

# INHERITED RETINAL DYSTROPHY IN THE RAT

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## ABSTRACT

Retinal dystrophies, known in man, dog, mouse, and rat, involve progressive loss of photoreceptor cells with onset during or soon after the developmental period. Functional (electroretinogram), chemical (rhodopsin analyses) and morphological (light and electron microscopy) data obtained in the rat indicated two main processes: (a) overproduction of rhodopsin and an associated abnormal lamellar tissue component, (b) progressive loss of photoreceptor cells. The first abnormality recognized was the appearance of swirling sheets or bundles of extracellular lamellae between normally developing retinal rods and pigment epithelium; membrane thickness and spacing resembled that in normal outer segments. Rhodopsin content reached twice normal values, was present in both rods and extracellular lamellae, and was qualitatively normal, judged by absorption maximum and products of bleaching. Photoreceptors attained virtually adult form and ERG function. Then rod inner segments and nuclei began degenerating; the ERG lost sensitivity and showed selective depression of the a-wave at high luminances. Outer segments and lamellae gradually degenerated and rhodopsin content decreased. No phagocytosis was seen, though pigment cells partially dedifferentiated and many migrated through the outer segment-debris zone toward the retina. Eventually photoreceptor cells and the b-wave of the ERG entirely disappeared. Rats kept in darkness retained electrical activity, rhodopsin content, rod structure, and extracellular lamellae longer than litter mates in light.

## INTRODUCTION

Cell biology has many facets, including the study of cell reactions in disease. Disease may reveal a range of cell functions, features of structure, and interrelations between cells not recognized under other circumstances.

Inherited degenerative disorders present a particularly difficult challenge. Cells of a given class degenerate at some point in their life span with no known immediate cause. Inherited degenerations of retinal photoreceptor cells are known in mouse (5, 20, 32, 34, 35), rat (1, 23), dog (22, 28), and man (7, 38). These retinal diseases are promising to study as models of the general class of inherited degenerative disorders, since more information is available on function, chemistry, and structure of photoreceptors than of most cell types.

The chemistry of photoreception provides a molecular explanation for the loss of vision in vitamin A deficiency (16, 39). Vitamin A aldehyde, retinene, is the photosensitive prosthetic group of visual pigments such as rhodopsin. Opsin serves both as the protein moiety of these visual pigments and as a structural component of the rod outer segment. Rats raised on a vitamin A-deficient diet supplemented with vitamin A acid maintain general good health but gradually lose vision because vitamin A acid is incapable of functioning in the visual system (17). The photoreceptor cells of these animals gradually disappear while other cells of the retina remain intact.

The retina in severe vitamin A deficiency is strikingly similar in histological appearance to the retina at advanced stages of all the inherited

degenerations of photoreceptor cells. These similarities in pathology have led several workers to suggest that an inherited defect involving an enzyme dealing with vitamin A or other component of the visual cycle might be the cause of the inherited lesions (7, 40, 41). A further hypothesis, based on histological and tissue culture studies in the mouse, suggests that the defect in the inherited diseases results from genetically determined synthesis of abnormal opsin (31).

To obtain chemical data bearing directly on these hypotheses we turned to the mutant rat, for the rat has more retinal tissue than the mouse, and the rods develop more fully before the degenerative process sets in (23). The present report describes an integrated analysis of retinal function, chemistry, and cell structure in the mutant rat. The data show that, at least in this species, the disease clearly involves neither a primary defect of vitamin A or of rhodopsin metabolism nor a synthesis of an abnormal opsin. The disease does involve some features hitherto unrecognized in an inherited degenerative process and relevant to our eventual understanding of normal retinal development and function.

## MATERIALS AND METHODS

### *Animals*

The animals were offspring of two breeding pairs of white-coated and tan-hooded, pink-eyed, dystrophic rats maintained at the Royal College of Surgeons, and generously given to one of us (R. L. S.) by Professor A. Sorsby. These animals were descendants of the University College strain in which the spontaneous retinal degeneration was originally described by Bourne, Campbell, and Tansley in 1938 (1). Bourne and Grüneberg (2) showed that the disease is inherited as an autosomal recessive.

The control animals were inbred albino rats of the Harvard University colony. The Harvard rats grew somewhat faster and attained heavier weights than did the affected strain, but both strains developed their visual responses similarly up to the time the disease became manifest in the dystrophic animals. On occasion, rats from both strains were taken from their natural mothers and given to be raised by foster mothers of the other strain. These animals developed according to their genotypes; the control rats still grew somewhat bigger and the dystrophic animals were still diseased.

### *Electroretinography*

For recording the electroretinogram (ERG), the animals were dark adapted overnight and

anesthetized with Nembutal injected intraperitoneally. For animals over 100 grams, doses of 5 mg/100 grams were used. Lighter animals needed less anesthetic, 12-day-old animals weighing 25 to 30 grams received only 2 mg/100 grams. The rat was secured to a board fitted with a clay head holder and its eye was maximally exposed by drawing the eyelids apart with sutures. All the preparations were carried out in dim, red light.

The response was recorded by means of cotton-wick electrodes moistened with Ringer's solution. One electrode was placed on the edge of the cornea, the other on a shaved area on the cheek. The electrodes were connected *via* silver-silver chloride wires to a Grass capacity-coupled P-5 amplifier with a half-amplitude time constant setting of 0.1 cycle/second. The responses were observed with a DuMont type 350 oscilloscope and recorded photographically with a Polaroid camera.

The stimuli were 1/50 second flashes of white light derived from a zirconium arc, the intensity of which was controlled with neutral density filters and photographic wedges. The responses were recorded over a luminance range of 7 log units, starting with the lowest intensity.

The electroretinogram is an easy, convenient, and reliable method for measuring the visual response of an animal such as the rat. Upon stimulation by a flash of bright light, the eye responds initially with a cornea negative potential, the so called a-wave of the ERG (see Fig. 2). The a-wave is rapidly followed by a cornea positive c-wave. If the stimulating light is dim, only the b-wave of the ERG appears. The ERG is primarily an on-type response; that is, whether the light remains on or is turned off, the potential rapidly returns toward the base-line level. (In many animals, the b-wave is followed by a much slower, cornea positive potential, the c-wave; in the albino rat eye, the c-wave is only infrequently seen, so that it was not considered in these experiments.)

The precise sources of the components of the ERG are not yet well understood, but for the mammalian eye, especially, there is general agreement as to the areas in the retina where the potentials arise. The a-wave is thought to arise in the area of the outer segments of the visual cells, while the b-wave appears to originate in the synaptic zone between the visual and bipolar cells (3, 4, 25). Thus, to a first approximation, the a-wave may indicate events closer to the initiating photochemical event, while the b-wave may give more of an idea of the activity coming from the whole visual cell. Regardless of the validity of this interpretation, however, there is abundant evidence that the ERG can be used to evaluate the relative sensitivity of the eye, which is its primary use in the present experiments (12, 19).

## Rhodopsin Measurements

Animals were dark adapted overnight, anesthetized with an overdose of Nembutal (15 mg/100 grams), and their eyes removed in dim red light. The lens and cornea were dissected from the eye and discarded, and the back of the eye was cut in half. One animal (two eyes) sufficed for a measurement.

The eyes were hardened in 4 per cent alum for 10 to 15 minutes, washed twice in distilled water, once with phosphate buffer (pH 7) and extracted overnight at room temperature in 0.25 ml of 2 per cent digitonin in phosphate buffer (pH 7). After centrifugation at 20,000 RPM for 10 minutes, 0.01 ml of 1 M hydroxylamine was added to the extract and the absorption spectrum was measured in a Cary recording spectrophotometer before and after bleaching. The change in extinction at 493  $\mu$  measured the amount of rhodopsin present. The first extract removed about 80 per cent of the rhodopsin from the eyes and a second extract was routinely made.

## Microscopy

Without regard for light or dark adaptation, animals were anesthetized with Nembutal, and their eyes removed. (Usually, the eyes were removed for microscopy immediately following electroretinography.) The lens and cornea were cut away and the back of the eye was immediately immersed in a cold (0°C) 2 per cent solution of osmium tetroxide buffered to pH 7.8 with Veronal acetate containing 45 mg per milliliter of sucrose and 1 per cent  $MgCl_2$ . After 15 to 20 minutes in the cold, the fixation was continued for 1 hour at room temperature. The eyes were dehydrated through a series of graded acetone-water mixtures, and infiltrated with Araldite epoxy resin (18). The eyes were soaked for 5 to 7 days in the Araldite at 50 to 60°C before accelerator was added and the plastic allowed to harden. The whole back of the eye was embedded, in the attempt—only partially successful—to retain contact between the retina and pigment epithelium. No changes in technique were introduced once this study was under way, so that all specimens would be processed alike.

For light microscopy, large sections 2 to 4  $\mu$  thick were cut from the Araldite blocks from the more central portions of the retina with a Porter-Blum microtome. The sections were mounted on slides with Mayer's albumin and stained with either Giemsa blood stain (14) or the periodic acid-Schiff (PAS) stain (24).

For electron microscopy, thin sections as large in area as possible were cut with glass knives mounted on a Porter-Blum microtome. Areas were selected on the basis of the light microscope sections. The sections were picked up on carbon-coated grids and stained overnight on a saturated solution of uranyl

acetate dissolved in 50 per cent ethanol. The grids were rinsed in fresh ethanol-water mixture and examined with the RCA EMU 3-D microscope operated at 100 kv. Photographs were taken at magnifications between 3000 and 10,000 times. At every stage studied, overlapping micrographs at  $\times 3000$  were made from Bruch's membrane to the outer nuclear layer. Analysis of a pathological process requires more extensive sampling than is usually needed in a study of normal cytology, and our standards of technical quality were sometimes relaxed in order to permit the mapping of large retinal areas.

## RESULTS

### 1. Normal Visual Cell Development

The rat, like other rodents, is born blind and begins to develop vision at 9 or 10 days of age. At 9 days of age, the nuclear layers of the retina have formed, and small inner segments extend beyond the external limiting membrane. At the distal end of the inner segments, the connecting cilia are frequently seen extending toward the pigment epithelium but, as yet, little, if any, outer segment material is seen. (See Fig. 15 in reference 15). At 10 days of age, the outer segments are developing rapidly, and electrical activity can first be recorded from the retina. This initial electrical activity is very small (75  $\mu v$ ) and cornea negative; it consists of two waves, a fast initial negative wave followed by a slower negative potential.

By 12 days of age, the outer segments average 4.9  $\mu$  in length (Table I). Rhodopsin in significant quantities can be extracted from the eyes and the electroretinogram has developed considerably in size and form (Fig. 2). The ERG is still primarily negative, but the beginnings of the cornea positive b-wave can be seen, especially at the lower intensities. The ERG is still rather insensitive; its threshold lies about 2 log units above the adult level.

By 15 days, outer segments have attained an average length of 11.1  $\mu$  as measured on electron micrographs and 12.9  $\mu$  as measured with the light microscope (Table I). The rhodopsin content of the eye has increased to about 35 per cent that of the adult. The ERG has developed beyond the 12 day stage, and shows a prominent b-wave (Fig. 2). The threshold of the response is now about 1 log unit higher than in the adult.

Fig. 1 shows the increase in rhodopsin content of the eye with age (circles), and also compares

the increase of rhodopsin with the increase in length of rod outer segments (crosses). The comparison is difficult to make accurately because the rods do not grow synchronously. The sample was biased in favor of the longer rods, especially when measurements were made with the light microscope on sections thicker than one rod diameter, in which it was difficult to delineate individual rods. A further difficulty is that in early stages of rod formation, the outer segments do not develop in an even row. Outer segments form at different distances from the outermost photoreceptor nuclei so that the outer segment zone appears wider than it actually is. Measurements of rod length on the electron micrographs are also somewhat biased in favor of the longer

23.5  $\mu$  by 39 days of age. The rhodopsin content of the eye likewise increases to the adult level of about  $8 \times 10^{-4}$   $\mu$ moles per eye, and the ERG increases in size to 1200 to 1,500  $\mu$ v, but retains essentially the same form.

## 2. Visual Cell Development in Dystrophic Rats

### ELECTRORETINOGRAPHY

Measurements of the electrical activity of the eyes of the affected animals were begun at day 12. At both 12 and 15 days the ERG of the diseased animals was normal in size, form, and threshold. (Fig. 2).

TABLE I  
*Length of Rod Inner and Outer Segments at Various Ages in Normal Rats*

Age (days)	Type of specimen	Number of Rods measured	Length ( $\mu$ )		
			Inner segments Range	Outer segments Mean $\pm$ S.D.      Range	
12	Electron micrograph	14	9-14	5.5 $\pm$ 2.5	2.5-12*
15	Electron micrograph	23	14-21	11.2 $\pm$ 2.8	5.5-17
	Light microscope section	20	14-19	12.9 $\pm$ 2.5	9-16
18	Light microscope section	20	14-17	18.0 $\pm$ 2.2	12.6-21
22	Light microscope section	20	11-15	21.6 $\pm$ 1.9	15.9-23.9
39	Light microscope section	10	12-16	23.5 $\pm$ 2.0	20.5-24.5

\* A single outer segment measured 12  $\mu$ ; no other exceeded 8  $\mu$ .

rods, since short rods were not distinguished from obliquely sectioned rods and probably were not sufficiently sampled. If these discrepancies are considered, the lengths of outer segments probably should fall even closer to the curve of rhodopsin concentration than are plotted in Fig. 1. It is our impression from these data, however, that it would have been even better to plot volume rather than length of rod outer segments against rhodopsin concentration during development of the visual cell, but we were unable to assess the volume accurately.

By 18 days of age, the rhodopsin content has increased to about 50 per cent of the adult level. The ERG at this stage is almost adult in size (1,000  $\mu$ v) and form. The threshold also is less than 0.25 log unit above that of the adult.

After 18 days the outer segments continue to lengthen until they reach an average length of

The first electrical changes in the affected animals are detected at about 18 days, although in some instances they are very slight indeed (Fig. 2). (The ERG's in Fig. 2 were all recorded from the animals whose retinal histology is shown in Fig. 3.) At 18 days, the threshold is raised slightly, but otherwise the response appears to be within the normal range.

By 22 days the ERG threshold has risen 1.5 log units above the normal level and the ERG exhibits a distinctly different form; the negative a-wave is depressed and the positive b-wave does not rise as sharply as in the normal. At the higher stimulus intensities, the b-wave is still close to normal in size.

At 27 days, these changes are accentuated; the threshold has continued to rise, and the a-wave potential to shrink, although again at the higher intensities the b-wave is close to normal in size.

