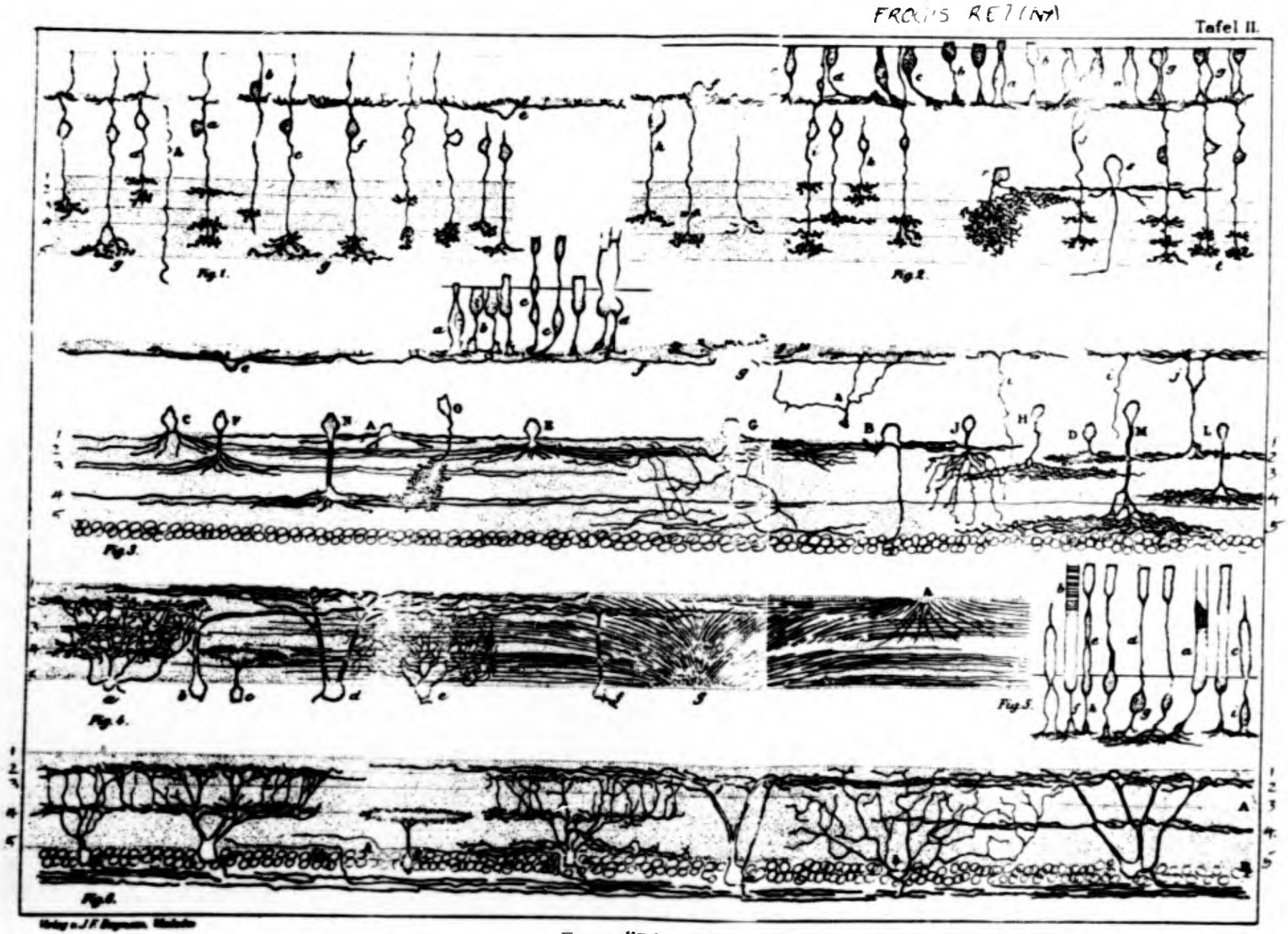
Fig. 2. a Cone nucleus; b nucleus of the common rod; c nucleus of an oblique rod; c2 nucleus of an oblique rod whose descending fiber ends in a point shape without a terminal swelling; d very long fibers originating from an oblique rod; f dislocated bipolar cell; g Landolt club; h large or outer bipolar cell; i bipolar cell with three lower ramifications; j large bipolar cell; r diffuse ramified spongioblasts; s neural spongioblasts of Dogiel.

Fig. 3. a Nucleus of a very thick cone; b rod nucleus; c nucleus of a club-shaped rod; d twin cones; e small horizontal cell; f fiber which has the appearance of an axis cylinder; g large horizontal cell with finger-shaped branches; h,i ascending fibrils, which ramify in the outer plexiform layer; j thick or outer bipolar cell; A amacrine cell, which spreads out its branches in the first sublayer; B neural spongioblast; C and E asterisk-shaped amacrine cells going to the second sublayer; D amacrine cell with tortuous tuft in the second sublayer; F and H amacrine cell going to the third sublayer; L and N amacrine cell going to the fourth sublayer; M amacrine cell going to the fifth sublayer; O and J diffuse amacrine cells; G amacrine cell which branches in two layers (double-stratified).

Fig. 4. a Quadrupally-stratified branched ganglion cell; b and f ganglion cells which branch in the second sublayer; d ganglion cell going to the first sublayer; c ganglion cell with granulose tufts in the fourth sublayer; e ganglion cell with diffuse, delicate branches; g radiating fibrils spreading out in two layers, possibly arising from a ganglion cell; h descending, fibrilar, radiating splintering of a protoplasmic stem, which probably arises from an asterisk-shaped amacrine cell.

Fig. 5. a Inner segment of a common rod; b outer segment with dark cross striations; d club-shaped rod with thread-shaped base; e club-shaped rod with conical, thick foot whose nucleus (b) lies beneath the external limiting membrane; i cone nucleus.

Fig. 6. Ganglion cells: a giant cell which ramifies in the second sublayer; b ganglion cell with diffuse, ramifying tuft; c giant ganglion cell which spreads out in two layers (second and fourth sublayers); d triple-stratified, ramifying ganglion cell; f and g medium-size and small double-stratified, ramified ganglion cells; e ganglion cell with granulose tuft in the fourth sublayer; h and k nerve fibers which appear to infiltrate the inner plexiform layer.



From "Die Retina der Wirbelthiere" by S. Ramon y Cajal (R. Greeff, ed.), Wiesbaden, 1894

Fig. 8. Cells of the retina of the frog

Ramon y Cajal noted five principal horizontal synaptic levels in the IPL, and numbered then 1 through 5, starting at the top. In the present study, although the levels are not always easy to identify, it appears that steady illumination resulted in heavy staining of level 4 (our Fig. 4) or levels 4, 3, 2 and perhaps 1 (our Fig. 5). Flickering illumination resulted in heavy staining of level 2 (our Fig. 7) or levels 2 and 4 (our Fig. 6). From Ramon y Cajal's work it appears that the cells whose processes occupy level 4 and no other level are certain amacrine cells (his Fig. 3N, L) and a small ganglion cell (his Fig. 4c and Fig. 6e). Levels 4, 3, and 2 are occupied by the dendrites of a ganglion cell (his Fig. 4a). Level 2 alone is occupied by processes of certain amacrine cells (his Fig. 3E, D), the teledendron of the large bipolar cell (his Fig. 3, j), and the dendrites of certain ganglion cells (his Fig. 4, b, f). Levels 2 and 4 are jointly occupied by dendrites of certain bipolar cells (his Fig. 6f, g, and c).

Functionally, the ganglion cells of the frog's retina have been divided into five classes by Lettvin et al[2,3] These classes are tabulated below.

Table I. Functional Classes of Ganglion Cells in the Frog's Retina

Class No.	Class Name	Phenomenon it Detects	Hartline's Class	Abundance	Conduction Rate of Axons	Size of Field
1	Sustained edge	Edge	ON	Many	Slow	1° - 3°
2	Convex edge	Bug	--	Many	Slow	2° - 5°
3	Changing contrast	Event	ON/OFF	Less	Fast	7° - 12°
4	Dimming	Adaptation	OFF	Few	Fast	10° - 15°
5	Darkness	Darkness	--	? Few	?	Large

The second and third stimulation conditions used in the present selective staining study both presented the same total amount of light to the same size area on the retina. The difference was that in one condition the illumination was steady, in the other it flickered at 3.3 cps. The flickering illumination would be expected to excite the ganglion cells of class 3, which respond to any change of light distribution in the region of retina to which each is connected (its receptive field), and class 4, which respond to any dimming of light on the receptive field. Both these classes of ganglion cells have axons that conduct nerve impulses at a fast rate. Therefore, the axons are probably large in diameter, and, from the relations observed in other neurons, the ganglion cell bodies are probably large, also.

From the fact that both these classes of ganglion cells have large receptive fields, it can be expected that both will have large horizontal spreads of their dendrites. The only ganglion cells in Ramon y Cajal's drawing which show both large cell bodies and large dendrite spreads are those of his Fig. 4, b, d and his Fig. 6a, c, g. Of these cells, all except that of Fig. 4,d have their dendrites in exactly those synaptic levels which stained heavily in the flicker-illuminated retinas. From this it seems likely that the selective staining procedure of this study stained most heavily the dendrites or dendrites synapses of those ganglion cells being most actively stimulated.

This conclusion can be tested by seeing whether it also holds true for those retinas which were steadily illuminated. There are no ganglion cells that respond to the presence of steady, uniform illumination. However, classes 1 and 2 ganglion cells respond continually to the presence of contours (light gradients) in their receptive fields. Such contours were present in the stimulus in the form of the image of the coiled lamp filament seen through the opal glass and the edges of the opal glass. These two classes of ganglion cells can be expected from the size of their receptive fields and the conduction rate of their axons to have small dendritic spreads and small cell bodies. The only ganglion cells in Ramon y Cajal's drawings which have those characteristics are those of his Fig. 4, a, c, e and his Fig. 6, e. Of these, all but the one of his Fig. 4, e have dendrites in exactly those synaptic levels which were stained most heavily in the steadily illuminated retina. The conclusion is confirmed.

The retinas kept dark-adapted did not show any heavy staining of the IPL, even though the class 5 ganglion cells (darkness detectors) would be expected to be active. But this may be because those ganglion cells are few or because their dendrites are diffusely located in the IPL (such as Cajal's Fig. 4,g and Fig. 6b).

Another piece of knowledge that can be extracted from this selective staining study is the type of information carried by the large bipolars. According to Ramon y Cajal (his Fig. 3, j) most of these bipolar cells have a large dendritic spread at the outer plexiform layer and a large teledendronal spread in the upper levels of the IPL. It is an upper level (level 2) which contains the dendritic endings of both the event and the adaptation detectors. Since any change in adaptation distribution would also qualify as an "event", the event detectors can be expected to connect to cells conveying information as to the adaptation state of the visual receptors in the various parts of the receptive field. Therefore, it can be expected that the synaptic level common to the event and the adaptation detectors (level 2) is the location of the teledendrons of the cells carrying adaptation information. The large bipolar cells, because of their large spread of teledendrons in just that level, are the cells which best meet this criterion, and therefore are most likely the carriers of adaptation information.

It should be noted that according to Ramon y Cajal a small percent of large bipolar cells differ from the ones discussed above in having smaller dendritic and teledendronal spreads and in having their teledendrons terminate

at levels other than level 2 (e.g., his Fig. 2, h, j). The function of these large bipolars cannot be determined at present.

Some further information can be extracted regarding the other classes of detectors. Only the class 1 and 2 ganglion cells were expected to be active during steady illumination. As mentioned above only two types of ganglion cells described by Ramon y Cajal (his Fig. 4a, c) correspond to the selective staining and functional characteristics of these two classes. Since class 1, the edge detector, is known functionally to have the smaller receptive field, it probably is the ganglion cell with the smaller dendritic spread, (his Fig. 4c). Ramon y Cajal found this type of ganglion cell was extremely common, and the edge detector is found functionally to be very common. This leaves his Fig. 4a as probably the bug detector.

The edge detector has its dendrites in level 4 of the IPL, therefore, this level contains the terminations of the cells carrying information as to light intensity gradients or edges at the receptors. The amacrine cells' terminations there are too widespread to provide the small receptive field of the edge detector. This leaves only the small bipolar cells (his Fig. 1) as the carriers of edge information.

The bug detectors, also, synapse at level 4 and get edge information. This is reasonable, since they report the presence of a particular kind of edge (that of a convex dark region) in their receptive field. The other synapses of the bug detector ganglion cell in levels 2 and 3 must provide the additional information needed to distinguish this particular edge. Level 2 has already been shown to carry information as to the state of adaptation of the region, and thus could signal the presence of a darker region within an edge. What information is carried by level 3 is not known.

There is a certain type of amacrine cell which according to Ramon y Cajal sends a radiating pattern of very long processes, some longer than a millimeter, within level 4 of the IPL (his Fig. 3, N). Since that is the level of edge information, and that type cell would integrate such information over a large area, it may be that such a cell type is concerned with recognition of the granularity (edgedness) of a light pattern. Where such information would be transmitted by an amacrine cell, which lacks an axon, is not known, so this suggestion cannot be readily tested.

Other sections of the retinas tested were stained with acridine orange. This did not show any readily recognizable differences in staining between the steady and flicker illumination conditions, but both of these showed more intense staining of the nuclei and cell bodies than did the dark-adapted condition.

In summary, even this exploratory study of selective staining has shown the great value of such techniques for unraveling the relations between cell connectivities and information processing function. Grateful acknowledgment is made of the inspiration provided by Dr. Jerome Lettvin, who in one paper[4] and his unpublished studies anticipated a number of these findings.

D. Topology of ganglion cells

A sample study was made of the exact spatial arrangement of a ganglion cell and its processes. A light adapted retina of Rana catesbiana (bull-frog) was stained by the Golgi technique and sectioned serially. Two particular ganglion cells which had staind heavily were traced microscopically through the sections. The locations of all points of branching or inflection were measured in three dimensions and from these measurements the top, front, and side views of the cells were drawn to scale (Figs. 9 and 10). The work is extremely tedious and lengthy --- these two figures represent six man-weeks of effort.

At first it was attempted to vitally stain the living, isolated retina with methylene blue dye and examine the retina immediately with the light microscope. It was hoped that this procedure would minimize the distortion produced by mechanical processes such as embedding, sectioning, mounting, and shrinkage during fixation. This technique was abandoned because the neural cell processes did not stain heavily enough to be traced for their entire lengths.

Serial sections of the retina stained with methylene blue gave better visualization, but were still not adequate. By far the best results to date were obtained with the following modified Golgi technique.

A Rana catesbiana was light adapted for 20 minutes in a white enameled pail from a 100 watt light bulb. Both eyes and the brain were removed, hemisected and placed in a 50 cc 1% cobalt nitrate-6 cc formalin (pH 3.1) fixing agent for 24 hours at room temperature. The tissue was removed and washed in distilled water for 20 minutes, then placed in a 1% solution of $AgNO_3$ at 37°C for 24 hours. Then the tissue was washed in distilled water and placed in hydroquinone reducer (Cajal's) for 16 hours. The tissue was removed and placed in 2% celloidin and methyl benzoate for 48 hours at room temperature. It was then placed in benzene for four hours at room temperature, transferred to fresh benzene for four hours at room temperature, then placed in 50%/50% benzene-paraffin for 15 minutes. After one hour in paraffin, it was imbedded by paraffin/celloidin (2%) at 54°-56°C. It was then serial sectioned, in 15 micron thicknesses.

The serial sections were examined with the light microscope and one of the selectively stained ganglion cells and its processes were traced through a group of adjacent sections. An American Optical Microstar microscope was used, and observation progressed (using a 15x eyepiece) through 10x, 20x, 43x objectives and a 97x oil immersion objective. After all structures of the ganglion cell were identified, separate sketches of the portions of this cell in each section were made using the 97x oil immersion objective and a 10x eyepiece with a reticule. Each unit on the reticule grid corresponded to a 10μ distance at the plane of focus (as determined with a stage micrometer). The drawings were made on graph paper to correspond with this grid and depth measurements were made in microns by means of a calibrated depth of focus adjustment. Using points of reference in the sections to

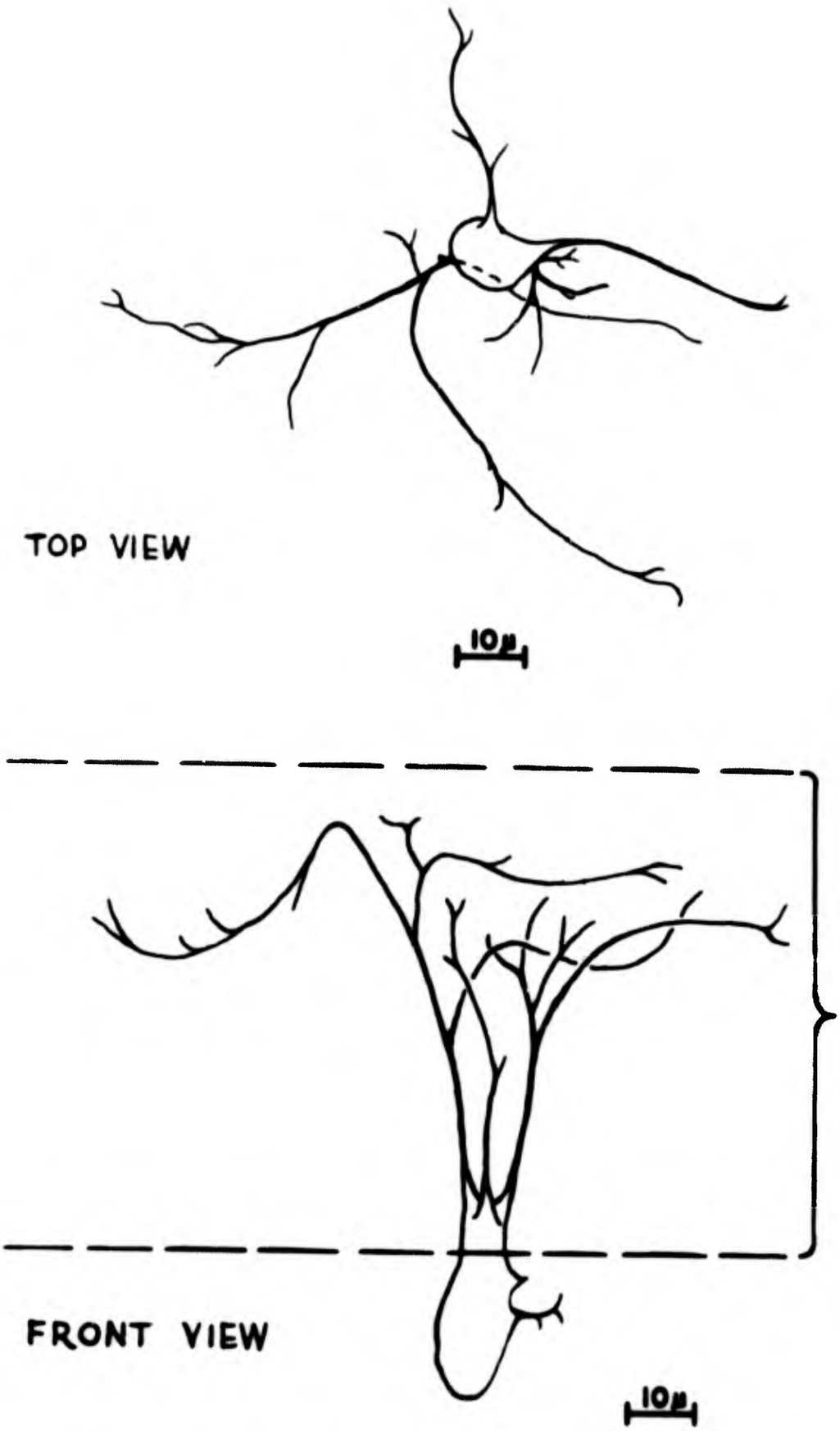
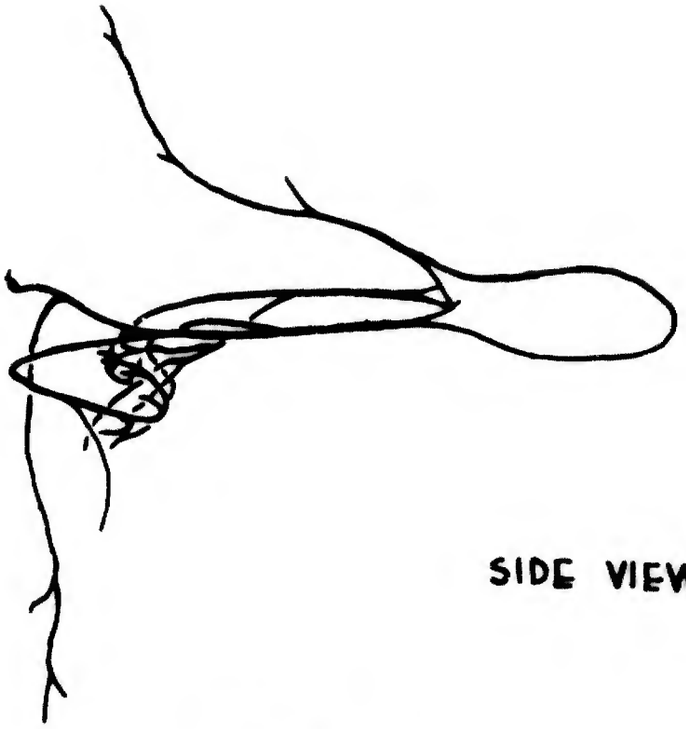