By 32 days, the b-wave has shrunk considerably, and no a-wave at all is seen. From here on the electrical activity of the diseased eye continues to decrease (40 days). At 60 days no positive waves are seen, only a peculiar slow negative wave elicited at very high intensities. At 1 year of age, no electrical activity is detected in the eye at any intensity of stimulus.

rhodopsin in their eyes as do the controls. The rhodopsin content increases rapidly in both groups until about 30 days of age, at which point the dystrophic animal still has about 1.7 times as much rhodopsin as the control. After 30 days of age, the rhodopsin level in the dystrophic animals falls sharply, so that by 40 days of age their eyes contain only about 50 per cent of the normal

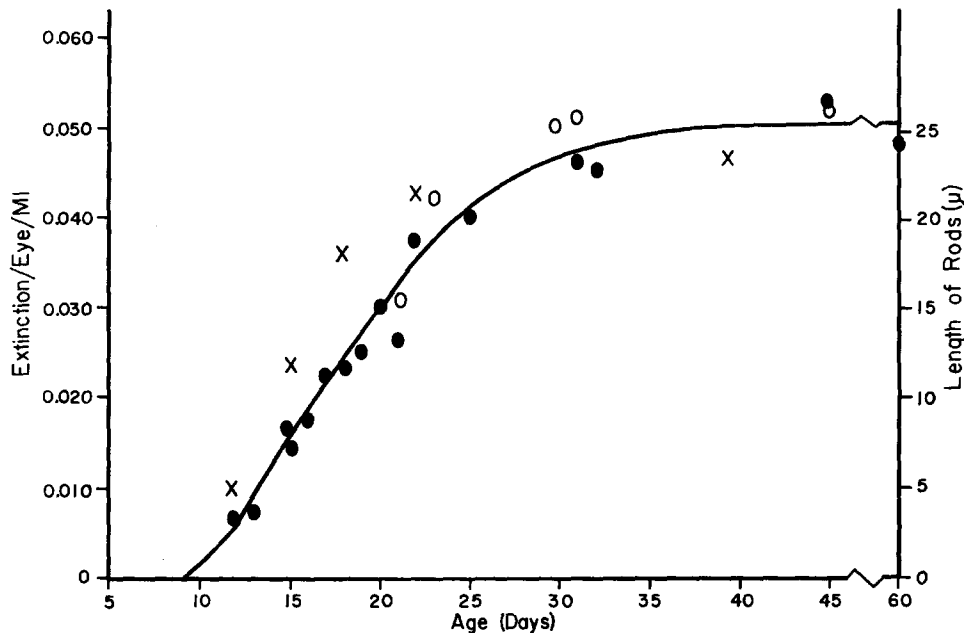


FIGURE 1

Increase of rhodopsin content of the eye with age (circles), compared with increase of outer segment length (crosses). Filled circles: animals raised under ordinary laboratory conditions of illumination; open circles: animals raised in darkness.

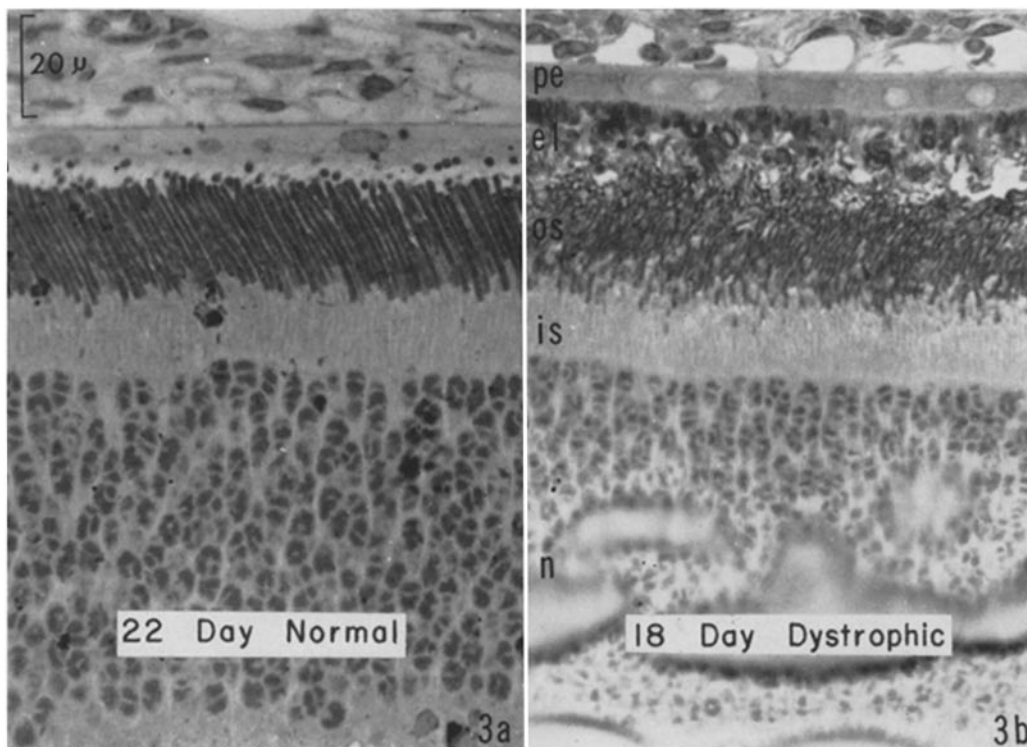
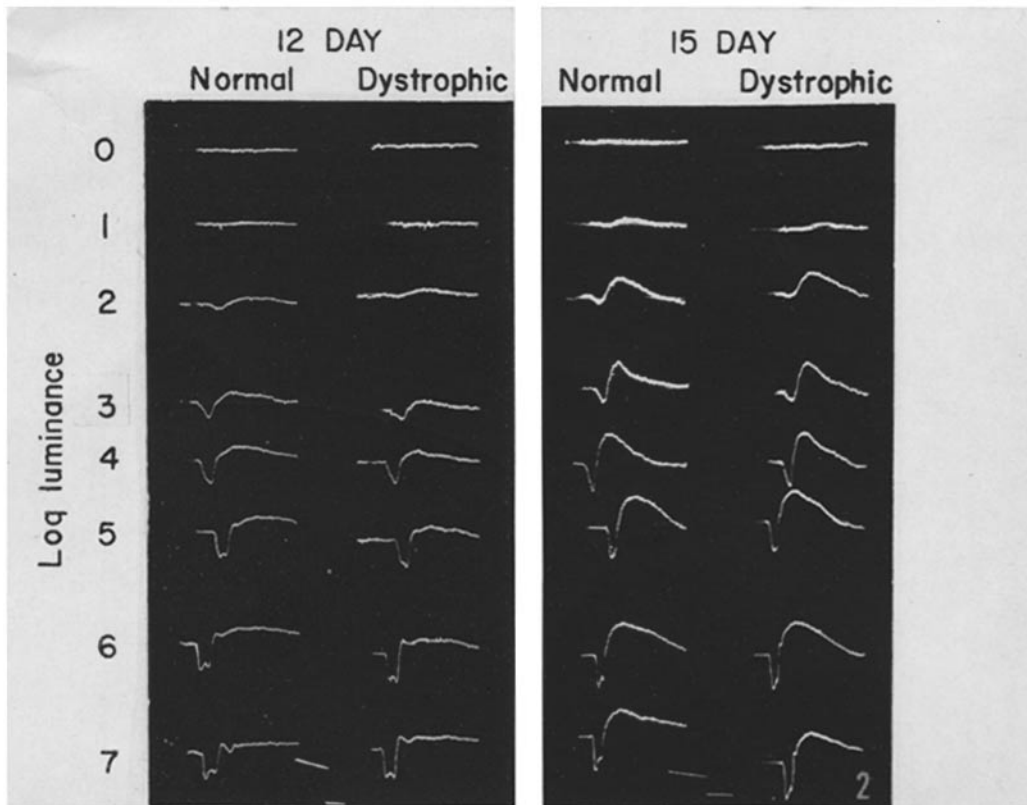
#### RHODOPSIN MEASUREMENTS

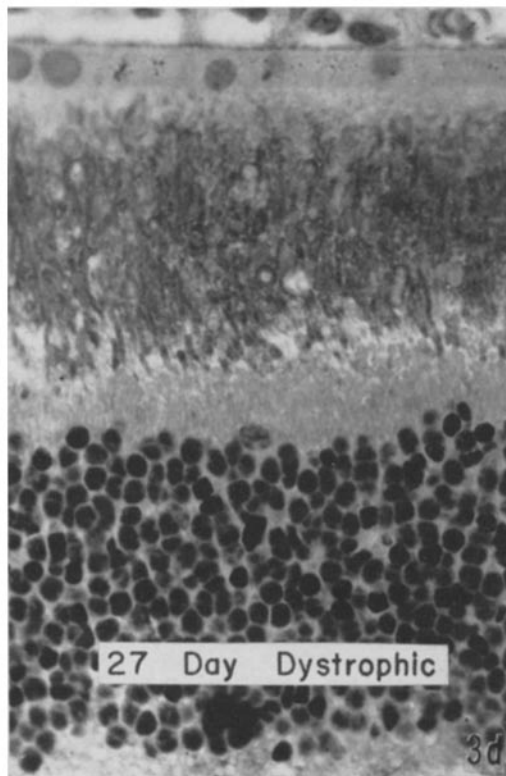
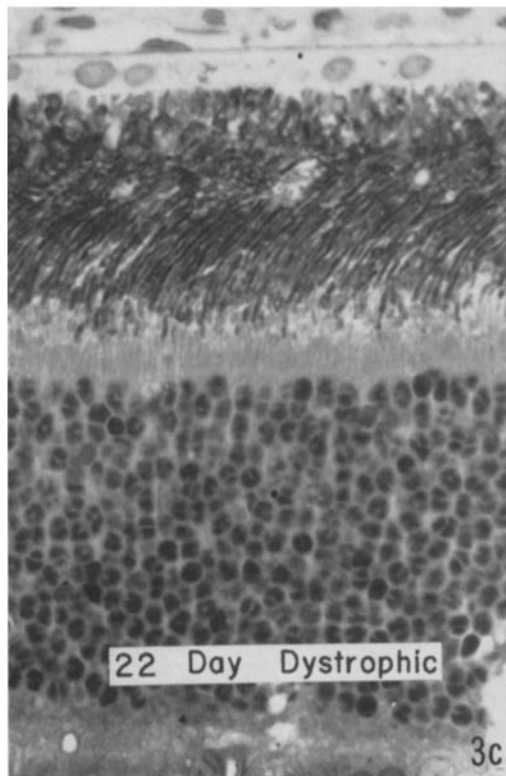
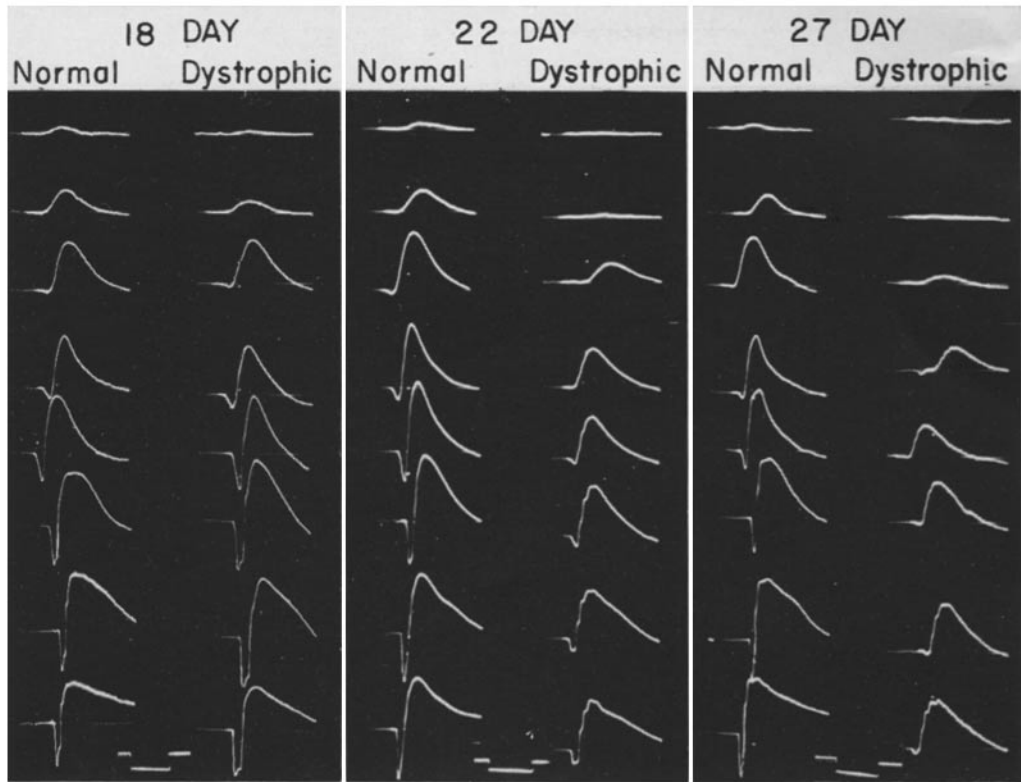
Measurements of rhodopsin were made on dystrophic animals between the ages of 15 and 60 days, in order to answer two major questions about the disease: (a) Is the gradual fall of visual sensitivity due to a loss of visual pigment as in nutritional night blindness? (b) Do these animals synthesize normal rhodopsin, as judged by its absorption spectrum?