Fig. 9a



INNER
PLEXIFORM
LAYER

GOLGI STAIN

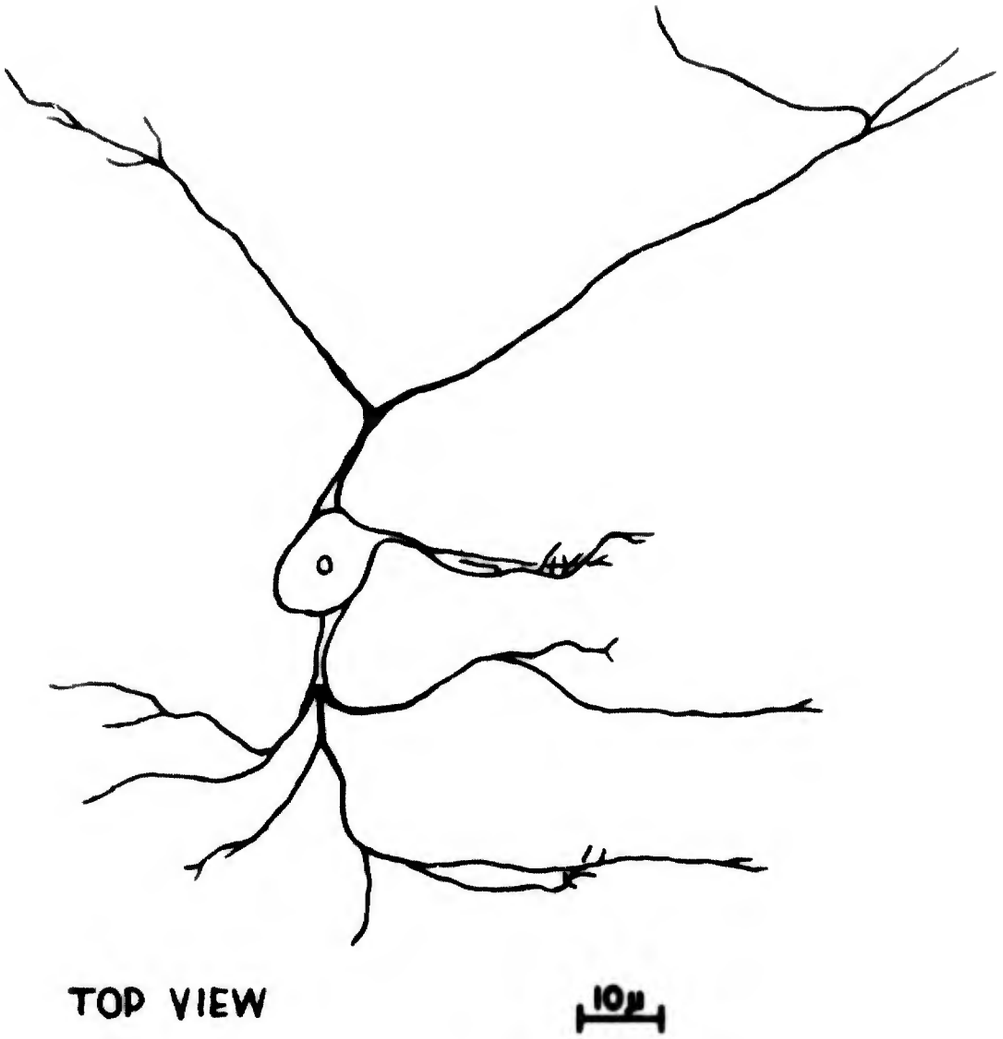
LIGHT ADAPTED RETINA

Rana catesbiana

TRIAL 6 18

CELL HEIGHT IN FRONT AND SIDE VIEWS 2 X SCALE

Fig. 9b



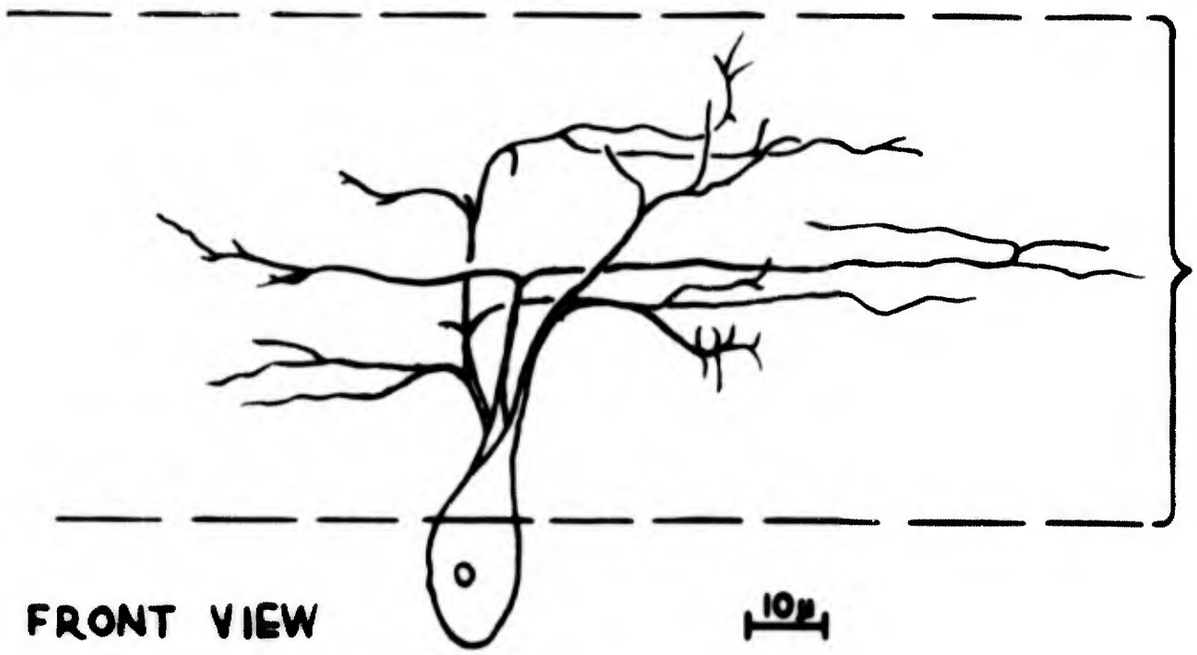
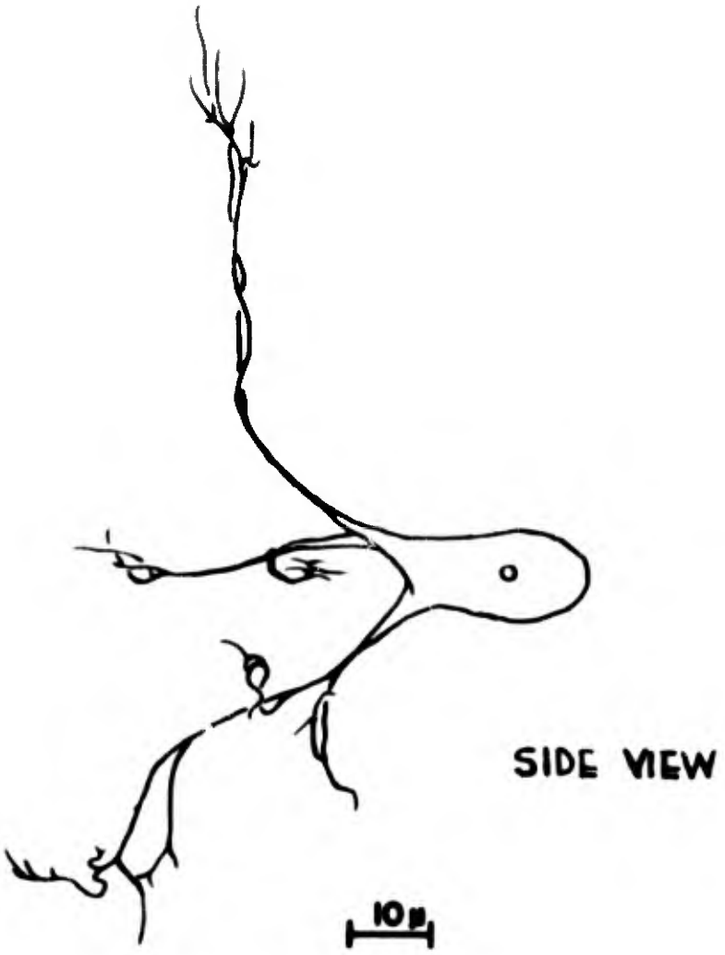


Fig. 10a



INNER
PLEXIFORM
LAYER

GOLGI STAIN

LIGHT ADAPTED RETINA

Rana catesbiana

TRIAL G 18

CELL HEIGHT IN FRONT AND SIDE VIEWS 2 X SCALE

Fig. 10b

determine orientation and alignment of the individual drawings, composite drawings were made of a side view of the cell; that is, a view of a plane perpendicular to the inner surface of the eye. From this composite drawing and the depth readings a top view are derived, (that is, a view of a plane parallel to the inner surface). These drawings of one such ganglion cells are shown in the attached figures.

Figure 9 is of a ganglion cell which has almost all its dendrites in level 2 of the IPL. It resembles the cell of Ramon y Cajal's Fig. 6,a (Fig. 8), and is probably an adaptation detector (see Section C previous). The top view shows that its dendritic field is only roughly circular.

Figure 10 is of a ganglion cell which has most of its dendrites in levels 2 and 4 of the IPL. It resembles the cell of Ramon y Cajal's Fig. 6,g (Fig. 8), and is probably an event detector. It can be seen that its dendrites are not symmetrically arranged. Thus, the top view shows that the dendrites at the top of the view go only to synaptic level 4 (edge information), while the dendrites at the bottom of the view go primarily to synaptic level 2 (adaptation information). This should make the ganglion cell responsive to adaptation changes on one (top) side of its receptive field and to edge changes on the other (bottom) side. Presumably, such a ganglion cell would respond to dimming, but not light redistribution in the top half of the field, and vice versa for the "bottom" half. Such a detector would respond especially well if the dark image of an animal's body fell on the "top" half of the field and the moving legs were imaged on the "bottom" half.

E. Ultrastructure of the frog's retina

1. Techniques of electron microscopy

The retinas whose electron micrographs appear in this report were prepared by osmium tetroxide or potassium permanganate fixation and by embedding in either Epon or Maraglas epoxy resins. The osmium fixation has been described in Section II B. The potassium permanganate fixation procedure is the same except for the fixative used. The fixative is made up from stock solution #1, which consists of 8.35 ml of 0.1 N HCl in 1000 ml of doubly distilled water, and stock solution #2, which consists of 19.02 gm of 0.14 M sodium acetate and 28.86 gm of 0.14 M sodium veronal brought to 1000 ml with doubly distilled water. Equal parts of stock solutions #1 and #2 are added and adjusted to pH 7.4 with Tris buffer to make the veronal acetate solution. The osmium fixative is prepared by adding equal parts of the veronal acetate solution and a 2% aqueous solution of osmium tetroxide and then chilling. The potassium permanganate fixative differs only in that a 2% aqueous solution of potassium permanganate is used instead of the osmium tetroxide.

The Maraglas embedding procedure was described in Section II B. The Epon embedding procedure was the same through the dehydration. The tissue is immersed in propylene oxide for 15 minutes. It is then placed for 15 minutes in propylene oxide containing about 10% by volume of Epon embedding

mixture which was thoroughly stirred in. The tissue is then immersed in the full strength Epon mixture for 2 to 3 hours. Capsules are filled with the mixture and the tissue strips laid on the surface of the mixture. After the tissue has settled to the bottom, the capsules are placed in a pre-heated oven at 60°C for 24 hours or longer.

The Epon embedding mixture is formed from stock solutions A and B. Stock solution A is 62 ml of Epon 812 in 100 ml of dodecenyl succinic anhydride (DDSA). Stock solution B is 100 ml of Epon 812 mixed with 89 ml of methyl nadic anhydride (MNA). The solutions are kept refrigerated until just before use, when they are warmed to room temperature, or at least above dew point, mixed together thoroughly, and then 2% by volume of benzyldimethyl amine is added.

The blocks of embedded tissue were trimmed to a square pyramidal shape and sectioned on a Porter-Blum MT-1 ultramicrotome with a Dupont diamond knife having a 45° cutting angle. The sections floated onto a water surface, were picked up on carbon-coated copper grids and air dried. The grids were then immersed in a saturated solution of uranyl acetate in 70% alcohol for 45 minutes. Each grid was then grasped at one edge with the tips of fine forceps, lifted out, rinsed first with clean 70% alcohol and then with doubly distilled water squirted gently onto it at a 45° angle from a plastic squeeze bottle. The grids were then immersed for 30 minutes in a small covered container holding a lead citrate solution. The solution is made by combining in a 50 ml volumetric flask 1.33 gm lead nitrate, 1.76 gm sodium citrate, $Na_3(C_6H_5O_7)\cdot 2H_2O$, and 30 ml of distilled water. The flask is shaken for one minute and allowed to stand for 10 minutes. This is repeated 3 or 4 times to permit complete conversion of the lead nitrate to lead citrate. Then 8 ml of 1.0N sodium hydroxide is added and the mixture is diluted to 50 ml with distilled water, then mixed by inverting the flask several times. The lead citrate dissolves, and the stain is ready to use.

After staining, the grids are washed again as described above. After air drying the grids are ready for placing in the electron microscope.

An RCA-3F electron microscope with a double condenser lens was used at 50 or 100 kV with Ilford No. 40 glass photographic plates, 2" x 10" in size. Grateful acknowledgment is made to Drs. D. Scarpelli and M. Greider of the Pathology Department of this University for allowing the use of the electron microscope and for advice during the development of some of these techniques.

The retinas were all from leopard frogs (Rana pipiens) which had all been treated the same prior to fixing the retinas. This has been described in Section II B.

Retina G58 was fixed with osmium tetroxide and embedded in Maraglas. Retina G60 was fixed in potassium permanganate and embedded in Maraglas.

2. Illustrative electron micrographs of the frog's retina

The legend for each appears opposite the micrograph.

Fig. 11. Electron micrograph (G58) is a portion of two cuboidal pigment epithelial cells. Numerous lamellated structures (L.S.) 4 to 5 microns long are shown in the basal halves of these cells whereas smaller similar structures are found in the apical halves. The typically round nucleus (N), a large complement of mitochondria (M), and a large lipid droplet (L.D.), bound by a dense peripheral region, are commonly found in this type of cell. Pigment granules (P.G.) which have a wide range of sizes and shapes, occasionally appear to be surrounded by a dense protein-like ground substance (arrow at center). Other pigment granules appear to float freely in the cytoplasm, while still others are located in the pigment epithelial cell processes (arrow at bottom), which extend as far as the inner segments of the receptors. A large number of vesicles (V), some of which appear in close apposition to lamellated bodies (arrow at left), make up the smooth endoplasmic reticular system and are generally believed to contain metabolic substances (enzymes?) transported by the cell.[5] These vacuoles are so numerous that they appear to take up a greater volume of space than the ground substance of the cytoplasm.[5] Such a vast system of endoplasmic reticulum indicates a highly active cell. Some of the vacuoles may be attributed to fixation artifact.

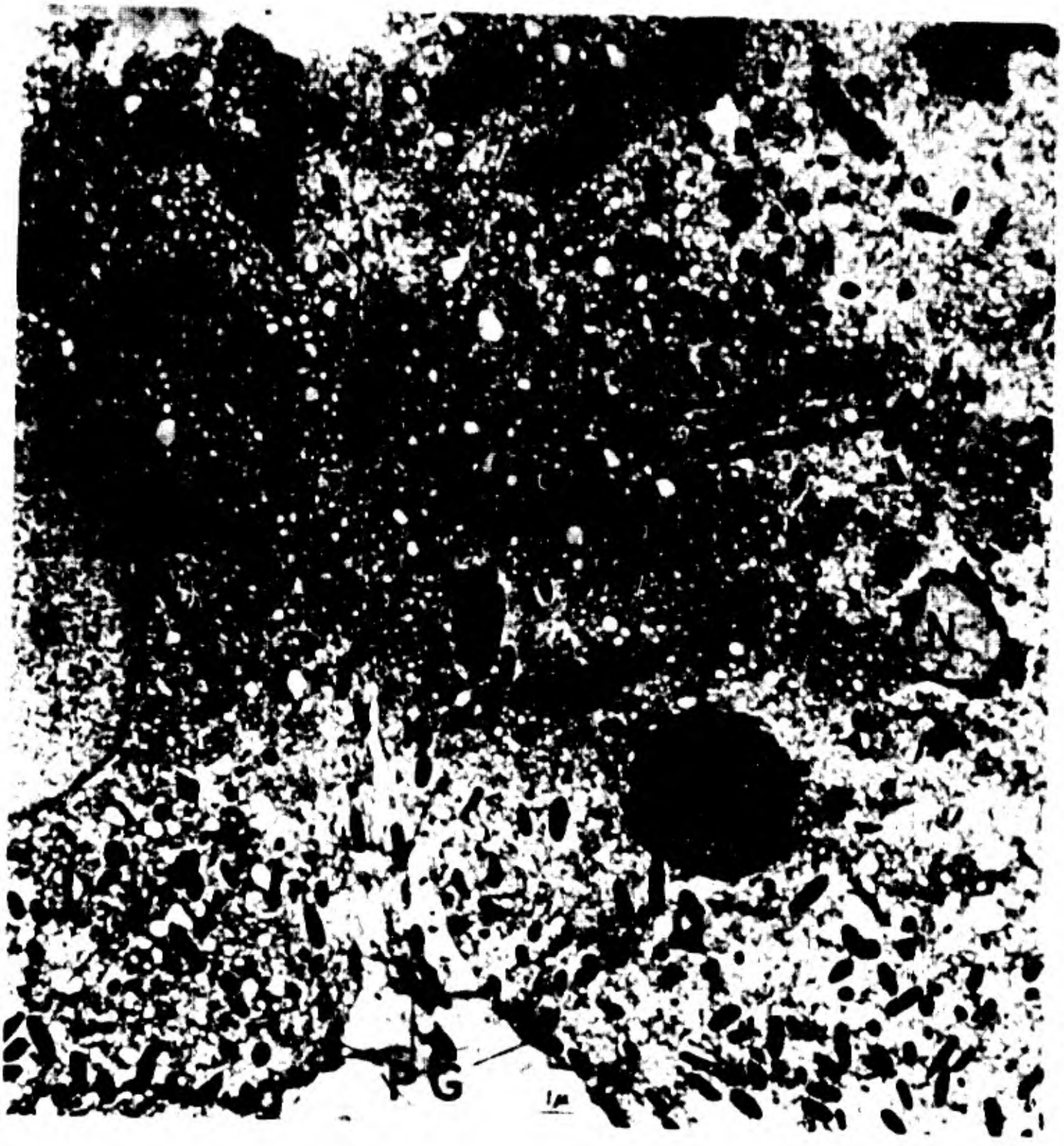

N
PG
1μ

Fig. 12. Electron micrograph (G58) is a dense osmiophilic lamallated structure found in a pigment epithelial cell. This myelin figure is approximately 3μ in diameter and has a membrane periodicity of 80Å - 100Å. The membrane interspace is slightly more than the width of a single membrane and the total distance covered by two membranes and one interspace is approximately 160Å to 200Å. Therefore, the membranes in this structure must approach the thinnest membranes resolved with osmium-fixed material.[6]

Several clefts are formed by apparent separation of the lamellae. This may be artifact caused by polymerization stresses. This myelin-like figure appears to be surrounded by a unit membrane which also encloses some ground substance (arrow). At the lower right side of the figure is a Golgi complex (G) with its associated vesicles (compare the size of these membranes with those of the myelin figure). Dense granules of glycogen-like particles are also seen in the matrix of the cytoplasm (g).

Fig. 13. Electron micrograph (G58) shows a junction of three pigment epithelial cells. Numerous vacuoles (V) ranging from less than 0.1μ to 1.0μ across are shown in the matrices of these cells. (The contents of the vacuoles are probably metabolites or synthetic materials produced by the cell.) Some of the vacuoles (smooth E.R.) are almost empty while others appear to be completely filled with an amorphous material (arrow). The pigment epithelium plays an important role apart from the phenomenon of pigment migration in that those metabolic substances, which are essential to visual perception, pass through the pigment epithelial cells.[2] Numerous tonofilaments (f) in single strands and in aggregates (arrows) appear in the peripheral region of the cell. These filaments are commonly found in most types of epithelial cells and are presumed to add support to the soft cell. Glycogen-like particles (g) and mitochondria (m) are also present in these cells. In the lower right corner of the micrograph is a folded membrane system resembling a Golgi complex in which the membranes surround vacuoles at the edges of the folds.

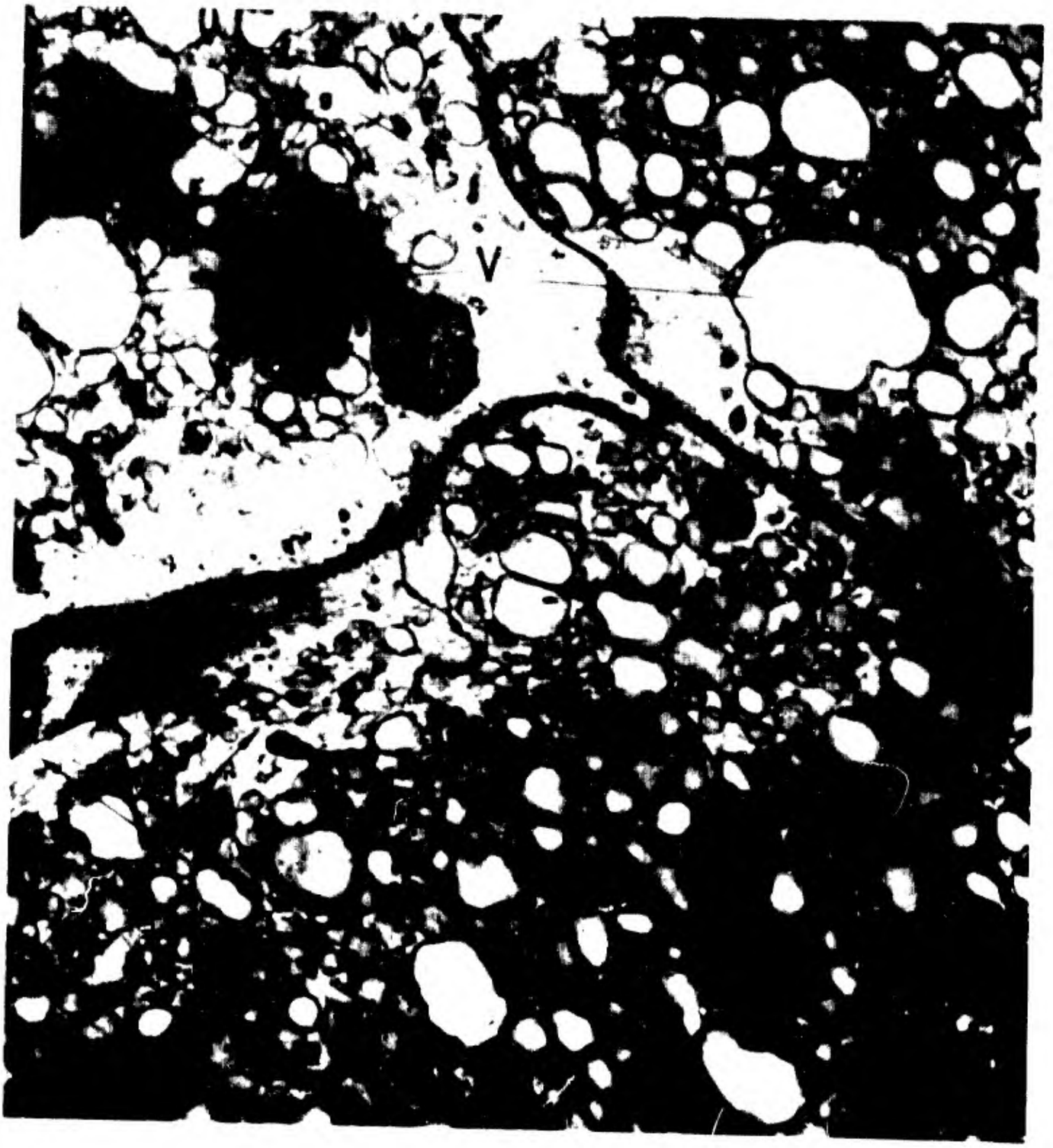

V

Fig. 14. Electron micrograph (G58) is an atypical outer segment of a retinal rod which extends to the pigment epithelium. Along one side of the outer segment (O.S.) is a lamellated system of membranes (M) which lie perpendicular to the rod discs. This membrane system extends for more than 25μ along the outer segment, and ends with its tip near the mid-line. From the pigment epithelium cells (P.E.) processes (p) which contain pigment granules are seen extending into the interstitial space outside of the outer segments. These processes have been observed to extend as far as the visual cell inner segments and the villous processes of the Müller cells. The pigment granules are often seen in close contact with the outer segments.[7] Upon illumination of the retina free radicals are generated in these granules.[8] It may be that these free radicals of the granules react with the outer segments, either chemically or electrically, in the response of the rods to light. Also, since it is believed that the pigment epithelium supplies metabolites for the outer and inner segments, as well as the general retinal metabolism,[5] the pigment processes (which also contain E.R.) could serve as convenient vehicles for the transport of metabolites in addition to the migrating pigment granules.

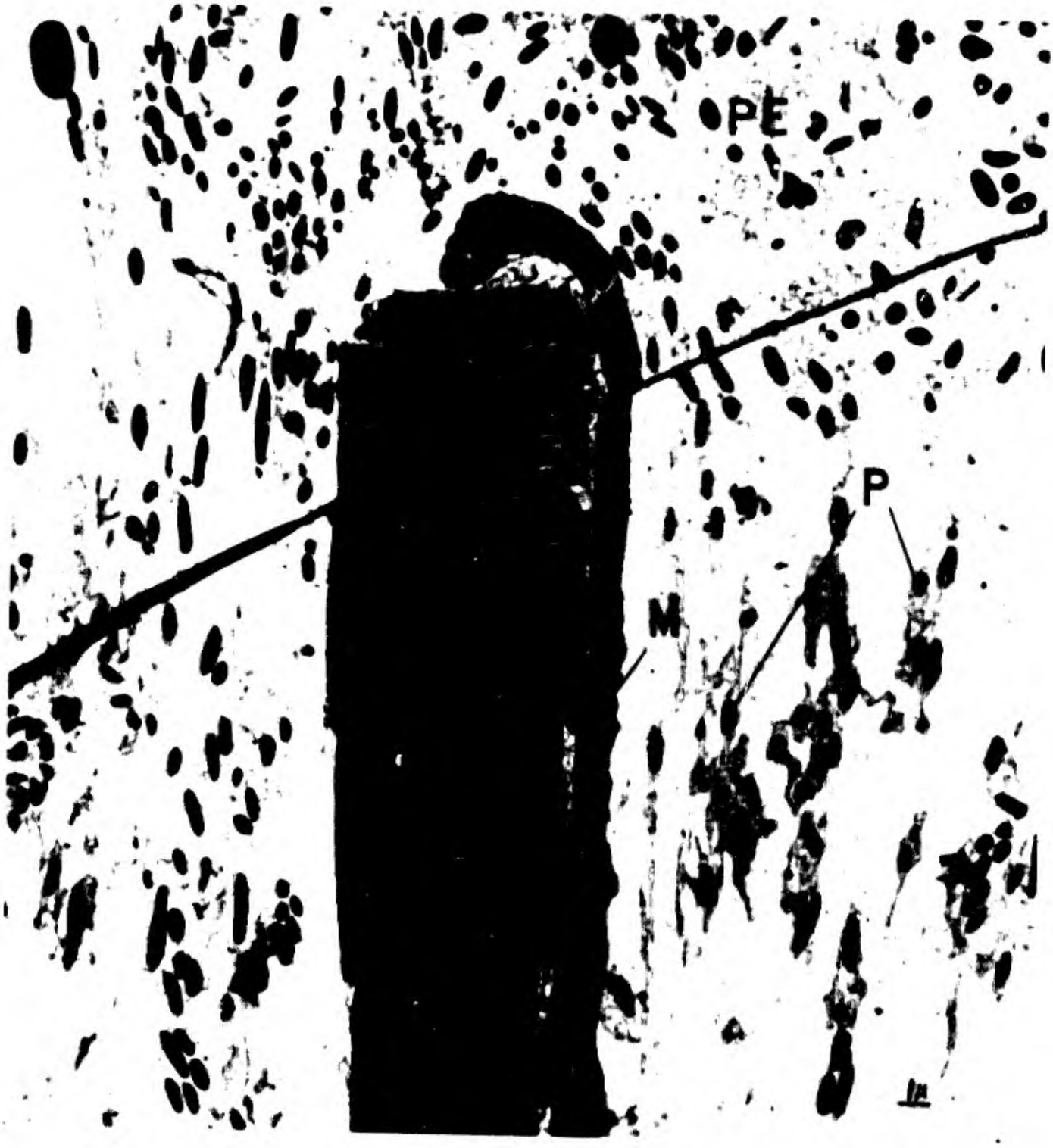

PE
P
M

Fig. 15. Electron micrograph (G58) is a more detailed picture of the lamellated membranes of the peculiar retinal rod.

The perpendicular membrane system appears to be a folded system of about eighteen triple layered membranes (arrow). These membranes have the same membrane width and interspace as do the rod discs (see Fig. 16 for disc membrane dimensions). Note how the plasma membrane (arrow at right) continues around the folded membrane system. The large vacuoles (V) located between the two sets of lamellae suggest that the outer segment is undergoing further development of its lamellar organization. Fissures (F) which separate stacks of rod discs are clearly shown where the disc membranes form loops (lower arrow). Other fissure-like structures, which either are the beginning of fissure development or are the result of a linear irregularity of the membranes, are also seen (fl).