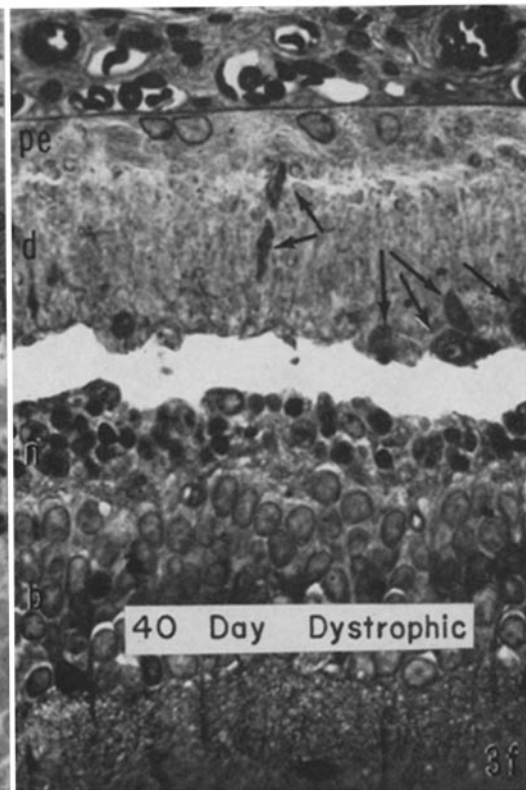
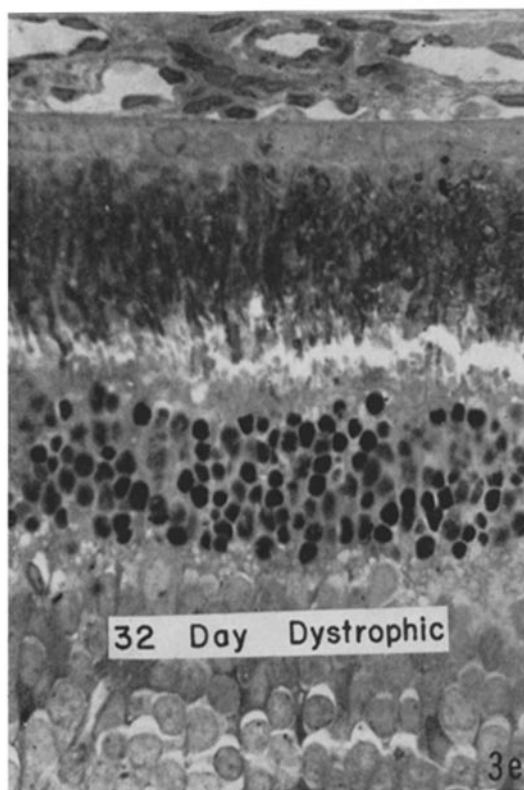
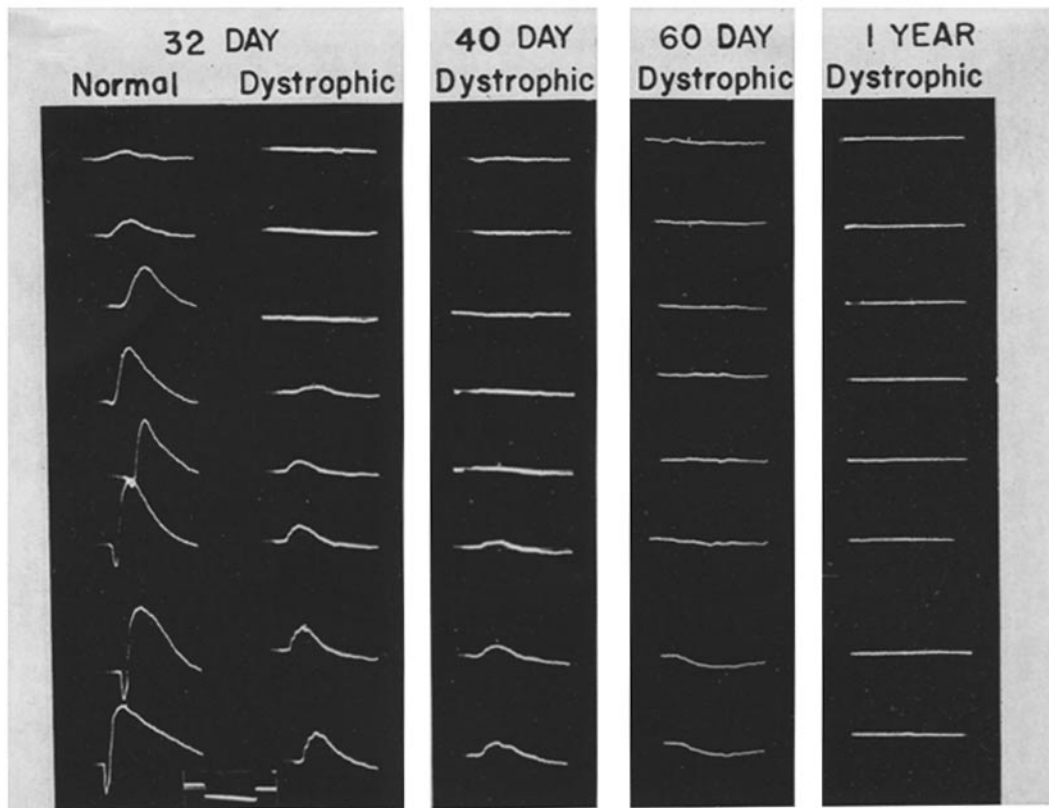
The measurements of rhodopsin content in dystrophic and normal animals are shown in Fig. 4. To our surprise, the affected animals synthesize considerably more rhodopsin than control animals. This difference is recognizable at about 14 to 15 days. By 20 days of age, the dystrophic animals have about twice as much

amount. By 60 days almost no rhodopsin remains in the dystrophic eyes.

It has recently been found that the decline of rhodopsin in vitamin A deficiency can be almost wholly prevented by keeping the animals in the dark (Dowling, unpublished observations). Apparently, rhodopsin is a very stable molecule; if not bleached by light, it holds onto its retinene (vitamin A aldehyde) even when the animal is otherwise wholly depleted of vitamin A. It seemed that in the dystrophic animal the loss of rhodopsin after 30 days of age might reflect an inability to resynthesize rhodopsin after bleaching. To test this point, dystrophic animals were kept, beginning at 10 to 20 days after birth, in a dark room, illuminated when necessary with dim red light.







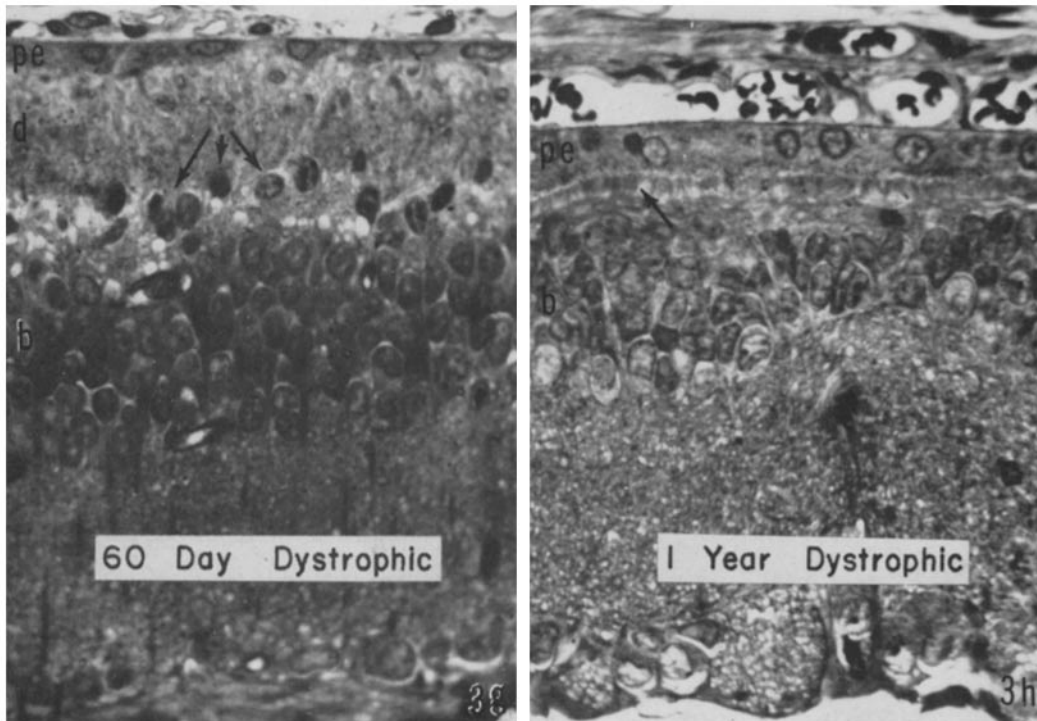


**FIGURE 2**

Upper half of preceding three pages. Electroretinograms (ERG's) recorded from normal and dystrophic animals. The ERG's were elicited with 1/50 second flashes of white light over an intensity range of 7 log units. A 200  $\mu$ v calibration signal is shown at the bottom of each set of records. The duration of the oscilloscope trace was 0.5 sec in all cases. The corneal positive b-wave is displayed upward in accordance with ERG convention. The ERG's of the control and affected animals developed normally to day 18. Then the ERG's of the dystrophic eyes showed rise of ERG threshold and selective a-wave loss (days 22 to 27). Later, the b-wave of the affected animals declined and gradually disappeared (days 32 to 60); the dystrophic animals were then blind.

**FIGURE 3**

Lower half of preceding three pages and this page. Retinal histology of normal (22 days) and dystrophic eyes from animals whose ERG's are shown in Fig. 2. The initial histological change in the affected eyes is an accumulation of outer segment-like material between the photoreceptor outer segments and the pigment epithelium (days 18 and 22). The inner segments and photoreceptor nuclei show evidence of deterioration by days 22 to 27, and thereafter rapidly degenerate and disappear (days 32 to 60). Pigment epithelial cells break away from Bruch's membrane and migrate through outer segment-debris zone (arrows, days 40 and 60). At 1 year the retina appears relatively intact except for complete absence of photoreceptor cells. Figs. 3 *a* to *e* are stained with the PAS method, Figs. 3 *f* to *h* with Giemsa. The poor focus of photoreceptor cell nuclei in the 18-day retina is artifact, as is the separation of photoreceptor cells from the debris zone in the 32- and 40-day retinas. Labels: *b*, bipolar layer; *d*, debris derived from degenerating extracellular lamellae and photoreceptor outer segments; *el*, extracellular lamellae; *is*, photoreceptor inner segments; *n*, photoreceptor nuclei; *os*, photoreceptor outer segments; *pe*, pigment epithelium; arrow on Fig. 3 *h*, prominent apical microvilli on pigment epithelial cells. All figs.  $\times 650$ .



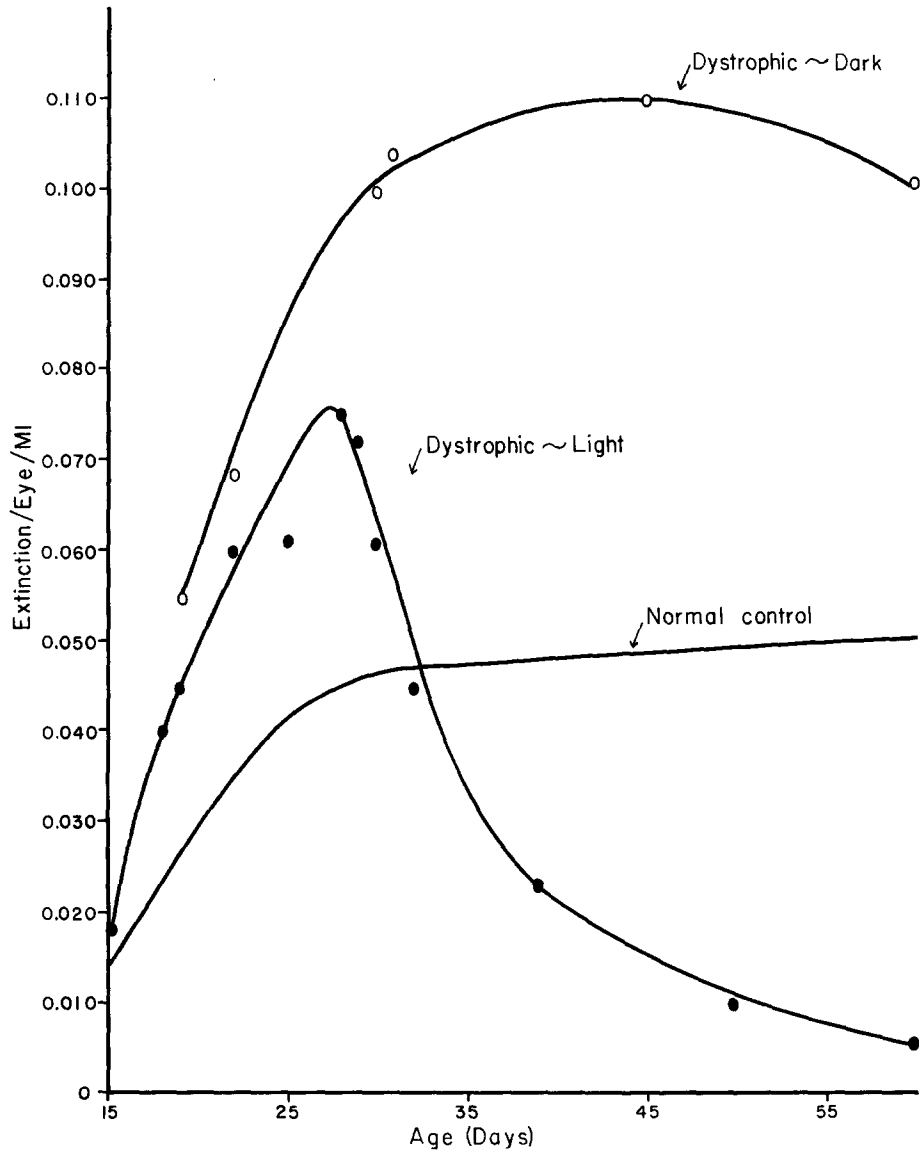


FIGURE 4

Rhodopsin content of eyes from dystrophic animals raised under ordinary laboratory conditions of illumination (filled circles) and in darkness (open circles), compared with rhodopsin content of eyes from normal animals (normal curve is taken from data of Fig. 1).

The results of these experiments also are shown in Fig. 4. Dystrophic animals raised in the dark were found to have even more rhodopsin than litter mate dystrophic animals raised in the light; at 30 days of age they had over twice as much rhodopsin as the normal controls. The dystrophic animals raised in the dark did not lose much

rhodopsin between 30 and 60 days of age, in contrast to those raised in the light.

The rhodopsin in the dystrophic animals has an entirely normal spectrum. Fig. 5 shows an original chart from the Cary recording spectrophotometer with measurements made on three animals 30 days old. The tracing on the right is

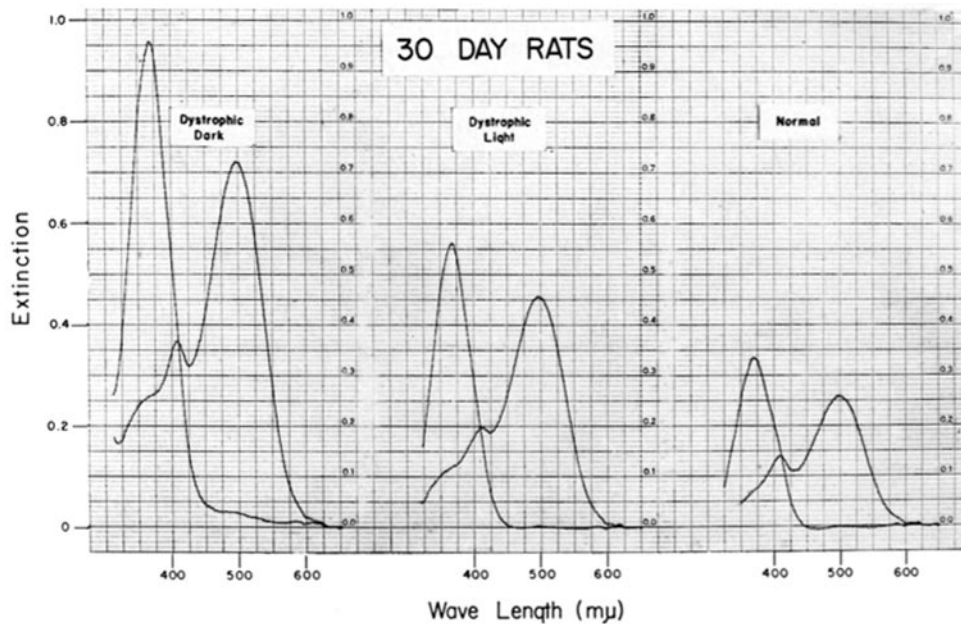


FIGURE 5

30 day rats. From right to left are absorption spectra of rhodopsin extracted from normal animal and dystrophic animals raised under ordinary laboratory conditions of illumination (Dystrophic light) or in darkness (Dystrophic dark). After white light bleach in the presence of hydroxylamine, the 493  $m\mu$  peak of rhodopsin disappears, and the retinene oxime band appears at about 360  $m\mu$ .

from the control animal, the middle record from a dystrophic animal raised in the light, and the left record is from a dystrophic animal raised in the dark. All three curves show the typical absorption spectra of rat rhodopsin, each with its  $\alpha$ -band absorption maximum at 493  $m\mu$ . The second band at about 410  $m\mu$  represents the Soret band of some heme contaminant. After bleaching in the presence of hydroxylamine, the absorption band at 493  $m\mu$  disappears, and the typical retinene oxime band appears at about 360 to 370  $m\mu$ . The ratio of extinctions of retinene oxime to rhodopsin are similar in all three cases, again confirming our impression that the rhodopsin produced by the dystrophic animals is normal.