In the top part of the electron micrograph are pigment processes (P.P.). Two to three pigment granules (P.G.) are located within the plasma membrane. In addition, each granule is surrounded by another unit membrane which indicates its lack of contact with the cytoplasmic ground substance of the pigment cell. Glycogen-like particles are also found in the processes.

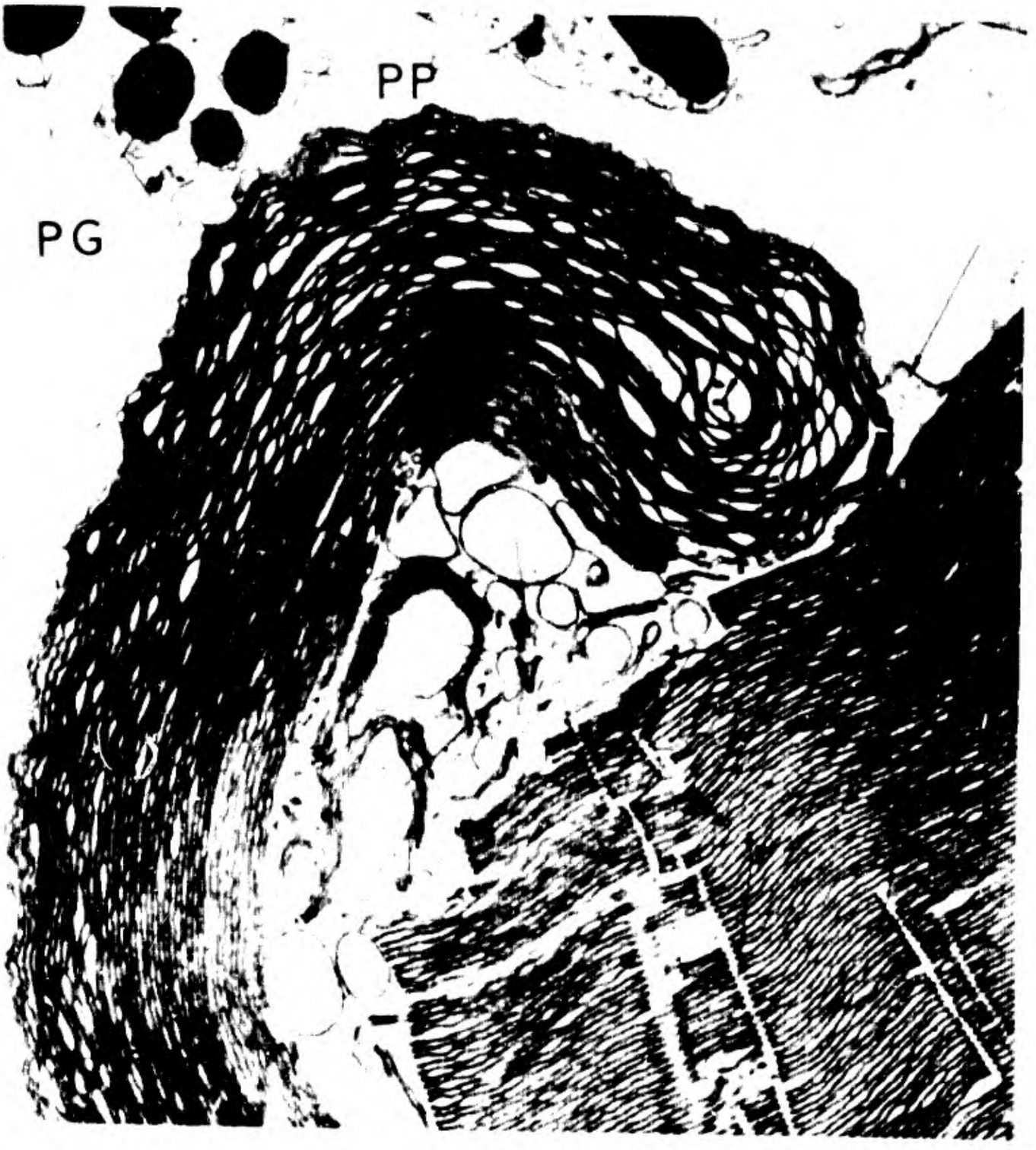

PP
PG

Fig. 16. Electron micrograph (G58), is another rod outer segment shown in detail to illustrate the regularity of the outer segment discs. The piles of flattened discs are formed by two dense membranes, each of which is about 70Å thick, enclosing a less dense space which is about 150Å thick at the middle of the disc and about 250Å thick at the peripheral loop making up the edge of the disc. Numerous single membrane structures are located in the peripheral border of the rod. Many appear to be flattened, others are round. These organelles are believed to be developing forms of the discs that make up the outer segment.

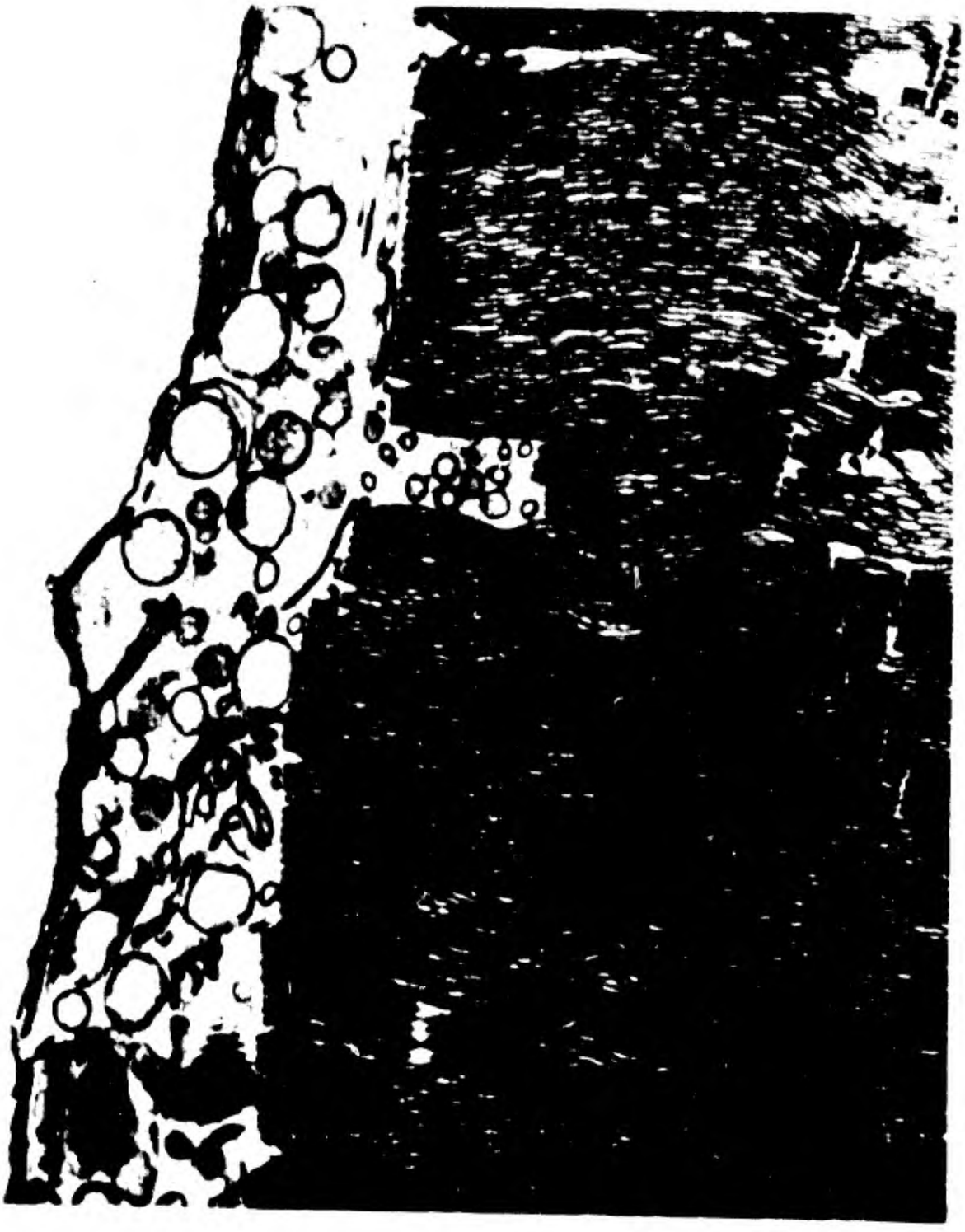

0.5 μ

Fig. 17. Electron micrograph (G58) is an accessory cone cell. The lamellar organization of the outer segment of the cell appears to be undergoing further development. Notice how the vesicles are arranged in a linear formation. There appears to be a row of vesicles for each triple-layered membrane adjacent to them. This could mean either the development of the membrane system or its breaking down, but is probably the development, since this preparation came from a young, apparently well-nourished frog. The connecting cilium (CC) with its filaments and basal plate are clearly visible in the micrograph. Fragments of a ciliary rootlet (arrows) are also visible. The calyx (C) of the cone cell extends along the left side of the outer segment for approximately 2μ. A number of mitochondria (m) and glycogen-like particles (p) are found in the inner segment. Two pigment granules, (PG) bound by a membrane which sometimes can be seen to be continuous with the endoplasmic reticulum, are enclosed within the plasma membrane of the pigment process.

Fig. 18. Electron micrograph (G58) is of a principal cone cell which appears to be undergoing development. In the outer segment of a mature cell one finds a well-organized lamellar formation. Here, there are numerous vesicles in no particular organizational pattern and a few lamellae arranged in a bizarre fashion at the tip. The outer segment is approximately 6μ long and the ciliary fibrils (f) extending upward from the connecting cilium (CC) are at least 5μ long. The oil droplet (OD) is rather small and is located in the distal portion of the inner segment. Well developed mitochondria (m) and glycogen-like particles (p) are also located in the inner segment.

To the upper left and lower right of the cone cell are portions of apparently well-developed rod cells.

Fig. 19. Electron micrograph (G56) is a portion of a rod inner segment on the left and a cone inner segment on the right. These cells are identified by the characteristic types of mitochondria that are found within their inner segment. The cone has very well-defined cristae in the mitochondria (arrow) while the cristae of the rod mitochondria (arrow) are poorly defined.

The rod inner segment has a large accumulation of glycogen-like particles (G.P.), most of which are arranged in rosettes. Smooth vesicles (SER) of endoplasmic reticulum are also found among the glycogen rosettes.

The cone cell has smooth and rough (RER) varieties of endoplasmic reticulum. There is also an abundance of R.N.P. granules, most of which are associated with the rough endoplasmic reticulum. Neurofibrils are seen along the right border of the cone cell (lower arrow).

Although the cells are contiguous, they are separated by an interspace of approximately 100 - 150Å. All the cells of the frog's retina are separated by a similar interspace when fixed in osmium tetroxide (OsO_4).[9,10]


0.5M

Fig. 20. Electron micrograph (G56) is a cross section of three visual cells. Two of the cells are sectioned through the nucleated region and show thin fins (F) of cytoplasm extending radially from the cell body. Where the fins or cell body come in contact with another cell, at the level of the outer limiting membrane, there is a dense condensation of substance on the cytoplasmic sides of the membranes which forms a terminal bar (arrows). There the interspace between plasma membranes of adjacent cells is considerably less than normal. These terminal bars provide mechanical stability and probably prevent movement of substances from the aqueous space above the limiting membrane into the gaps between the visual cells. The fins increase the amount of visual cell surface exposed to the aqueous space.

Located in the aqueous space between the two visual cells and among the villous processes (VP) of the Müller cells are ciliated endings, called Landolt clubs (L.C.), of the small bipolar cells. Within the plasma membrane is a cilium which has nine groups of filaments arranged in a circular formation. Each group of filaments appear to form a triad of fibrils. The central group has not been resolved.

A cone cell in the upper right corner is identified by its characteristic mitochondria.


0.5μ

Fig. 21. Electron micrograph (G56) is an enlargement of a region like that shown in Fig. 20. The cilium extension (C.E.) from a Landolt club is seen in cross section at the upper right. At this level it shows 9 groups of peripheral filaments, but seems to be losing its central groups.

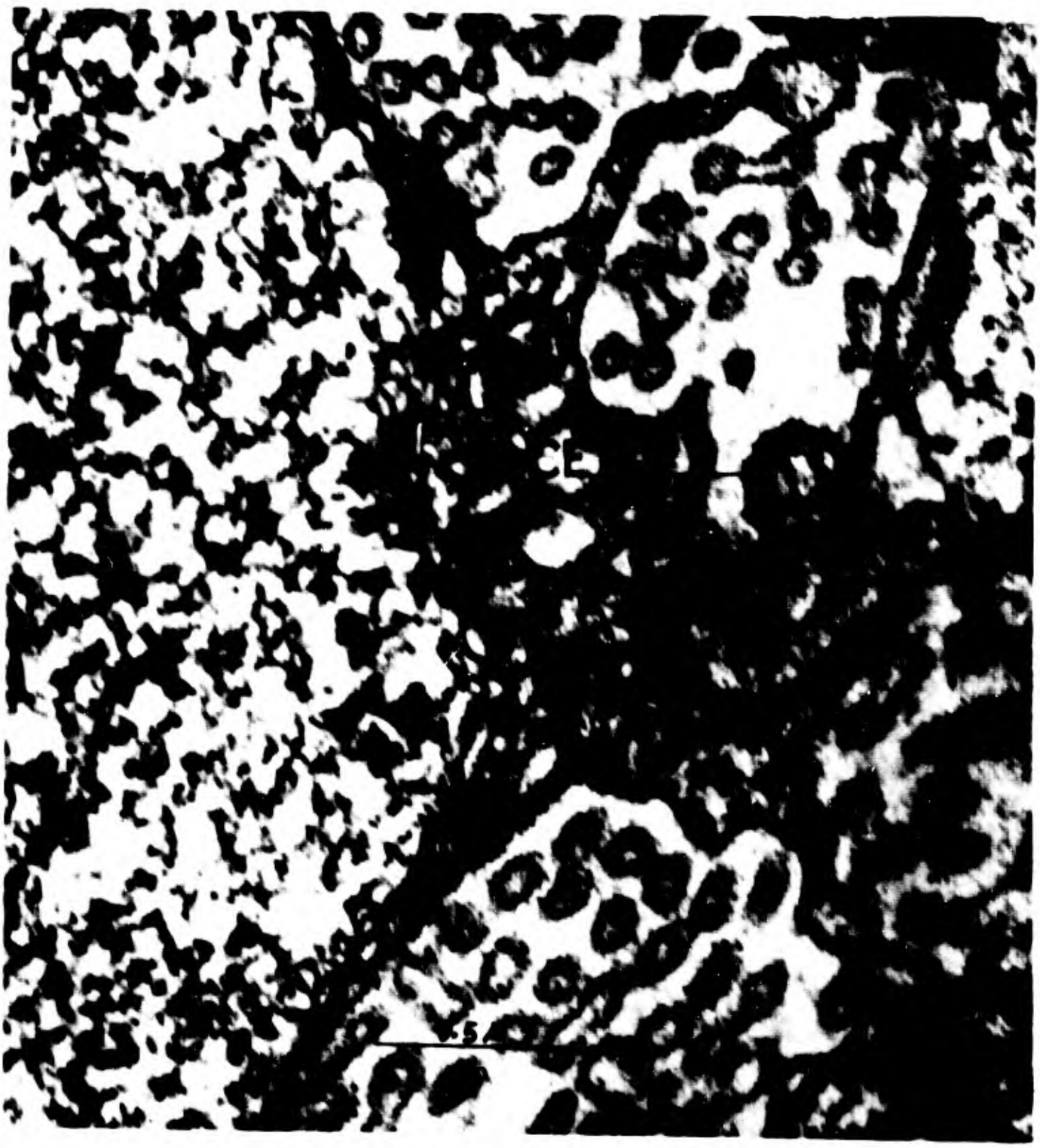


Fig. 22. Electron micrograph (G58) is a region of the retina near the outer limiting membrane. Portions of two visual cell myoids (My) are visible on each side of the micrograph. At the top center, a Landolt club ending appears in two portions. The isolated tip extends up among the villous processes (VP) of the Müller cells (MC) and contains a cilium cut at an oblique angle. The main process of the Landolt club (L.C.) contains the other centriole (Ce) and has numerous vacuoles (V) and mitochondria (m) within. The Müller cells, which lie in close proximity to the Landolt club and visual cells, but can be distinguished from neurons by the absence of neurofilaments, have a large deposition of glycogen-like particles (p) as well as mitochondria (m on left). Note how the Müller cells fold into thin vertical strips of cytoplasm (F).

The outer limiting membrane consists of dense condensations of osmiophilic material on the cytoplasmic sides of the plasma membranes, called terminal bars. The terminal bars are believed to serve as adhesion plates which maintain rigid cell-to-cell spatial relationships. Note that the terminal bars (T) are formed only from a Müller cell to a visual cell; a Müller cell to a Landolt club; or a Müller cell to a Müller cell. Terminal bars are also found between visual cells, as in Fig. 20. They have never been found between Landolt club and visual cell, those two cells always being separated by Müller cells right up to the outer limiting membrane.


VP
M
m
V
LG
F

Fig. 23. Electron micrograph (G58) is a topographic view of the visual cell nuclear region showing many of the structures already mentioned. The section is oriented with the sclerad direction toward the top left. Several visual cell nuclei (N), myoids (M), and synapses (S) are shown. Landolt clubs (L.C.) with their ciliated endings, and Müller cells with their cytoplasmic folds (F) and villous processes (VP) are also clearly shown.

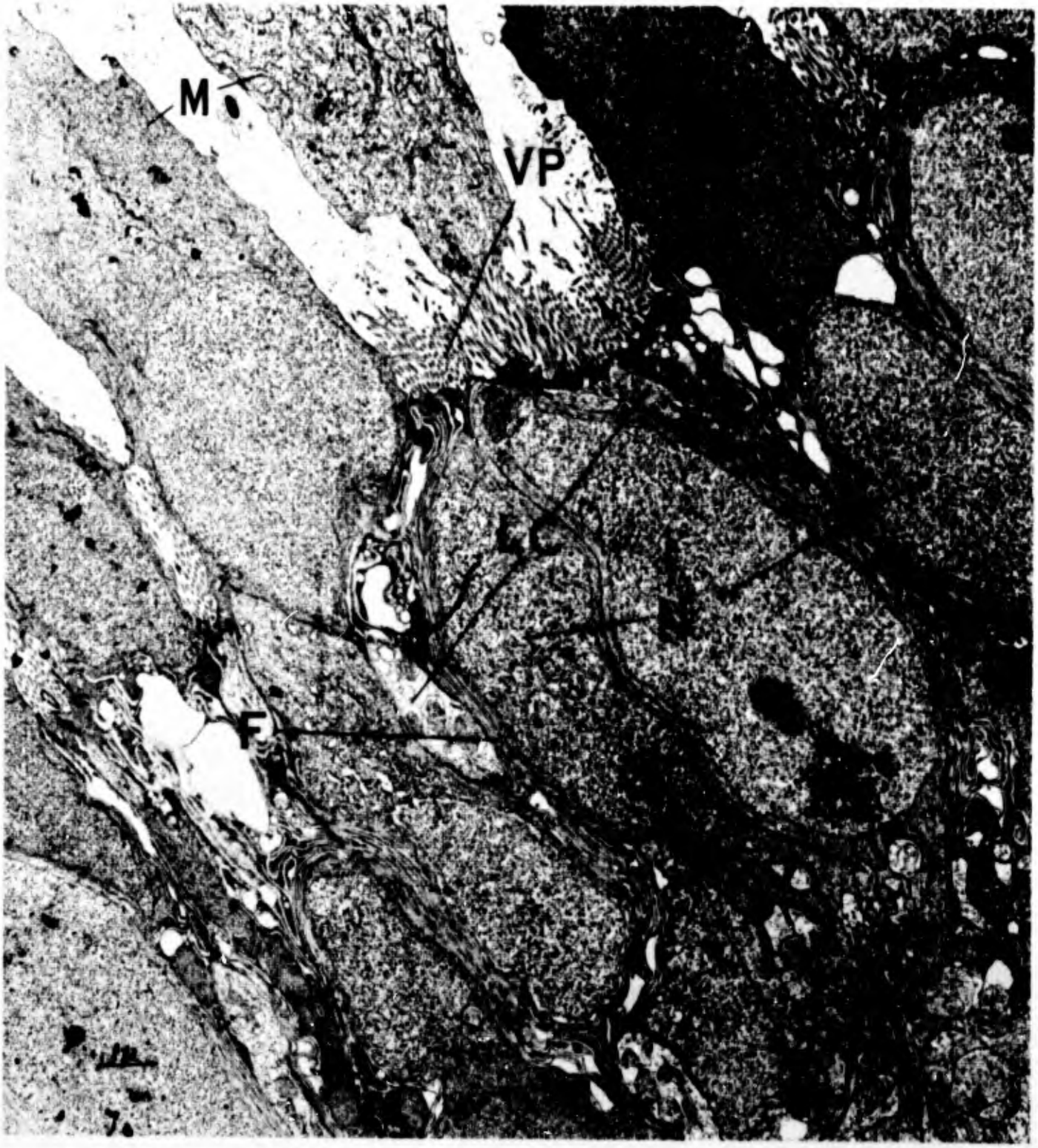

M
VP
F

Fig. 24. Electron micrograph (G58) shows portions of two Landolt clubs (L.C.) located between two visual cells (V.C.). Each club is separated by Müller cells (M.C.) from other cell types. Only a thin strip of Müller cell separates the right-hand club and visual cell. This Landolt club has been traced for more than 16μ. Its widest portion measures about 2μ across and is located near the synaptic region of the visual cells. Its outer ending is visible in this micrograph. It is dart-shaped and tapers off into the ciliated portion (C), which contains the fibrils of the cilium surrounded by a scant rim of cytoplasm. At the bottom, the Landolt club appears to branch (B) toward the right through a gap (ga) in the Müller cells and possibly contact the visual cell below the nucleus (N). Numerous neurofibrils (nf) are seen within the Landolt clubs as well as the visual cells. The lefthand Landolt club contains the type of mitochondria (m) which are characteristic for this kind of neuron. A high concentration of glycogen-like particles (g) appear in the Müller cells, visual cells and the Landolt clubs in this region of the retina with this particular embedding and staining procedure. The fin (f) of a visual cell is seen top right among the villi (V) of the Müller cells.


F
VC
MC
B

Fig. 25. Electron micrograph (G58) shows the unique structure of the Müller cells (M.C.) in the region of the visual cell nuclei (V.C.N.). Much of the cytoplasm of the Müller cells are bound in thin sheets which are folded many times. This is especially true between the visual cells and between Landolt clubs and visual cells. The folds of cytoplasm contain an abundance of dense granules which resemble in size and shape the glycogen particles commonly observed in amphibia with this type of preparation (lead complexing stains).

Numerous vacuoles are scattered throughout the folds. Some of these vacuoles have been traced directly to the aqueous space between the visual cells, among the villous processes. Since the vacuoles are restricted entirely to the Müller cells, it is probable that there was a difference in tonicity between the Müller cells and neuronal elements.

The mitochondria (m) are an unusual variety. Rarely do they have well developed cristae, but they nevertheless seem to resemble mitochondria in every other respect. They sometimes adhere to the plasma membrane of the Müller cell and are thus in direct proximity to the visual cell. It has been suggested that metabolites are transferred there from the Müller cell mitochondrium to the visual cell.[11]


MT

Fig. 26. Electron micrograph (G58) is a visual cell — bipolar cell synapse. This micrograph includes most of the common characteristics of this specialized synapse.

Extending into the synaptic region from the upper right is the pre-synaptic endfoot of a visual cell. Notice the large complement of synaptic vesicles (V), which range from 400Å to 200Å in diameter. Three rather long synaptic ribbons (SN) about 250Å in width, which sometimes appear as double bands (arrow) show the characteristic alignment of synaptic vesicles in rows along each side. Just beyond the lefthand tip of each ribbon is a thickening and increased density of the cell membrane which comprises the synaptic interface. Where there is a change in thickness, staining properties, or separation of the plasma membranes, or where one finds a special structure adjoining the plasma membrane, it seems probable that in that region specialized cell-to-cell transfer occurs. This transfer might be of chemical or mechanical or electrical energy.[12] Those surface contacts, which are capable of the rapid intercellular transfer of activity, are called "synapses."[13] At the left center is a bipolar cell process (bcp) with its swollen ending making direct synaptic contact with the visual cell. There seems to be a lack of synaptic vesicles in that process, which is the post-synaptic element of the synapse.

Each synaptic membrane appears to be about 70Å to 100Å thick and they are separated by a space of approximately 150Å to 300Å. Below, another neural process (NP) shows neurofilaments (nf), glycogen-like particles (p) and other inclusions.