#### LIGHT MICROSCOPY

Fig. 3 shows representative histological sections to illustrate the sequence of pathological changes. The control retina (Fig. 3 a) is from a 22-day-old animal. The visual cells appear perfectly normal and mature except that the outer segments are still about 2  $\mu$  shorter than those of an adult

animal, and many of the nuclei still contain several chromatin masses instead of the single large mass characteristic of adult photoreceptor nuclei.

In the diseased animals, we first see changes in the electrical activity of the eye at 18 days of age. At this time, the most prominent histological abnormality is a considerable increase in the thickness of the outer segment layer to almost double that of the control (compare Figs. 3 b and 3 a). This thickened outer segment layer is divided into two discrete zones. The lower one (labeled *os*) shows typical outer segments, although in this section they appear somewhat disarranged. The upper layer (*el*), however, contains no typical rod structures, and consists instead of disorganized particles of various sizes and shapes. This material is about as dense as the rod outer segments below, and stains as deeply. It will be recalled that the diseased animals at this stage have about twice as much rhodopsin in their eyes as normal animals. The inner segments (*is*) and nuclei of the visual cells (*n*) and the pigment epithelium (*pe*) look normal at 18 days.

At 22 days, the enlarged outer segment layer is still divided into two zones (Fig. 3 *c*). The inner zone composed of rod outer segments is well oriented in this specimen and again appears normal. The outer zone is made up of large chunks of outer segment-like material. At this stage, however, the inner segment layer is reduced in width (compare with Fig. 3 *a*). Some of the inner segments appear to be rounding up, suggesting that other inner segments are disappearing. The first abnormalities in size and intensity of staining of visual cell nuclei are seen in some sections. It will be recalled that at this stage the ERG first becomes grossly abnormal.

By 27 days, the outer segment layer appears much more homogeneous (Fig. 3 *d*). It has now attained its maximum width and still stains like the outer segments. The inner segments have degenerated considerably at this stage. Many of them have disappeared, and the remaining ones have rounded up. Photoreceptor cell nuclei are now rapidly degenerating; they stain more darkly than normal, and vary in size, some being very small and a few much larger than normal. The zone of photoreceptor nuclei is slightly reduced in width.

At 32 days, the outer segment layer is still wide and homogeneous, while the inner segment layer is even further reduced (Fig. 3 *e*). Many photoreceptor nuclei have disappeared without trace, and many more are degenerating. The pigment epithelium still appears normal.

By 40 days of age, the inner segments appear completely gone, and the photoreceptor nuclei have been reduced to only 2 to 3 layers (Fig. 3 *f*). The layer of outer segment debris at this stage no longer stains as deeply as previously and, with the PAS method, is virtually unstained close to the pigment epithelial layer. It will be recalled that at this stage the rhodopsin is rapidly disappearing from the eye. The pigment epithelium now appears grossly abnormal. Its cells have lost their uniform cuboidal arrangement along Bruch's membrane, and both cytoplasm and nucleus appear irregular in outline and larger in volume than normal. Some of the cells have departed from Bruch's membrane and are seen scattered through the layer of outer segment debris. In Fig. 3 *f*, the four cells at the bottom of the debris layer (arrows) probably are all pigment epithelial cells.

At 60 days, few, if any, photoreceptor cell nuclei remain (Fig. 3 *g*) and the photoreceptor-bipolar synapse zone has disappeared. The b-wave of the ERG has also disappeared by this time. The nuclei between the bipolar cells and the rod debris layer mostly represent migrating pigment epithelial cells. Pigment cells which are still located adjacent to Bruch's membrane appear elongated and flattened. The zone of outer segment debris has narrowed somewhat, and stains weakly with the periodic acid-Schiff and Giemsa methods. At 90 days the debris zone is almost unstained.

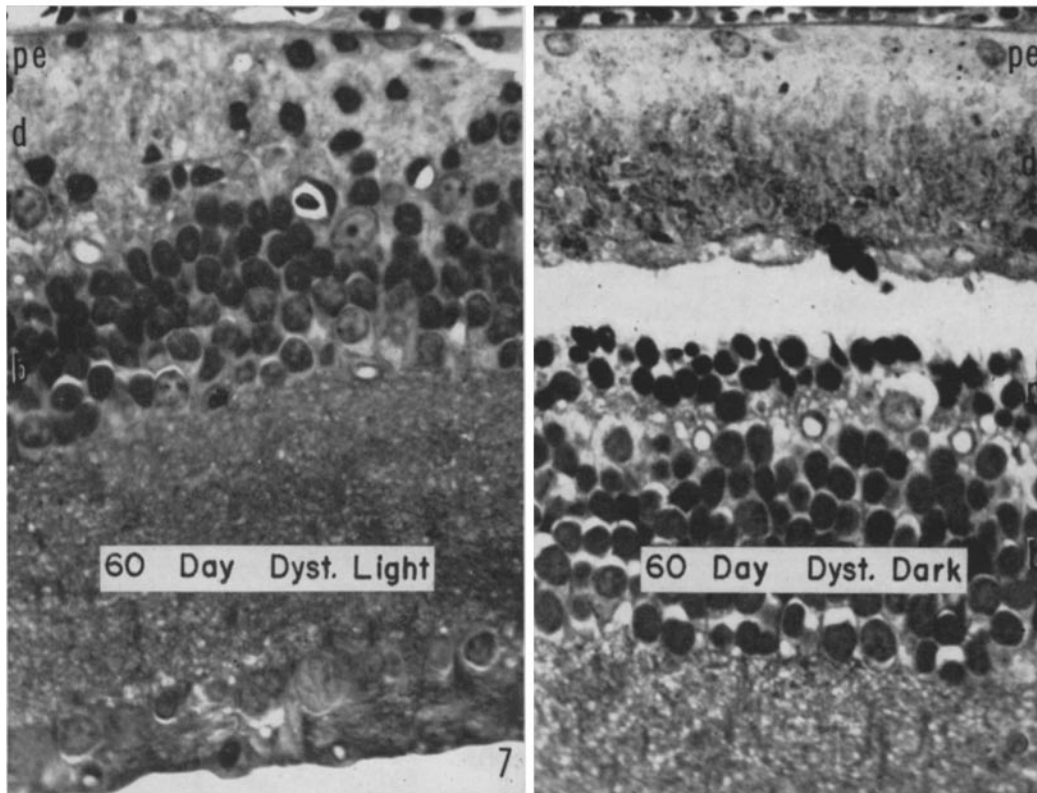
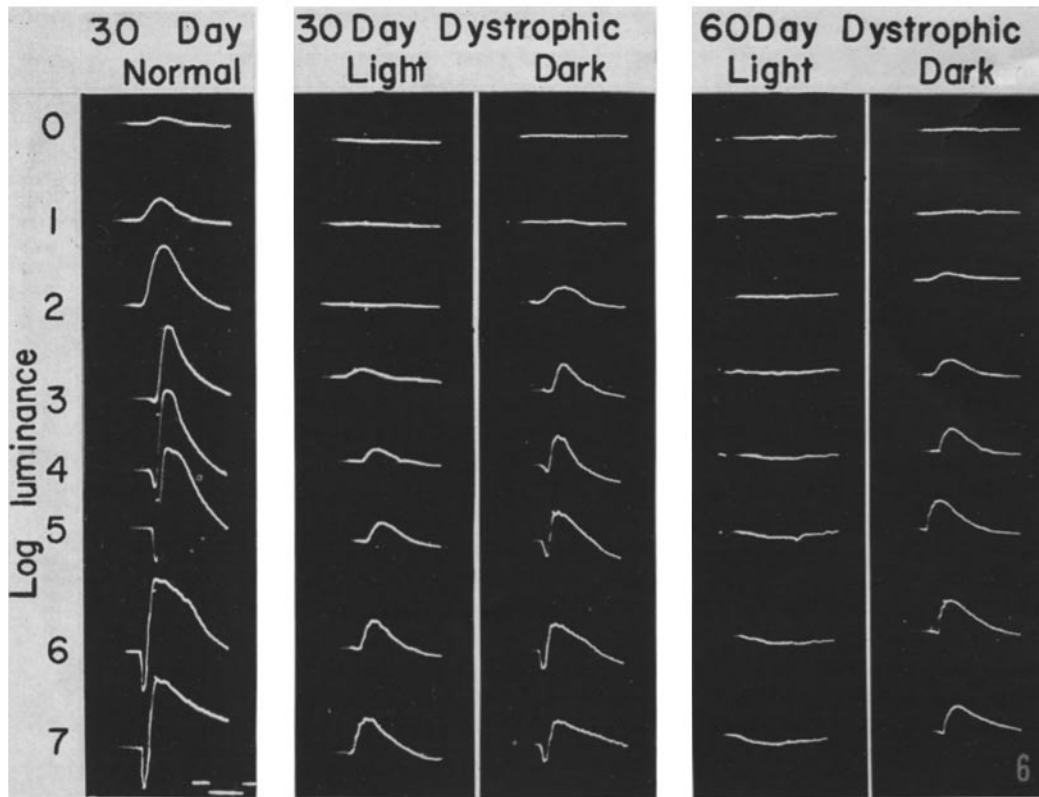
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#### FIGURE 6

Upper half of facing page. Effect of light and darkness on ERG degeneration in dystrophic animals. The ERG's were evoked with 1/50 sec flashes of white light over an intensity range of 7 log units. At the bottom of the control record is a 200  $\mu$ v calibration signal. The sweep duration is 0.5 sec. Maintenance of dystrophic animals in darkness delays the deterioration of all components of the ERG. At 30 days, the dystrophic animal kept in darkness is more sensitive to light than the animal kept in the light, and also displays a prominent a-wave. At 60 days, the dystrophic animal maintained in the dark continues to show a sizeable b-wave, while its littermate kept in the light displays no b-wave.

#### FIGURE 7

Lower half of facing page. Retinal histology of 60-day-old litter mates whose ERG's are shown in Fig. 6. The retina of the animal kept in the light shows complete loss of photoreceptor cells and migration of many pigment epithelial cells across the debris zone. The retina of the animal kept in darkness has retained a few rows of photoreceptor cell nuclei and a well defined photoreceptor-bipolar cell synapse zone. The separation between the photoreceptor cell nuclei and the debris zone is artifact. Labels: *b*, bipolar layer; *d*, debris zone; *n*, photoreceptor cell nuclei; *pe*, pigment epithelium.  $\times$  650.



During the months that follow, the zone of rod debris gradually disappears. The appearance of the retina after 1 year is shown in Fig. 3 *h*. The bipolar and ganglion cell layers are well preserved, though the number of cells in these layers may be decreased. The layer of outer segment debris is almost entirely gone, and an irregular row of pigment epithelial cells with prominent apical microvilli (arrow) is present between the bipolar cells and Bruch's membrane. No large numbers of pigment epithelial cells appear to have penetrated into the layer of bipolar cells (*b*). As the debris has disappeared, the migrated pigment epithelial cells seem to have been displaced back towards Bruch's membrane.

We have noted that the loss of rhodopsin can be prevented in dystrophic animals by raising them in the dark. It is of considerable interest to inquire whether the electroretinographic changes and histological deterioration of the retina are alleviated by darkness. We have performed this experiment twice with separate groups of animals and both experiments gave identical results. The data from one of these experiments are shown in Figs. 6 and 7.

The animals were litter mates. Half of them were put into the dark at 10 days of age and the others kept in ordinary laboratory conditions of illumination. On the left of Fig. 7 is a histological section from a 60-day-old dystrophic animal raised in the light. The degree of degeneration is close to the 60-day stage shown previously (Fig. 3 *g*); the ERG is similarly deteriorated (Fig. 6). The retina of the animal kept in the dark (Fig. 7, right) also has degenerated considerably, but is more comparable to the 40-day stage of the disease (Fig. 3 *f*). The photoreceptor nuclei have not yet entirely disappeared; there is a prominent photoreceptor-bipolar synapse zone, and, in line with this, a significant b-wave can still be elicited from the eye (Fig. 6). The layer of outer segment debris

in this retina is larger than that of 60-day-old dystrophic animals raised in the light, and also stains considerably darker. The pigment epithelial cells also show the changes typical of the 40-day stage.

It appears, therefore, that darkness can, to some degree, retard the deterioration of the dystrophic rat retina, at least through 60 days of age. It is interesting to inquire if raising animals in the dark delays the onset of the disease or slows its course. We have recorded the ERG's of animals raised in light and darkness at 30 days of age, and have found that the animals raised in the dark show less deterioration of the ERG (Fig. 6). At 30 days of age the ERG threshold of dark dystrophic animals was elevated only 1.5 log units above normal as compared with 3 log units in animals raised in the light. The a-wave of the ERG was also significantly more prominent in the animals raised in the dark, but the size of the b-wave at high intensities of stimulation was depressed equally in light and darkness. We plan to perform further experiments to test the course of the disease in the dark.

#### ELECTRON MICROSCOPY

To permit comparison and orientation, a low power electron micrograph of a pigment epithelial cell and the distal tips of several rod outer segments from a normal 15-day-old albino rat is shown in Fig. 8. The separation of rod outer segments from the pigment epithelial cell is primarily artifact; in the intact eye, the rod outer segments probably are in contact with the pigment cell. In the intact eye an interstitial mucin is interposed between pigment cell and rod outer segments; possibly this aids in holding the retina to the back of the eye (30). With aqueous fixatives such as OsO<sub>4</sub> the mucin may be dissolved away, allowing the retina to detach from the back of the opened eye. In the rat, the retina almost al-

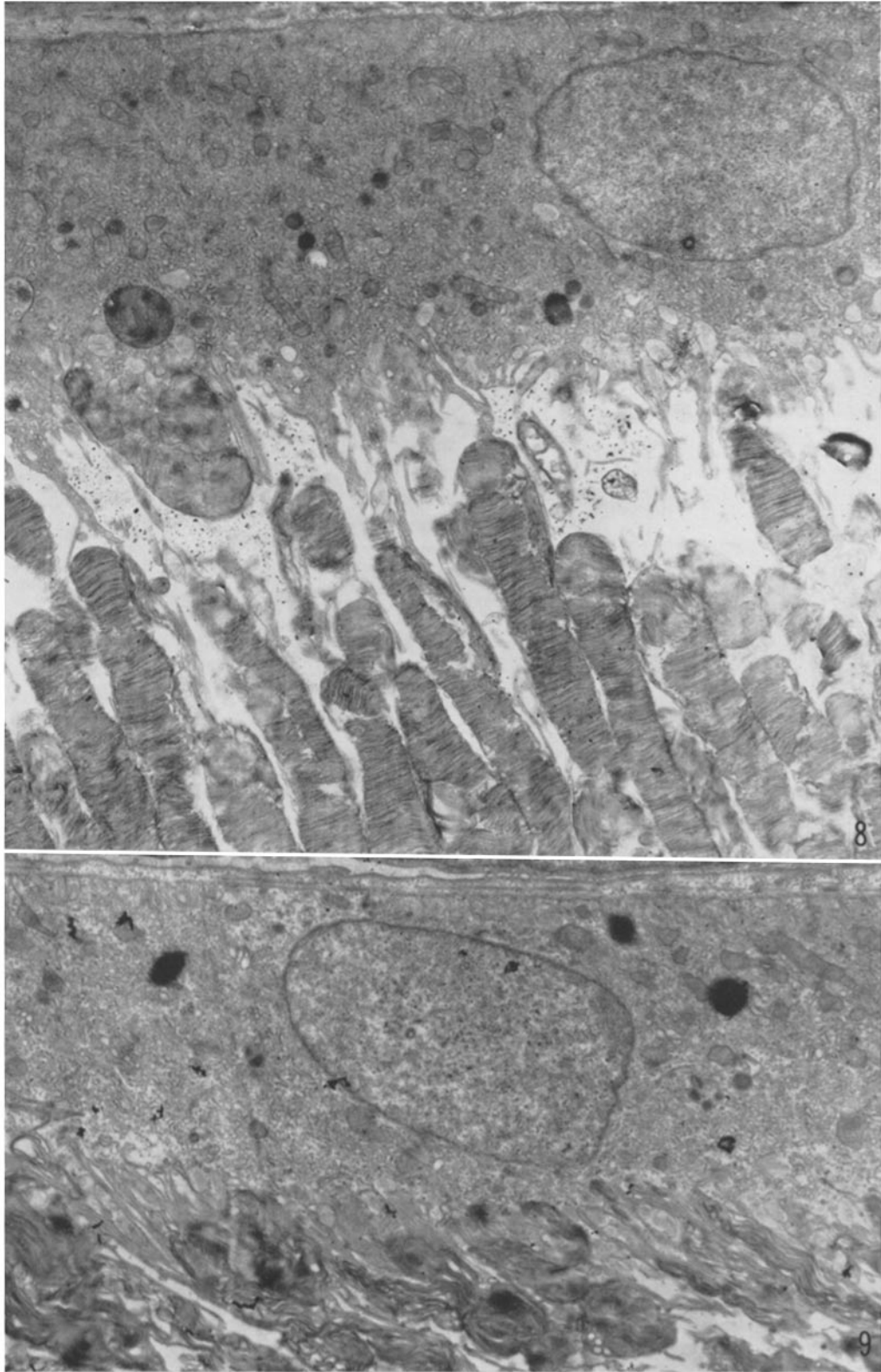
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#### FIGURE 8

Low power electron micrograph of pigment epithelium and distal tips of rod outer segments from a normal 15-day albino rat. The separation between rod outer segments and pigment epithelial cell is probably artifact.  $\times 7000$ .

#### FIGURE 9

Pigment epithelial cell from a 15-day dystrophic animal showing accumulation of extracellular and unorganized lamellae between villous processes along lower border of cell.  $\times 7000$ .



ways comes away intact; few rod outer segments stick to the pigment epithelial cell (Fig. 8).

In a dystrophic 12-day-old rat eye, disorganized outer segment material is already present, and by 15 days it is abundant (Fig. 9). Membranes of this material may be arranged in elongate bundles or sheets parallel to and closely apposed to the pigment epithelial processes, or they may be disposed in more tortuous patterns between the microvillous processes and the rod outer segments. The pigment epithelial processes and cytoplasm, and the rod outer segments appear normal.

In the dystrophic eye at 18 days, light microscopy showed a retina in which the photoreceptor outer segment layer was about twice as wide as normal, and was divided into an inner zone, containing typical rod outer segments, and an outer zone composed of pieces of rod-like material. The low power electron micrograph montage extending from Bruch's membrane through to the inner segments (Fig. 10) confirms this interpretation. Photoreceptor inner (*is*) and outer (*os*) segments, obliquely oriented but appearing normal, are seen in the lower half of the picture. Despite the oblique section, the regular size and shape of the outer segments is readily apparent. Along the top of the micrograph, two apparently normal pigment epithelial cells (*pe*) are seen, adjacent to Bruch's membrane (*Bm*). (For a more complete description of the structure of the pigment epithelial cell of the normal albino rat, see reference 15). Between the pigment cells and rods is a zone of unorganized extracellular outer segment-like material (*el*) that has been laid down in enormous pieces as compared with the size of a normal rod outer segment. The large circular mass of material in the upper center of the micrograph, for example, is approximately 7  $\mu$  in diameter. The adjacent pigment cell is about 6  $\mu$  wide, whereas rod outer segments normally average only about 1  $\mu$  in diameter.

The extracellular outer segment-like material is entirely lamellar (Fig. 11). The lamellae consist for the most part of double membranes, whose dimensions and density are similar to those of the

normal rod disc. Most frequently the lamellae are arranged in large whorls, each seeming to be composed of one large intact sheet rolled up. This is only an impression; we have not yet been able to follow single lamellae for even a few complete turns.

Although this extracellular material has the lamellar structure of the rod outer segments, it seems to be in more intimate contact with the pigment epithelium than it does with the rod outer segments themselves (Figs. 9, 10, and 11). Frequently, a pigment cell process is partly enveloped in one of the giant whorls of lamellae. Direct contact between the typical rod outer segments and the large pieces of lamellae has not been seen, and we have noted that when separation of the retina from the pigment epithelium occurs during the processing of dystrophic eyes, the layer of lamellae consistently remains with the pigment epithelium rather than with the rods. Fig. 13 shows the separated pigment layer from an 18-day-old dystrophic animal, as viewed with the light microscope. The extracellular lamellar zone in the diseased eye has come away entirely with the pigment cell. The retina separated from this preparation, incidentally, appears normal; examination of retinas alone at this age gives no evidence of disease.

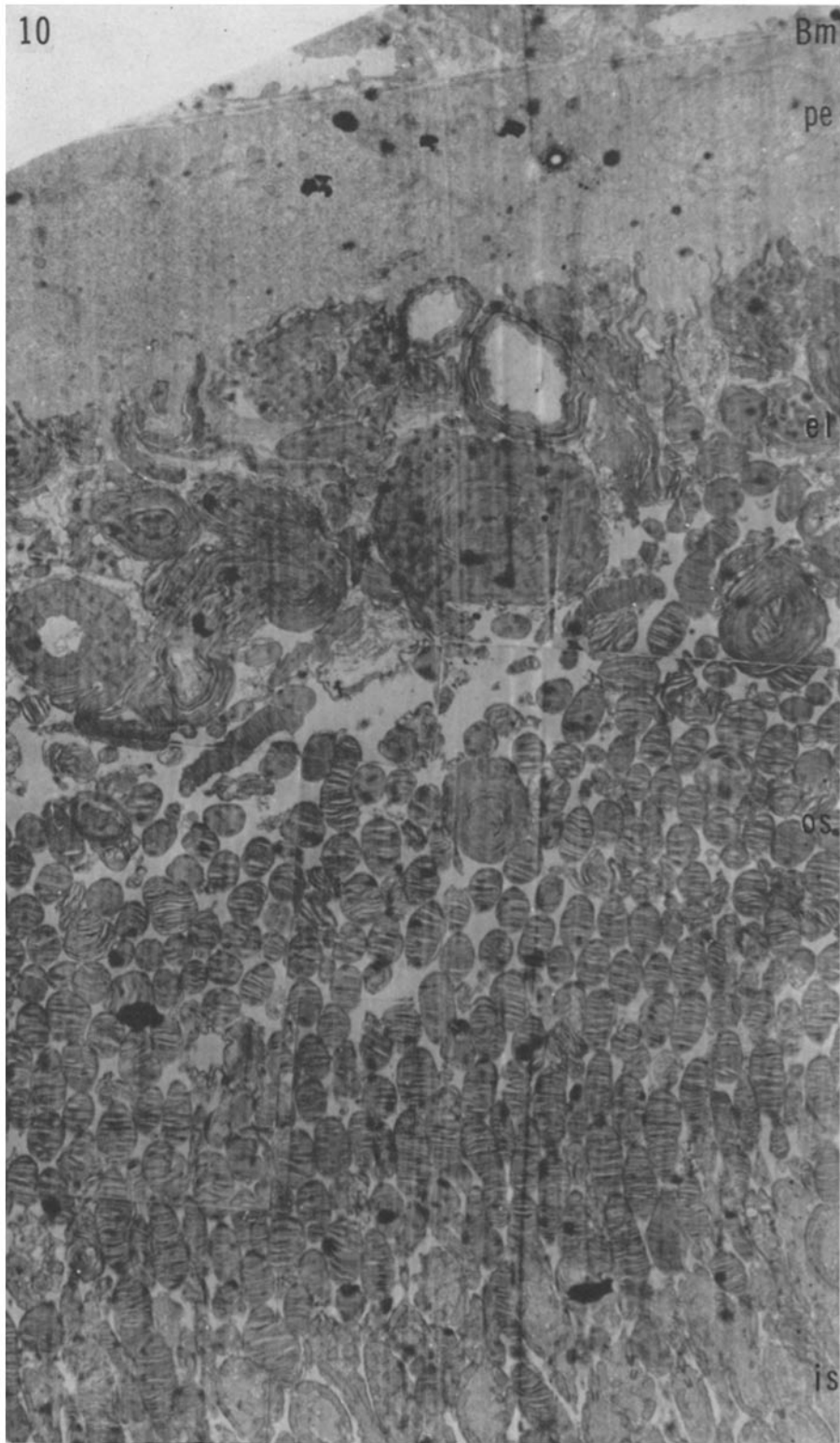
A further experiment established that the extracellular lamellae contain rhodopsin. The retinas of 18- and 20-day-old dystrophic animals were dissected away from the back of the eye and both tissue samples were analyzed for rhodopsin. The extracellular lamellae presumably remained with the pigment epithelium in the back of the dystrophic eye. In three normal animals of these ages, an average of 4 per cent of the total rhodopsin was found with the back of the eye, presumably due to contamination of rods adhering to the pigment epithelium (see Fig. 9). In the two dystrophic animals, 33 and 31 per cent of the total amount of rhodopsin was found with the back of the eye. The total rhodopsin content of these dystrophic eyes was 30 to 40 per cent above controls of the same age, indicating that probably

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FIGURE 10

Electron micrograph montage from 18-day dystrophic animal extending from Bruch's membrane (*Bm*) to inner segments (*is*) of photoreceptor cells. Giant pieces of extracellular lamellar material (*el*) have accumulated between normal-appearing pigment epithelium (*pe*) and obliquely sectioned rod outer segments (*os*).  $\times 3500$ .





all the extra rhodopsin in the dystrophic eyes is in the extracellular lamellae.

The synthesis of the extracellular lamellae and extra rhodopsin in the dystrophic eye must involve a marked increase in the synthesis of rod protein (opsin, etc.), and we might expect to see morphological evidence of this. Recently Lasansky and De Robertis (21) studied a retinal dystrophy in the mouse, and described a considerable increase in the number of ribosome-like particles in the inner segments of rods in the early stages of the degeneration. Figs. 12 and 14 show respectively low and high power micrographs of rod inner segments from the 18-day-old dystrophic rat retina. We observe no increase of ribosome-like particles or other cellular organelles in the inner segments, which appear entirely normal. The ciliary apparatus, from which the outer segment is developed, also appears normal, as does the fine structure of the rod outer segments (Fig. 14).

The formation of excess outer segment material in dystrophic eyes clearly commences considerably before day 18, when the ERG gives physiological indication of the disease process. Formation of extracellular lamellae has begun by the 12th day, when the rod outer segments are still very small. By the 15th day, extracellular lamellae are accumulating rapidly and biochemical measurements indicate that the dystrophic eyes have produced more visual pigment than the normals. Since the rod outer segments in these dystrophic retinas grow considerably in length between days 12 and 18, they must be differentiating and elongating in a normal fashion at the same time that the extracellular lamellae are accumulating between their distal ends and the pigment epithelium.

At 22 days of age, the pigment epithelial cells, extracellular lamellae and outer segments of the dystrophic retina look much as they do at 18 days. Many of the inner segments, however, have rounded up, a hint that other inner segments already have disappeared. Other than the rounded shape, no striking changes in fine structure of the

inner segments have been noted. By 27 days, many inner segments have disappeared, and almost all that remain are rounded (Fig. 15, compare with Fig. 12). Again the fine structure of the surviving inner segments looks about normal, and there is no hint as to why they are disappearing. Ribosomes, mitochondria, and connecting cilia look normal and are present in about normal numbers. We have noted, however, that in both the 22- and 27-day specimens the cytoplasm of the inner segments contains a number of large vacuoles that are not present in well preserved normal inner segments. The significance of this observation is not clear.