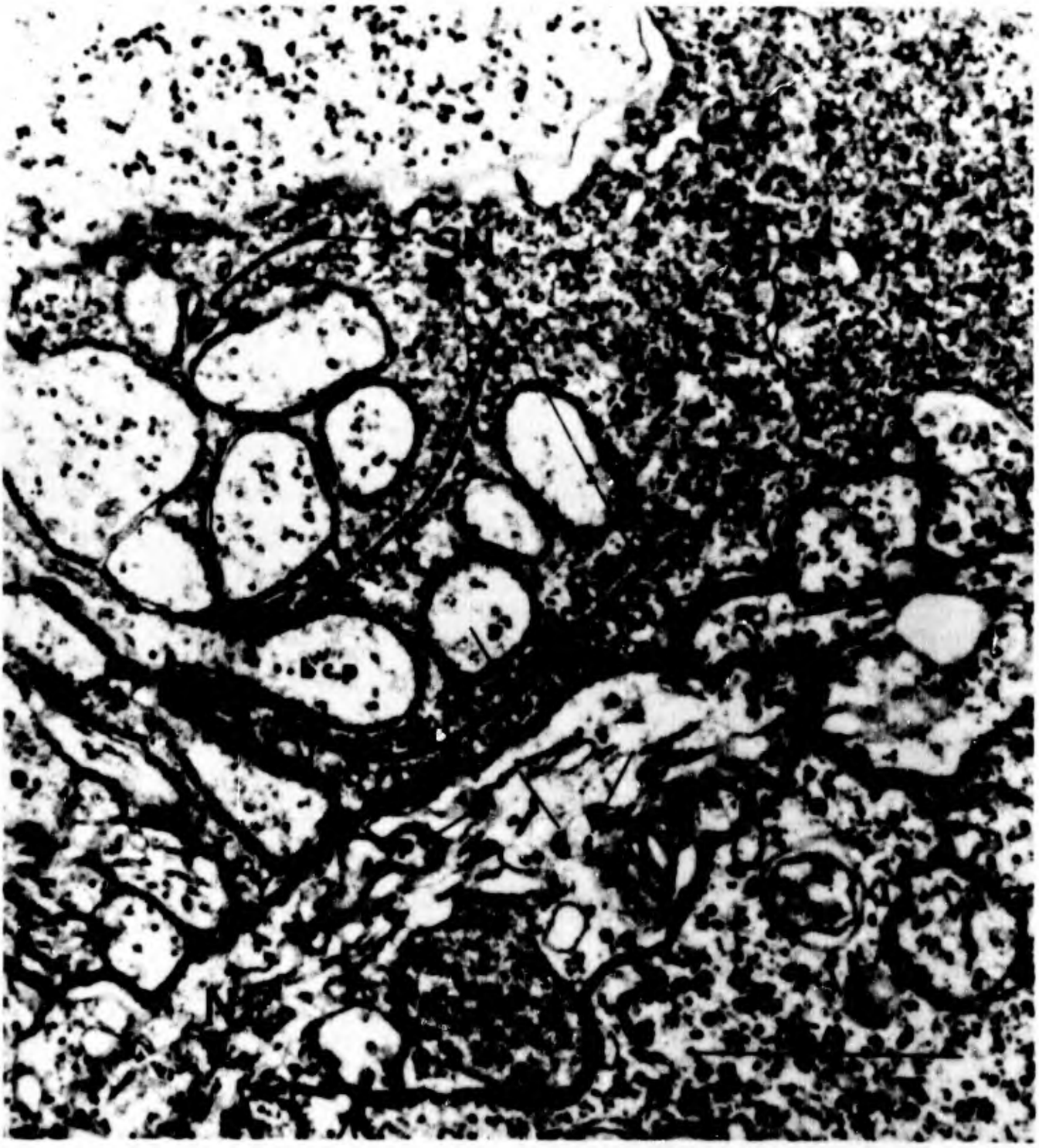


Fig. 27. Electron micrograph (G56) shows a region of the outer plexiform layer which includes a synaptic complex (SC), and a horizontal cell with many neural processes.

The horizontal cell, whose nucleus (N) is in the lower part of the micrograph, has processes (P) which have been traced for a distance of approximately 56μ. This particular type of horizontal cell (neuronal) is characterized by its numerous mitochondria (m) both in the soma and the distal processes. Other types of horizontal cell processes have fewer mitochondria but have a larger number of neuronfibrils (nf).

Fig. 28. Electron micrograph (G60) shows two bipolar cells (BPC) separated by Müller cells (M.C.). A group of horizontal cell processes are visible at the top right corner. This micrograph was of a preparation intended to demonstrate unit membranes. The "plasma membrane" in potassium permanganate ($KMnO_4$) fixed material, such as this, generally appears in cross-section as a pair of dense lines about 20Å thick separated by a light interzone about 25Å to 35Å across.[9] The membranes in this preparation appear to have these membrane dimensions in only a few places (arrows) but the matrices of the cells are conspicuously devoid of cytoplasmic detail.

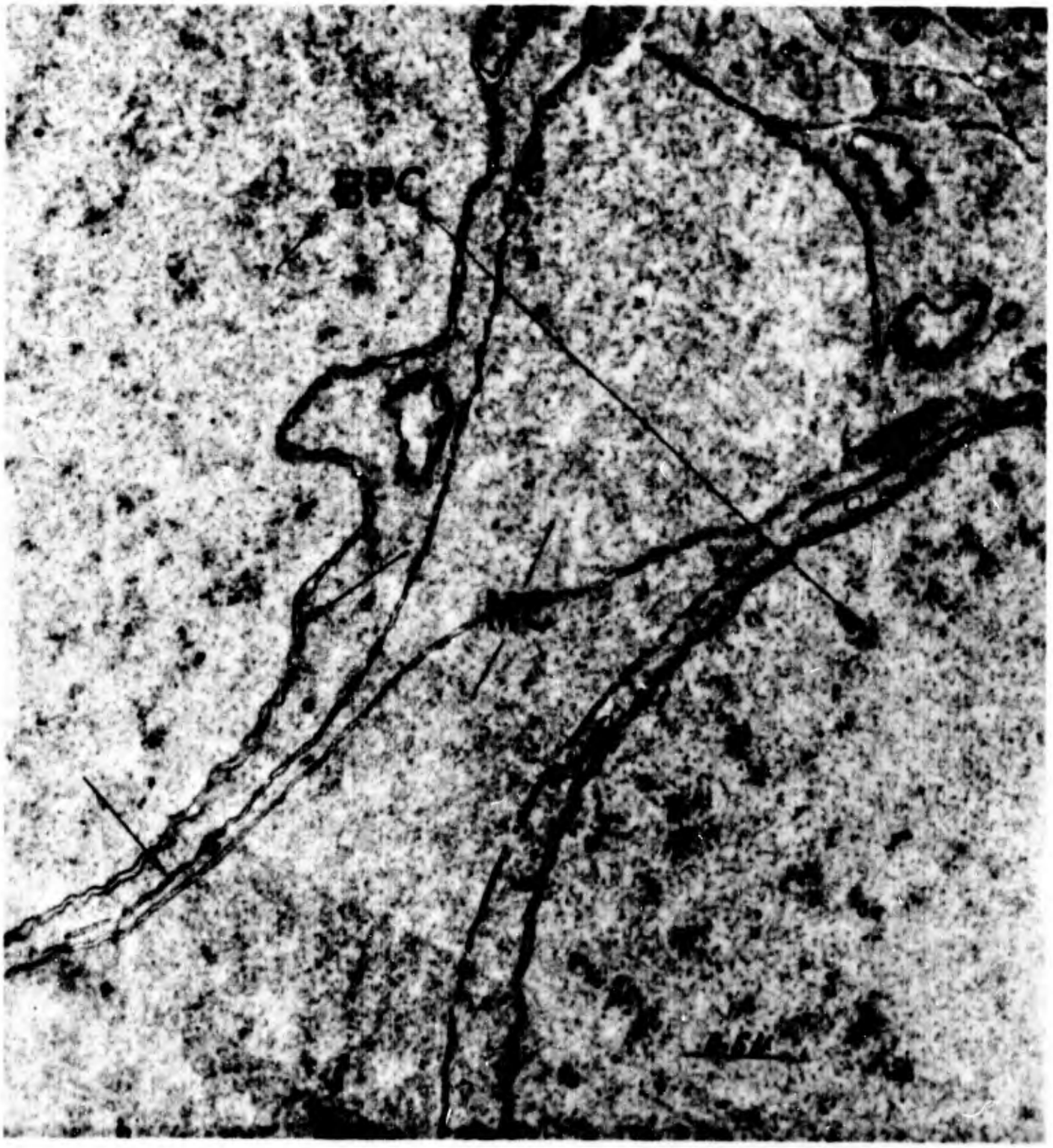

BPC

Fig. 29. Electron micrograph (G60) is of an amacrine cell (AC) bounded on three sides by Muller cells (MC) and on the fourth side by another amacrine cell. Unlike the bipolar cells, whose nuclei take up almost the entire soma, the amacrine cells have rather abundant cytoplasm. The nuclei (N) are quite frequently pushed over to one side and are occasionally lobate (arrow). In the cytoplasm one can see many vesicles of smooth endoplasmic reticulum (E.R.) and mitochondria (m). Globules of amorphous material of various densities are common in these cells; which suggests the production there of a neurosecretory material.

Notice the desmosomes or adhesion plates (D). One is located at an amacrine cell to Müller cell junction, and the other at an amacrine cell to amacrine cell junction. In the lower right corner of the micrograph is a dense radial fiber (rf) of Müller cell cytoplasm. This type of radial fiber extends vitrad through the inner plexiform layer to end with its footpiece at the inner limiting membrane.

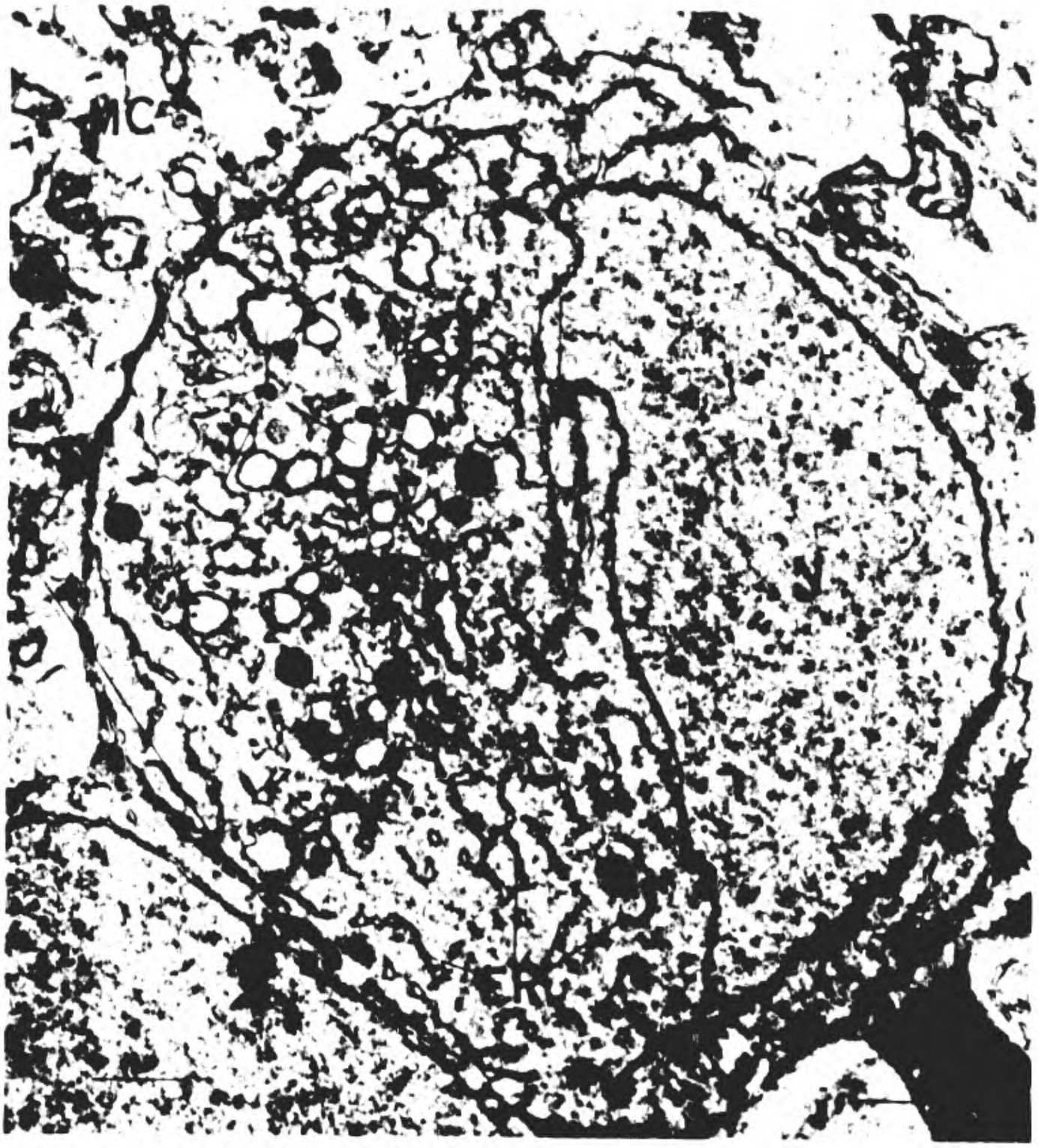

MC

Fig. 30. Electron micrograph (g58) is of the innermost region of the retina. Ganglion cells (G.C.), Müller cells (M.C.) and bundles of optic fibers (o.f.) make up the major portion of this region. The inner plexiform layer (IPL) is visible in the upper left corner. Vitrad to the internal limiting membrane (ILM) is a capillary. The lumen (L) of the capillary is surrounded by an endothelial cell which has a dense nucleus (N) located to the right.