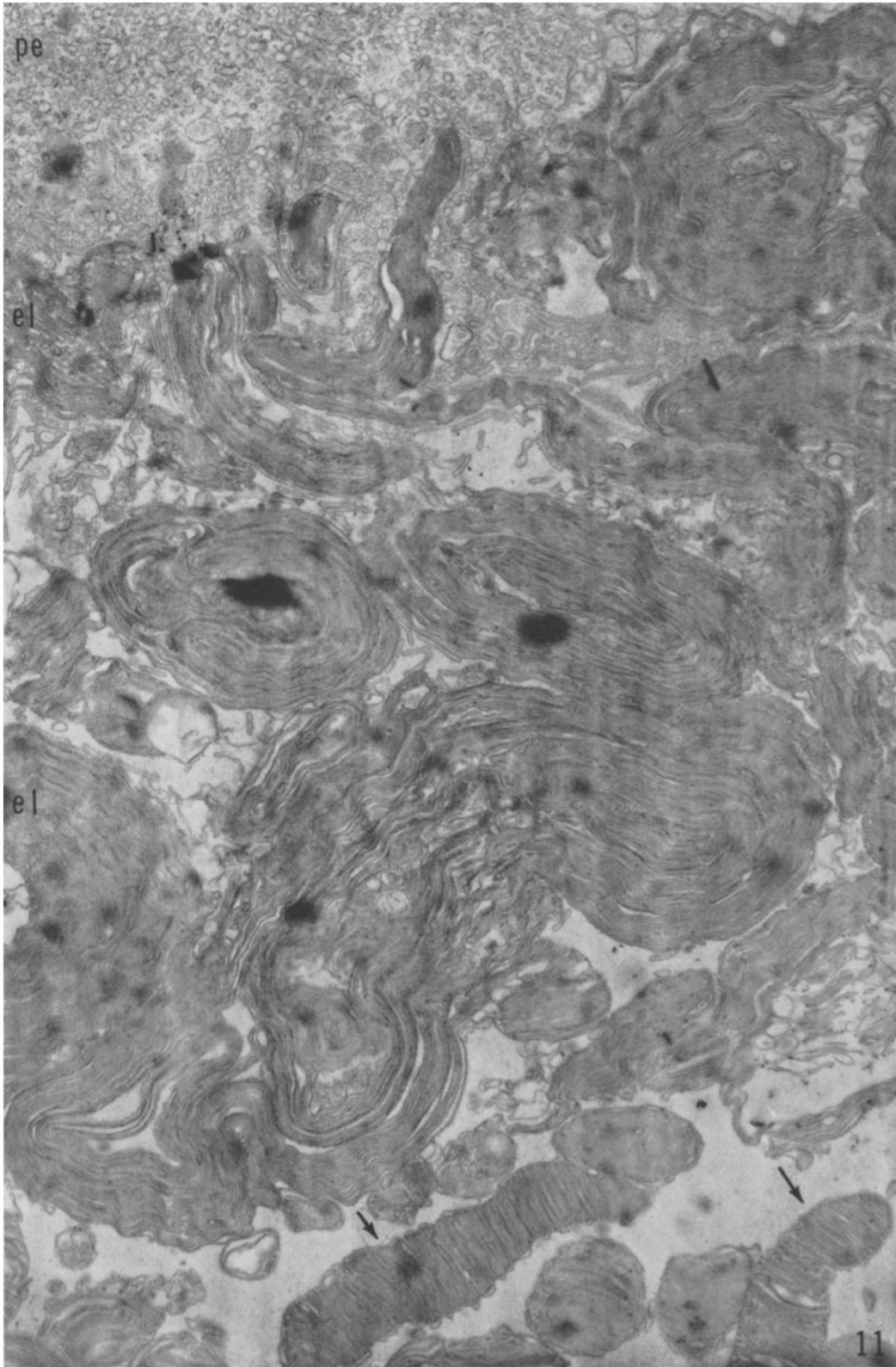
The outer segment layer at 27 days is no longer separated into two discrete zones of extracellular lamellae and rods. Fig. 16 shows a typical area in about the middle of the layer. Rods and lamellae are now mixed together. This pattern is seen throughout the outer segment layer, although toward the pigment epithelium the extracellular lamellae predominate while toward the retina there are more intact rods. Many of the rods in Fig. 16 are still quite intact and reasonably normal looking. Others show swelling of their component discs into vesicles and tubules, suggesting the rod breakdown process seen in vitamin A deficiency (14). Some of the extracellular lamellae also show swelling and vesicles, suggesting that they too may be undergoing some degeneration. By 32 days, when the rhodopsin level in the retina is declining very rapidly, the vesiculation of the rods and debris is particularly prominent.

Degeneration of photoreceptor cell nuclei was under way by day 22, and is proceeding rapidly by day 32. Fig. 17 shows a section extending from the remnants of inner segments through several layers of photoreceptor cell nuclei. Normal photoreceptor cell nuclei are finely grained, of uniform size, and of low contrast in electron micrographs (see Figs. 12 and 15). While degenerating, they begin to stain quite densely and become varied in size. Some of the degenerating nuclear masses become very large. In Fig. 17, the sequence

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FIGURE 11

Enlargement from Fig. 10. The lamellar extracellular outer segment-like material (*el*) is in close contact with apical processes of the pigment epithelial cell (*pe*). The lamellae are double, with dimensions and density similar to those of the discs in normal rod outer segments (arrows). The rod discs are enclosed within a surface membrane (left arrow), while the extracellular lamellae are not.  $\times 12,000$ .



of stages in nuclear degeneration is shown. The nucleus at the upper right appears essentially normal; the one at the top left shows slight increase in density, while those on the bottom are all quite dense, relatively homogeneous, and of various sizes.

Figure 18 shows the remains of the photoreceptor cell nuclei, the outer plexiform synapse layer, and part of the bipolar layer at 40 days (for orientation, compare with Fig. 3 f). Two relatively intact nuclei surrounded by some cytoplasm are seen at the top of the micrograph; the other photoreceptor cell nuclei present are in various stages of degeneration or remain only as fragments. The synaptic layer is thinner than normal but shows some intact synapses which probably do not deteriorate until the photoreceptor cell nuclei degenerate. The electrophysiological data support this impression, in that until the receptor nuclei entirely disappear some b-wave activity can be recorded from these eyes and the b-wave is believed to originate in the plexiform layer (3, 4). The nuclei at the lower center and right of the figure are bipolar cell nuclei which, like the photoreceptor nuclei, are quite regular in size and finely granular. The larger, more coarsely granular nucleus on the left may be a horizontal or Müller cell nucleus.

The pigment epithelium has appeared normal and now, at 40 days, begins to undergo certain changes. As noted in the light microscope, some pigment cells break away from Bruch's membrane and migrate through the debris layer. At the top of the electron micrograph montage in Fig. 19, two pigment cells have separated from the normal pigment cell layer and have presumably started to migrate. The gap seen here between the pigment cells may be an artifact, but we have observed such a space in other micrographs at this stage of pigment cell migration. At the bottom of the picture, two pigment cells (arrows) that have already migrated are easily identified by their nuclear pattern and the characteristic dense inclusion bodies in the cytoplasm (see reference 15). The inclusion bodies provide a secure criterion

for identifying the pigment epithelial cell, regardless of where in the retina it may be.

The outer segment layer also gradually changes character at this time. Generally, it becomes less electron-opaque and more homogeneous. Individual rod outer segments and bits of extracellular lamellae can still be recognized here and there, but the intervening space is becoming filled with cell processes. Whereas at earlier stages the space between rods or between particles of debris appeared empty in the electron micrographs (Fig. 16), now cytoplasmic processes of migrating pigment cells ramify extensively through the debris layer, and account in large part for its more homogeneous appearance.

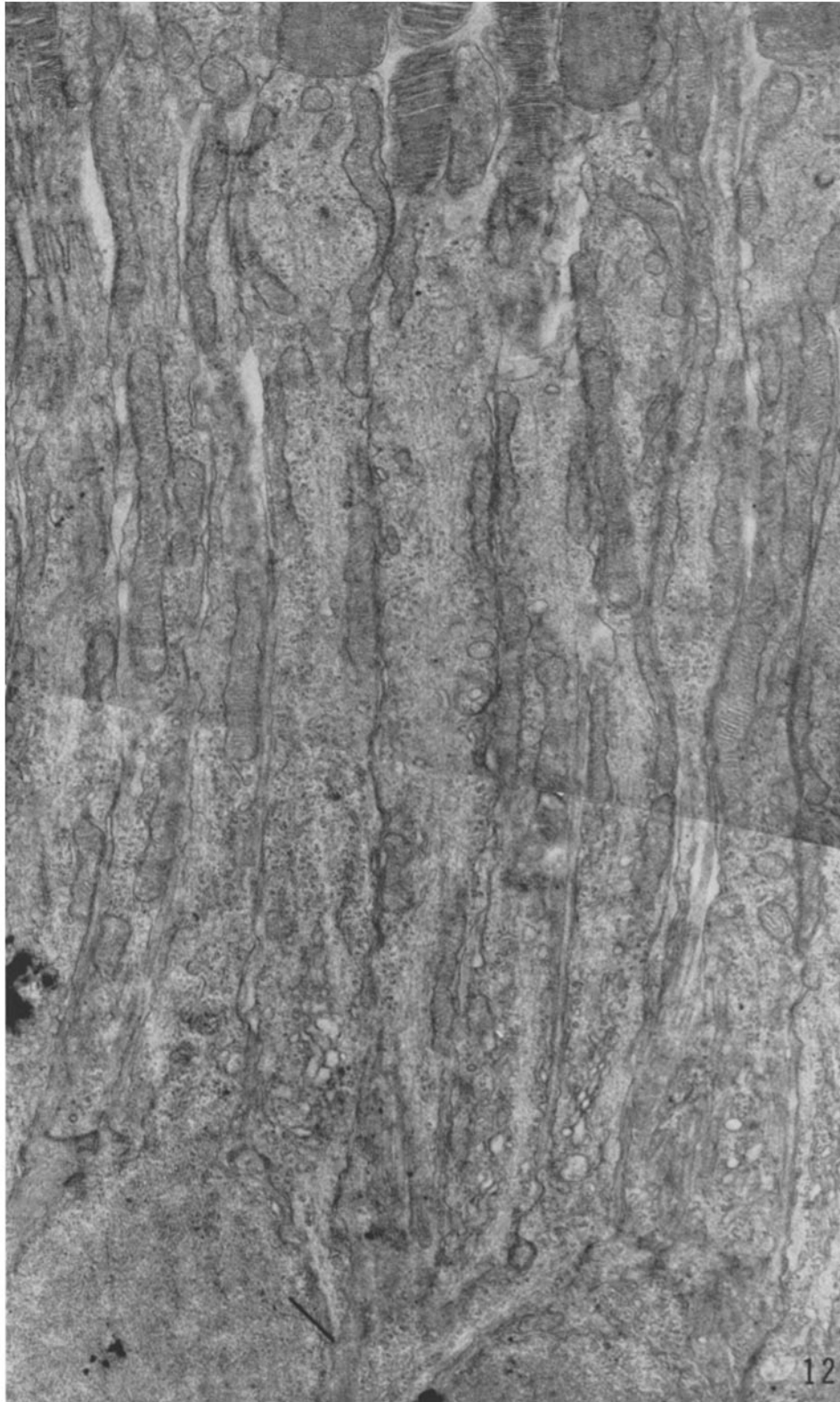
Fig. 20 shows the pigment epithelium and photoreceptor cells at 60 days. The inner segments, nuclei and synaptic processes of the photoreceptor cells have now entirely disappeared. The outer segment-debris layer is narrower, stains much less intensely, and appears more homogeneous than before. Discrete pieces of outer segments, however, are still seen. The pigment epithelial cells along Bruch's membrane have elongated parallel to the membrane and commonly overlap one another. In the normal eye, pigment epithelial cells have straight lateral borders which make a right angle with Bruch's membrane and never overlap. In Fig. 20, a migrated pigment epithelial cell is seen at the left center of the micrograph adjacent to the bipolar cell layer, and in the bipolar cell layer at the lower right corner of the micrograph, a piece of another pigment epithelial cell can be identified by its dark staining inclusion bodies.

Bruch's membrane has never been seen free of contact with a pigment epithelial cell at any stage of the disease, even when numbers of pigment epithelial cells have migrated to new positions some distance away. Possibly some division of pigment epithelial cells has occurred that has provided extra cells enough to keep Bruch's membrane coated. Fig. 19 shows two unusually small cells, possibly daughter cells, apparently breaking from the pigment cell layer. If it is true,

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#### FIGURE 12

Normal-appearing inner segments of photoreceptor cells from 18-day dystrophic animal. Two photoreceptor cell nuclei appear at the bottom of the micrograph; the proximal ends of several outer segments are seen at the top of the figure.  $\times 12,000$ .



however, that non-migrating cells spread out to cover Bruch's membrane, as suggested by the elongation of pigment epithelial cells in Fig. 20, no cell division need be postulated.

At higher magnification, the pigment cells along Bruch's membrane at 60 days are seen to differ considerably from their normal counterparts with respect to the fine structure of their cytoplasm (Fig. 21). Characteristic of the normal pigment epithelial cell cytoplasm is a very dense, entirely vesicular endoplasmic reticulum (Figs. 8, 9, and 11). In the pigment epithelial cell of the dystrophic eye at 60 days (Fig. 21) the endoplasmic reticulum is not especially dense and is frequently observed in the form of long tubules. Along Bruch's membrane, normal pigment cells have an extensively infolded surface membrane, which is not seen in the cells of dystrophic animals. The characteristic alignment of mitochondria just below the infolded surface membrane in normal cells is also not seen here and many of the cells have fewer of the processes that in the normal eye extend from the apical border of the pigment cell among the rod outer segments. In short, the pigment cells at this stage of the disease appear to have dedifferentiated to a considerable degree.

At 1 year of age, the region from Bruch's membrane to the bipolar cell layer appears as shown in Fig. 22. The outer segment debris material is nearly gone; only a small bit of it is seen at the top center of the micrograph. Along Bruch's membrane at the top right one finds, again, a thin layer of pigment epithelial cell cytoplasm. In the middle of the picture, pigment cells that presumably had migrated through the outer segment debris layer earlier in the disease have now apparently been displaced back toward Bruch's membrane. Only occasionally did we see a pigment epithelial cell in the inner layers of the retina (Fig. 20). Extending from the apical (lower) borders of the pigment cells in Fig. 22 are

remnants of epithelial cell processes, adjacent to some deteriorated synapse material. The nucleus cut off at the lower left of the micrograph belongs to a bipolar cell. Blood vessels do not reach the photoreceptor or pigment epithelial cells in the normal retina, but are frequently seen (*bv*) among the pigment epithelial cells at advanced stages of the disease. No vessels cross Bruch's membrane in our specimens.

Superficially, the pigment epithelial cells of dystrophic animals at 1 year of age appear more nearly normal than they did at 2 months. The pigment epithelial cells have tended to withdraw their processes by 1 year of age, and for the most part are again aligned in a single layer or two, though they do not form a continuous sheet of regular cuboidal cells as in the normal animal. In a light microscope section, however, the pigment cells look reasonably intact (Fig. 3 *i*), and, if earlier stages of the disease had not been seen, one might conclude that it affected only the visual cells.

## DISCUSSION

### *The Sequence of Changes*

The first sign of disease, already present at 12 days of age in the eyes of affected rats, is the accumulation of extra outer segment-like lamellae between the ends of the developing rods and the pigment epithelium. By 15 days rhodopsin is measurably increased in the dystrophic eye but up to about 18 days the photoreceptor cells of the dystrophic retinas continue to develop normally, as judged by the electrical activity of the eye and the appearance of the cells in the light and electron microscopes. Despite the apparently normal status of the photoreceptor cells, the amounts of both rhodopsin and extracellular lamellae may reach twice the normal values at 18 days.

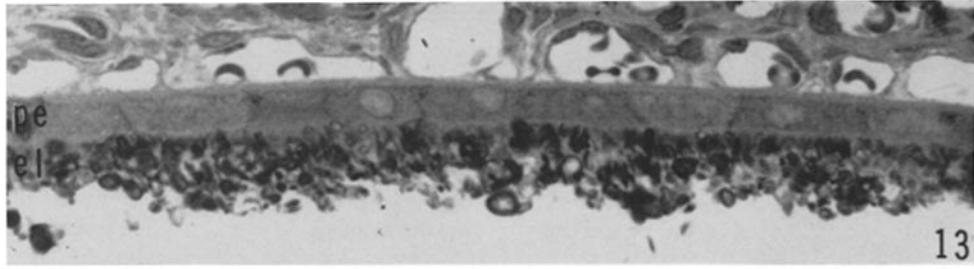
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FIGURE 13

Light microscope section of isolated pigment epithelium from 18-day dystrophic animal. The extracellular lamellar material (*el*) has come away entirely with the pigment epithelium (*pe*).  $\times 650$ .

FIGURE 14

Higher magnification micrograph of zone between outer and inner segments from 18-day dystrophic animal. Outer segments, inner segments, connecting cilia, and basal bodies all appear normal.  $\times 62,000$ .



By 22 days, the inner segments and some nuclei of the photoreceptor cells have begun to degenerate, and the ERG shows diminished sensitivity and selective diminution of the a-wave. The outer segments still look normal and the eye has continued to accumulate rhodopsin, principally in the extracellular lamellae. At about 27 days, the amount of extracellular lamellae and rhodopsin both reach a maximum. By 32 days the inner segments have almost entirely disappeared and the ERG shows further decrease in visual sensitivity and loss of the a-wave. With the loss of inner segments, the eyes apparently lose the ability to resynthesize rhodopsin, and from 30 days onward rapidly lose visual pigment if the affected rats are left in the light. Coincident with the loss of rhodopsin, both the typical outer segments and extracellular lamellae begin to degenerate. Through the second month after birth, the degeneration spreads through all parts of the visual cell. As the nuclei and synapses deteriorate, the b-wave of the ERG is lost and presumably the animal becomes completely blind.

We have no suggestions as to why the visual cells degenerate. The extracellular lamellae interposed between pigment cell and visual cell may constitute a barrier that prevents metabolites from reaching the photoreceptors, and this could be a factor in the degeneration of these cells. In the dystrophic mouse, however, no barrier has been recognized, and yet the photoreceptor cells degenerate the same as they do here in the rat.

Beginning at around 40 days, and concomitant with the loss of the photoreceptor cell nuclei, many of the pigment epithelial cells of the eye break away from Bruch's membrane, and migrate through the remnants of the outer segment-debris zone. Others seem to spread laterally and main-

tain a covering of Bruch's membrane. The pigment epithelial cells appear to dedifferentiate to some extent at this time.

The mechanism for disposing of the remnants of the degenerating photoreceptor cells and extracellular lamellae is unknown. Cytoplasmic processes of migrating pigment epithelial cells ramify extensively through the interstices between particles of debris (Fig. 19), but no evidence of phagocytosis is seen.

By 1 year the outer segment-debris zone has almost disappeared, and the migrated pigment epithelial cells regain positions near Bruch's membrane. Capillaries carrying blood from the retinal circulation are frequently encountered among the pigment epithelial cells. At 1 year, then, the retina displays an intact Bruch's membrane, a vascularized pigment epithelial layer, no visual cells, and intact layers of bipolar and ganglion cells.

#### *Relation of ERG Components to Structure of Photoreceptor Cells*

The ERG provides the best available index of retinal photoreceptor cell function (12, 25). The extracellular lamellae adjacent to the pigment epithelium seem to be composed of the same material as the rod outer segments and contain rhodopsin, but seem not to contribute to the electrical activity of the eye. Between 12 and 18 days, this lamellar material is rapidly accumulating in the affected eye, so that at about 18 days it may contain about as much rhodopsin as the still normal rods. The ERG, however, does not reflect this development; it remains normal, and then decreases when the inner segments and nuclei begin to degenerate. There is evidence that the a-wave originates in the outer segments of the

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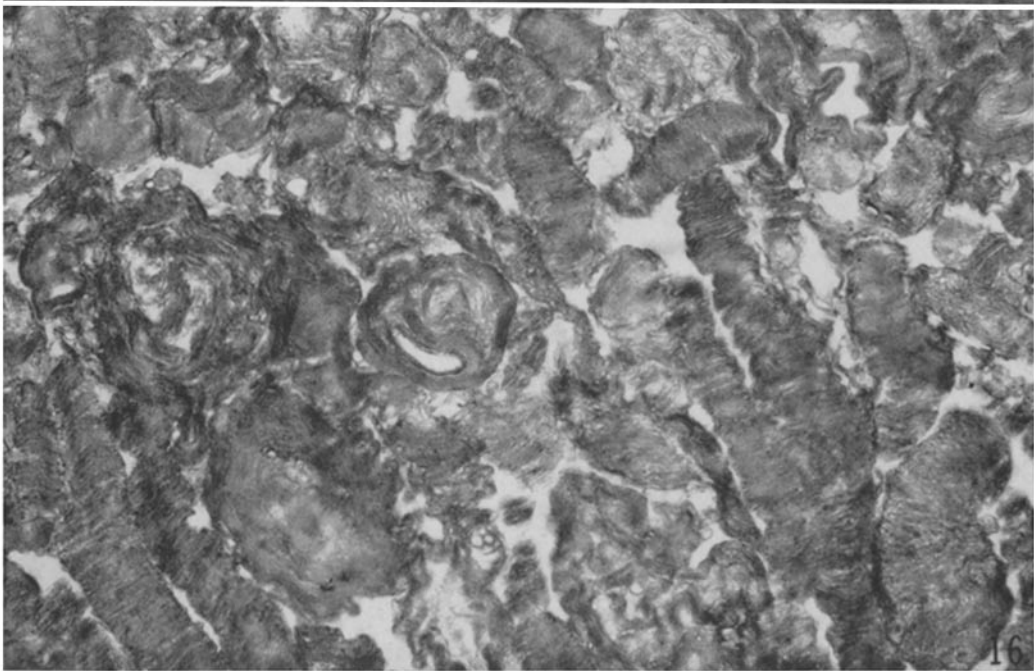
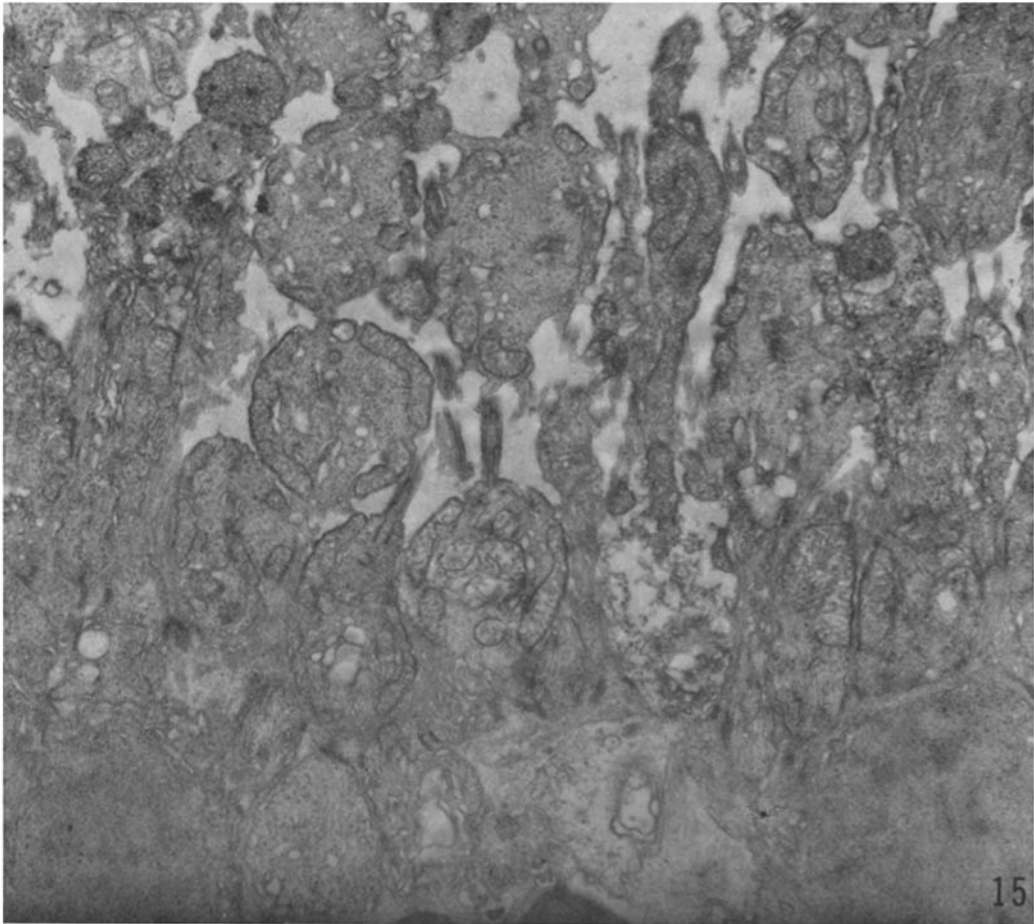
FIGURE 15

Typical inner segments from 27-day dystrophic animal. Many inner segments are degenerating or have already disappeared; the remaining inner segments characteristically have become rounded, but otherwise appear relatively normal. The photoreceptor cell nuclei at the lower corners of the micrograph also appear normal in this section.  $\times 9500$ .

FIGURE 16

A section taken in about the middle of the outer segment zone of a 27-day-old dystrophic animal. Extracellular lamellar material and photoreceptor outer segments are now mixed together. Some lamellae of each show vesiculation, indicative of early degeneration. Extracellular spaces between lamellar structures appear empty.  $\times 9500$ .





visual cell (3, 4, 25), so that particularly the a-wave might have been expected to enlarge in the diseased animals; but it did not do so. Presumably to generate electrical activity, the rod lamellae must be regularly organized and connected with the inner segment.

One of the first electrical changes noted in the diseased eye is a decline in the a-wave potential at about 22 days, when the inner segments are beginning to degenerate. The outer segments of the visual cell still look normal at this stage, and it is somewhat surprising that the a-wave potential should decline so early. Perhaps here, as suggested above in reference to the absence of electrical activity from unorganized lamellae, the loss of contact with inner segment diminishes a-wave activity. Alternatively, the a-wave may not arise in the outer segments; there is evidence that in cold-blooded vertebrates the a-wave may originate in the bipolar cells (37). The b-wave of the ERG disappears in the diseased eye in parallel with the disappearance of photoreceptor cell nuclei and of rod-bipolar synapses, as we would expect if the b-wave arises in the synaptic layer (3, 4), or in the bipolar cells (37).

An interesting relationship between visual sensitivity and rhodopsin content of the normal eye is brought out by a consideration of the 18-day data on rod length, rhodopsin content, and ERG threshold. The eye is almost maximally sensitive at this stage when the rod outer segments still have not attained their full growth (Table I) and the retina contains only about half the adult content of rhodopsin (Fig. 1). When the rhodopsin content in an adult rat eye with fully grown rods is lowered 50 per cent either by light adaptation or vitamin A deficiency, the threshold is raised approximately 2 log units, or 100 times (13, 16, 17). The situation observed here, in the immature eye, resembles that in an animal that has recovered from a severe vitamin A deficiency, but has lost half its rods (see Fig. 15 in reference 17). There also the sensitivity of the eye is close to normal,

although the retina has only about half the normal number of rods, and half the content of rhodopsin. It seems that the eye achieves its highest sensitivity when the retina contains a full complement of rhodopsin for the amount of outer segment material present, regardless, to a considerable degree, of either the length or number of outer segments.

#### *The Source of the Extracellular Lamellae*

The novel feature of the rat disease is the appearance of extracellular lamellae between the pigment epithelial cells and the rod outer segments. The material contains rhodopsin and resembles rods in electron opacity, and dimensions and spacing of the lamellae. The close relation noted between rhodopsin content and number of lamellae in both the normal eye (Fig. 1) and diseased eye (compare Figs. 3 and 4) suggests strongly that the rhodopsin molecule is an integral part of the double membrane structure found both in the normal rod disc and in the extracellular lamellae of the dystrophic eye. Determination of the origin of the lamellae material might help to clarify the site of rhodopsin synthesis in the eye.

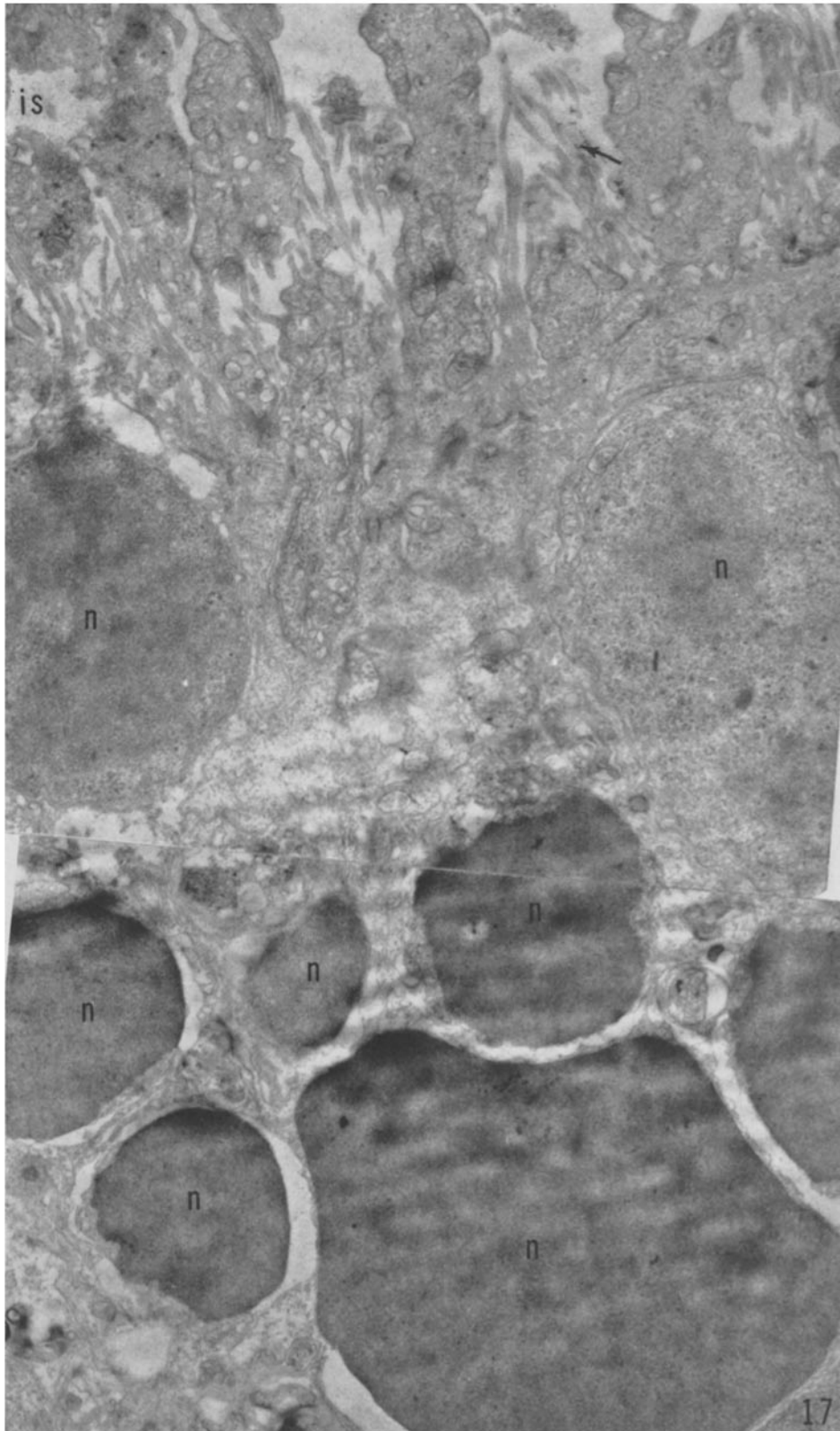
One might expect the debris to originate from the developing rod outer segment. However, we have not seen any connection between these lamellae and the rods at any stage. Extracellular lamellae are clearly present at 12 days, when the rods are only about one-fifth the adult length, yet even at this early stage we have observed no morphological links between the lamellae and the developing rods. A current view is that the rod lamellae are derived from the infolding and subsequent pinching off of the plasma membrane surrounding the outer segment and connecting cilium (11, 36). It seems very questionable, however, that the extracellular lamellae could have this source.