It is commonly known that nutrients which supply the retina are absorbed through the capillary walls into the retina proper. Note in the capillary wall, as well as within Müller cells, at the vitrad border of the retina, the accumulation of particles which may be glycogen or some other polysaccharide.


Pt
1M

III. ADAPTATION AND THE LANDOLT CLUB

A. Evidence for an adaptation signal

It is known that the threshold intensity of a light flash required for seeing the flash is raised by the presence of a background illumination. In a recent review[15] the evidence was summarized that the background light is not acting directly on the rods and cones. Further, that even indirectly it is not acting to raise the rods' thresholds for signal output in response to absorption of photons; at least, not in the processes prior to the one which is saturated by a bright light. Instead, a signal is spread in some way from the receptors that have absorbed photons (from the background light) and causes the signal outputs of the neighboring receptors (in response to photons from the test flash) to have less effect at the pool where the outputs are summed and the sum sent on further towards the brain as a signal of the brightness of the flash. The output-reducing signal will be called the "local adaptation signal" (LAS).

It is also known that following exposure to a bright light the threshold for visibility of a test flash is raised, and this threshold drops subsequently in the dark. At any instant during this dark adaptation the retina acts as though there were an equivalent background illumination present. If the threshold is measured against a bright background during dark adaptation, it is found that the real and equivalent backgrounds sum linearly. This and other evidence indicate that the LAS is the level of a neighborhood pool into which each photo-activated molecule of visual pigment adds an increment (usually the same for all molecules), while the level of the pool is steadily depleted as a function of regeneration of the visual pigment. The actual relation is

$$LAS + Z = Z\ 10^{an}$$

where Z is the equivalent LAS present in complete dark adaptation, a is a constant, and n is the total number of photo-activated and as yet unregenerated visual pigment molecules present in the neighborhood which contributes to the LAS pool. Such molecules will hereafter be called the "Y" molecules.

It has been shown by Dowling[14] that the same adaptation effect as by exposure to light can be produced by reducing the retinene content of the retina by Vitamin A deficiency. From this it would seem that the "free" opsin molecules left uncombined with 11-cis-retinene in the rods are the "Y" molecules.

Thus, there are two signals present at the level of the receptors: (1) A signal representing the number of visual pigment molecules just then activated by the absorption of photons, the "PAS." This corresponds to the "brightness" signal (or that aspect of the signals) reaching the brain. The

ratio between number of molecules photo-activated and the strength of the PAS is in turn controlled by the second signal, (2) the LAS.

There is evidence that the LAS, also, or a signal proportional to it, is sent on to the brain. The first is the finding of Alpern and Campbell[16] that during dark adaptation the slow phase of the pupil's dilatation, which follows a curve very similar to that of rod dark-adaptation, is controlled by signals originating from the retina containing the previously illuminated rods. They concluded that signals related to the amount of unregenerated rhodopsin reach the central nervous system even in total darkness.

The second evidence (reported by J. Lettvin) was the as yet unpublished finding that the OFF-type optic fibers of the frog's retina, the type also called dimming detectors or adaptation detectors, fire during dark adaptation at a rate that decreases with the same time constant as the regeneration of the bleached visual pigment. He believes that this agreement is not coincidental, but is a signal originated by the bleached pigment present (the Y molecules).

The third kind of evidence is summarized by Barlow and Sparrock.[17] An afterimage is visible following exposure to a bright light. At each moment during dark-adaptation the afterimage is matched in appearance by the retinally stabilized background light which raises the threshold for a test flash the same amount as the previous exposure to the bright light has raised it in the region of the afterimage. In other words, the positive afterimage is the sensation corresponding to the LAS.

It is thus rather certain that a signal corresponding to the LAS passes from the level of the receptors to the brain. Section II C of this report shows that in the frog's retina this signal is probably carried by the large bipolars having large dendritic spreads. (Ramon y Cajal reported that it was extremely likely that those bipolars connect to the cones).

It is not known whether any given receptor carries both the LAS and PAS at the same time, but it does appear from the evidence cited that each receptor must be capable of carrying either signal. It would seem from the high concentration of synaptic vesicles in the receptor synapses (see Fig. 26) that one signal is carried out of the receptor and across the synapse by the chemicals contained in those vesicles.

Then how is the other signal transmitted from the receptor? It has been suggested by J. Lettvin that there is produced a reduction of electrical resistance in the membrane of the outer segment of the receptor; that this allows an increase in the current passed through the receptor by an external source; that the current increase is the other signal. He suggested that the only structure in the retina which could carry this current outside the receptor is the Landolt club of the small bipolar cell. The anatomical study of Section II E supports this suggestion, and the evidence will be reviewed in the next section.

B. Anatomical evidence for a Landolt club-receptor current flow

A club-shaped structure extending from the outer plexiform layer almost to the outer limiting membrane was first noted by Landolt.[18] Ramon y Cajal and others identified it as an extension of a small bipolar cell. They reported its presence in the retinas of amphibia, birds, reptiles, but not fish. Dogiel reported it in mammals, but Ramon y Cajal could not find it there. Polyak[19] found in rhesus, chimpanzee, and human, occasional small bipolars ("mop" type) one of whose dendrites extended into the outer nuclear layer.

Our findings with electron microscopy of the frog's retina confirm that the Landolt club is an extension of a small bipolar cell. The Landolt club is separated from the visual cells by at least one pair, and often many pairs of Müller cell membranes from the synaptic region to the outer limiting membrane (Figs. 22, 23, 24). The visual receptors, also, are surrounded in that region by Müller cell membranes (Fig. 25). These membranes are of relatively high electrical resistance and act to insulate the receptors and Landolt clubs from each other.

The Landolt club extends past the outer limiting membrane into the aqueous space; the extension is a cilium. Both these findings are new. All types of cilia previously studied have been shown to be energy transducers (e.g., chemical to mechanical to electrical). The cilia which produce mechanical movement have the central pair of filaments present the length of the cilium. In the Landolt club the central pair appear incomplete (Fig. 21). Therefore, it is unlikely that the function of this cilium is movement.

Below the cilium the Landolt club has one or more swellings containing large mitochondria. It is suggested that these furnish the energy to maintain an electric current through permeable membranes of the cilium. Such a current would flow down the Landolt club, through its dendrites into its synapses with the neighboring visual receptors, up the receptor and out the fins of the inner segment, across the aqueous space, and into the cilium (Fig. 20).

C. Speculation concerning the PAS and LAS mechanisms

It may be that photo-activation of visual pigment, which forms part of the membrane of the outer segment, reduces the resistance of that membrane well below that of the fins, so that there is a marked increase in current flow. If it is assumed that this increased current increases the permeability to sodium of the dendrites, this would cause a current flow out the teledendron of the small bipolar, activating it, and this would provide a mechanism for transmission of the PAS.

The LAS might depend on the Y molecules, which were left by the photo-activation, acting to increase the permeability to sodium ions of the outer segment's membrane. This would cause a current flow down through the receptor and out its synapses, and cause chemical transmitter to be released at the synapse. This would excite the dendrites of the large bipolars, thus transmitting the LAS.

If it is assumed that the dendrites of the small bipolar are made more permeable to potassium ions by the chemical transmitter of their synapses, then this would act as a shunt to the sodium current from those dendrites to the teledendron. Since the sodium current is the PAS and the potassium current depends on the LAS, the effect is roughly to divide the PAS by the LAS.

The LAS could originate as well from neighboring receptors having Y molecules. They would chemically excite the neuronal horizontal cells, which in turn release chemical transmitter at their synapses with the small bipolar's dendrites and depolarize them to potassium. (Only glial type horizontal cells have been observed electrophysiologically; although the neuronal-appearing type have been observed anatomically by Ramon y Cajal, and in the present electron microscopic study. See Fig. 27).

That this mechanism is not very unreasonable can be argued from the recent finding of Cone and Platt[20] that the b-wave of the rat's electroretinogram has a latency that is little affected by adaptation conditions, though the amplitude is strongly affected. The b-wave has the same polarity across the retina as the voltage drop across the receptor caused by the hypothesized PAS current. It would be expected from the above mechanism that the latency of the PAS current would not be affected by the LAS, although its amplitude would be.

All these, not unreasonable, assumptions provide a mechanism that correlates with the findings of this and other studies. It provides a means of getting the LAS and the PAS to the brain. It makes the LAS dependent on the freed opsin. It makes the PAS dependent on the immediate photo-activation of visual pigment, and greatly reduced by the LAS from the same or neighboring receptors.

However, it should be pointed out that the actual retinal mechanism must be more complex. The finding of Section II C was that the small bipolars carried information as to the presence of edges in the light image at the receptors. The above suggested mechanism has them carrying information as to the brightness of the light. Therefore, further refinements will certainly be needed, and the mechanism discussed above should be regarded only as an attempt to point out some reasonable possibilities for relating the new anatomical findings to known retinal functions.

APPENDIX

Working Procedure for Making Casts of Frog's Eyes

By John H. Patterson

As is true when taking impressions of any type, one of the first things to consider is how to carry the impression material to the subject. Since this was, to my knowledge, the first time this kind of work had been attempted with frogs, there were no impression trays, or molding shells as they are called by opticians, available.

I experimented with and used satisfactorily, red thermal impression compound for making trays. The trays were made by shaping them around a 6mm steel sphere, giving a cup-like shape. A handle was then fashioned from a straight piece of the same material and fastened to the outside bottom of the cup.

Due to the fact that a frog's eye is shaped quite differently from most other animals, I found that I could not place the tray under the eye lids as is done when taking impressions of human eyes. Only the cornea is seen on a frog with the lids in place, and the lids are too small and tight to make it feasible to slip anything as thick as the rim of the cup-like impression tray under them. After much experimenting it was decided that in order to obtain an impression of a suitable amount of the eye surface to enable us to fashion contact lenses from the castings, the frogs would have to be sacrificed and the lids removed.

Frogs were selected that had been in the tank a long time and whose muscles had grown weak. They were considered unsuitable for other experiments. Since the goal was to obtain a cross section of sizes so that several stock-size lenses could be made, frogs of different sizes were chosen. They were pithed by passing a needle up and down the vertebral column, the lids removed with dissecting scissors, and the blood washed away. By keeping the frog moist, the eyes remained suitably firm for impression taking approximately half a day.

Since the frog is one of the animals that has the ability to retract its eye, something must be done to prevent this. One method is to place a ball of cotton inside the mouth directly behind the eye. Another, and probably better method is to hold the frog in such a manner as to pull the jaws ventrally and toward each other, thereby forcing the eye to extrude as far as possible. This may be done by grasping the frog's head along the right and left jaws between the thumb and forefinger of one hand. By applying pressure to the jaw on the same side as the eye with which you are working, that eye will be forced to extrude from the eye socket as much as possible. In this way a suitable amount of eye surface will be accessible leaving the other hand free to take the impression. The free hand can thus hold the

filled impression tray over the eye with a very slight pressure until the material has set.

Several impressions were taken of each eye, using an alginate impression material known as Jeltrate, on four different sized frogs. Castings were then made of Castite or Hydrocal and duplicates made by pushing the castings into modeling clay, lifting them straight out, then pouring plaster into the impression thus made. The castings were numbered from 0 through 4 according to size with 0 the smallest and 4 the largest.

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13 ABSTRACT The information handling properties of the frog's retina were studied by three techniques. (a) An attempt was made to investigate the mutual interaction of excitatory and inhibitory connections to bipolar cells by studying the averaged electroretinogram response to stimulation with various patterns of light. It was found that present techniques of electroretinography and photometry did not permit the required stability of measurement. (b) Light and electron microscope studies were made of the cell types and connections of the frog's retina. Illustrative micrographs are included in the report. The new findings were made that the Landolt club of the small bipolar cell is a mitochondria-packed process and that it terminates as a cilium in the aqueous space between the visual cells. It is suggested that the function of these structures is to detect the presence of bleached, unregenerated visual pigment molecules in the surrounding visual cells. (c) A technique was developed which seems to result in selective staining of the active bipolar-ganglion cell synapses. With it functional and anatomical studies were correlated to identify the four ganglion cell anatomic types which transmit information each as to the presence in its receptive field of (1) an edge, (2) a small dark image, (3) a changing light distribution, and (4) a dimming of light intensity. The large bipolar cells were found most likely to be carriers of "light adaptation" information, the small bipolars of "edge" information.

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Information handling						
Frog						
Retina						
Histology						
Selective staining						
Electroretinogram						
Receptors						
Cilium						
Retinal ganglion cells						

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