The only other plausible source for this material would seem to be the pigment epithelium, acting

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FIGURE 17

Photoreceptor inner segments (*is*) and nuclei (*n*) from 32-day dystrophic animal. As the nuclei degenerate, they become electron-opaque and variable in size. The inner segments have almost entirely disappeared at this stage. The villus-like processes between the remaining inner segments are the outermost extensions of the Müller cell processes (arrow), which form the so called fiber basket around the bases of the inner segments in intact retinas.  $\times 11,000$ .



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alone or in conjunction with the outer segments. Formation of the extracellular lamellae must involve a great increase in protein synthesis, but neither pigment epithelial cell nor photoreceptor shows increased numbers of ribosome particles or highly oriented membranes of endoplasmic reticulum, the usual hallmarks of extensive protein synthesis. It has not been shown, however, that ribosomes are involved in the synthesis of membranes. Perhaps production of lamellar structural constituents involves cellular mechanisms different from those involved in the production of protein secretory products so well described for exocrine cells (27).

The extracellular lamellae do seem to be in very intimate contact with the apical processes of pigment epithelial cells, from 12 days onward. The morphological relationship is reinforced by the repeated observation that the lamellae remain with the pigment epithelium when the retina and pigment cells are separated during fixation and preparation of the eye. Also strengthening this view is the finding that normal pigment epithelial cells of the adult rat possess membranous inclusion bodies that contain rod-like lamellae (15). Since recognition of the source of this extracellular lamellar material in the dystrophic eye likely would have considerable relevance for the source of normal outer segment lamellae, we are continuing to study this aspect of the disease.

#### *Phenotype and Genotype*

Retinal degenerations in several mammalian species have commonly been considered to be essentially one disease. The earlier investigators classified the disease as an abiotrophy, an inherited disorder involving loss of vitality of unknown cause on the part of a mature cell (9). Lucas *et al.* disputed this classification and argued that, in mouse, rat, dog, and probably man, degeneration sets in while the photoreceptor cells are still immature; they therefore preferred the

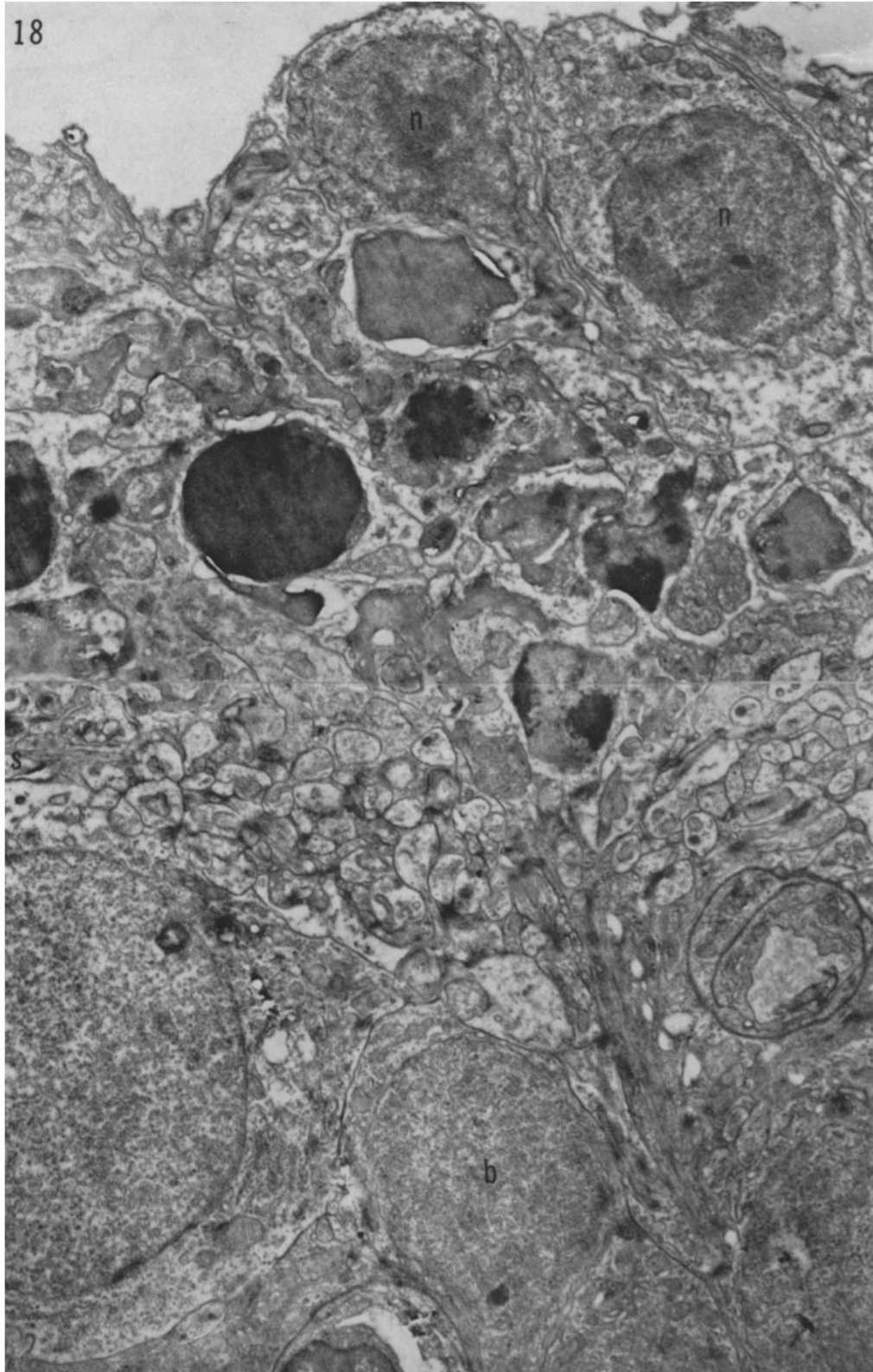
term "congenital anomaly of progressive character" (23). More recently, Sorsby and Williams (33) have suggested a more serviceable classification, intended primarily to encompass certain human retinal disorders: "aplasia" would refer to an arrested development severe enough to preclude normal function, and "dystrophy" would indicate a postnatal degeneration of a tissue which had developed sufficiently to function adequately until changes set in.

In Sorsby's terms, the rat disease becomes a dystrophy and the mouse disease an aplasia. In the rat, the ERG develops to a virtually normal adult state and the rods likewise attain almost normal form. In the mouse, the ERG is always abnormal (26) and the rod outer segments form only to a limited extent (26, 31). Further differences are the swift pace of degeneration in the mouse and the remarkable formation of rhodopsin-rich extracellular lamellae in the rat. The dog disease also is a dystrophy, in that rod outer segments form and only slowly disappear (22, 28), and an ERG has been recorded (29); however, in contrast to the rat dystrophy, no zone of extracellular lamellae is seen in the dog.

The question is, are these differences between rat and mouse diseases fundamental? Both show autosomal recessive inheritance and a common phenotype at the chronic stage of the disease. On the one hand, many genes would be expected to influence the formation and maintenance of a cell as complex as the photoreceptor cell. Many instances are known of a common phenotype resulting from different mutations, for example, postnatal degeneration of the organ of Corti in the mouse as a result of several independent mutations (10). A given tissue can express only a limited number of reactions; in the retina the late stage of chronic vitamin A deficiency (17) is difficult to distinguish histologically from advanced stages of the inherited degenerations of photoreceptor cells. On the other hand, a given mutant gene might be

FIGURE 18

Photoreceptor cell nuclei and synapses at 40 days. No inner segments are seen by this time. Two relatively normal-appearing photoreceptor cell nuclei (*n*) are seen at the top of the micrograph but the other photoreceptor nuclei show various degrees of degeneration. The synapse zone (*s*) is thinner than normal, but some synapses still appear relatively intact. At bottom of micrograph, the small nucleus in the center belongs to a bipolar cell (*b*); the large nucleus to its left may be either a Müller or horizontal cell nucleus.  $\times 6,000$ .



expected to produce different phenotypes in different species. Phenylalanine hydroxylase deficiency, for example, is expressed phenotypically as "phenylketonuria" in man, and "dilute" in mouse (8). Species differences such as the presence or absence of lamellar debris in a retinal disorder might reflect merely differences in the age of onset of the degeneration rather than a basically different disease process. The question of identity will remain open until one defines a more fundamental aspect of the phenotype, such as a primary enzyme defect.

### *Relation of Animal and Human Inherited Retinal Degenerations*

In human retinal degeneration, pathology has been studied only at late stages, and functional studies have so far not served to define the early features of the disorders. The term "retinitis pigmentosa," with its several modes of inheritance, probably represents several human diseases which have a common and relatively uninformative end

stage (7). With the recognition that the rat retinal dystrophy involves overproduction of rhodopsin, it becomes important to determine whether any forms of the human disease are comparable. Data on rhodopsin concentration can now be obtained in intact humans since the development of retinal densitometry by Rushton (6). Should a human counterpart of the rat disease be recognized, one would want to explore the possibility, by analogy with the rat, that the pace of the human disease might be slowed by sparing rhodopsin by wearing dark red glasses and avoiding bright light.

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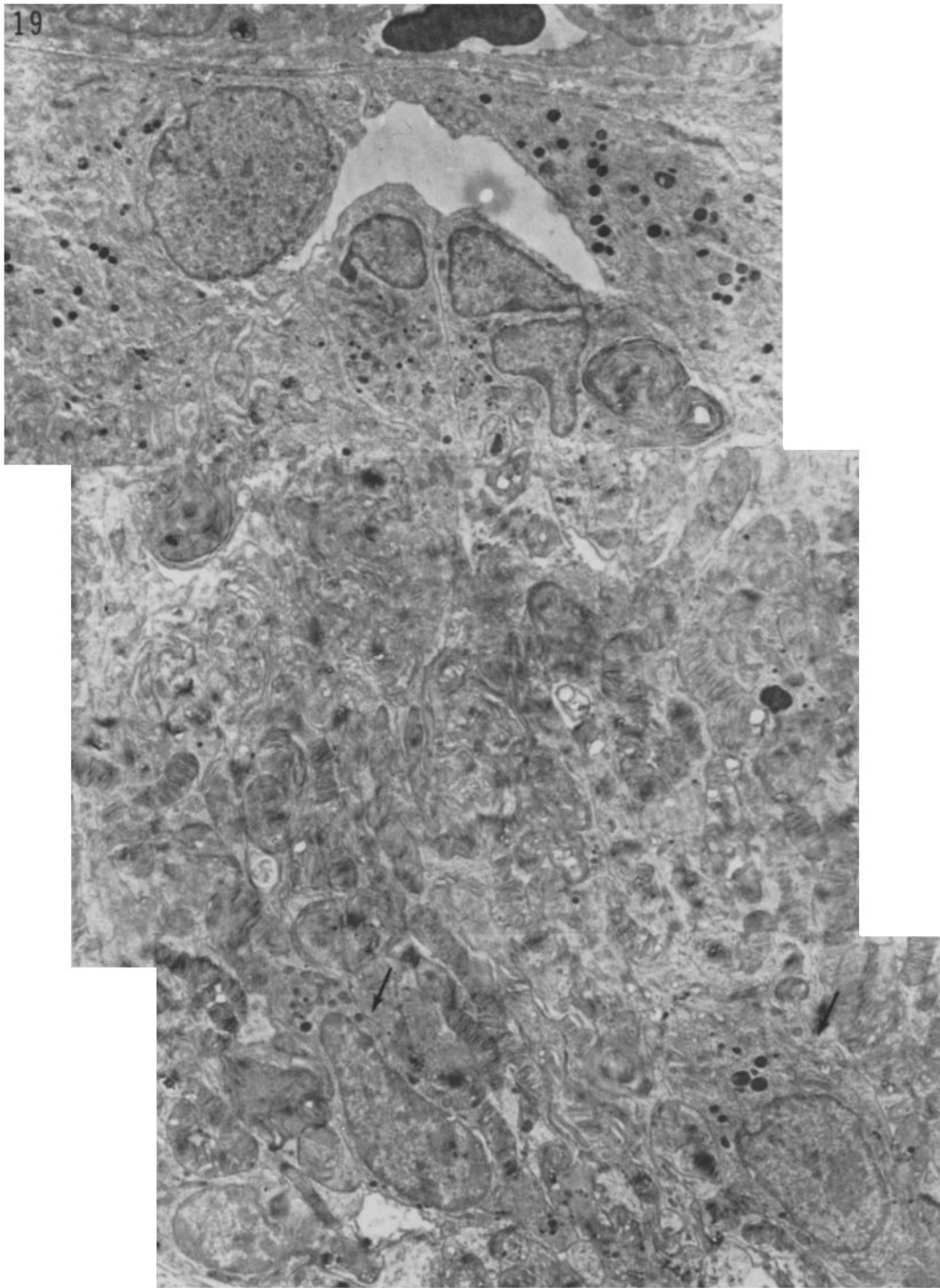
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FIGURE 19

Low power montage through pigment epithelium and outer segment-debris zone from 40-day-old dystrophic animal. At top of micrograph, two cells appear to be breaking away from pigment epithelial layer. At bottom of micrograph, two migrating pigment epithelial cells (arrows) are recognized by their characteristic inclusion bodies. The interstices between outer segments or pieces of debris are occupied by cytoplasmic processes of pigment epithelial cells, many containing inclusion bodies (contrast with Fig. 16).  $\times 5000$ .

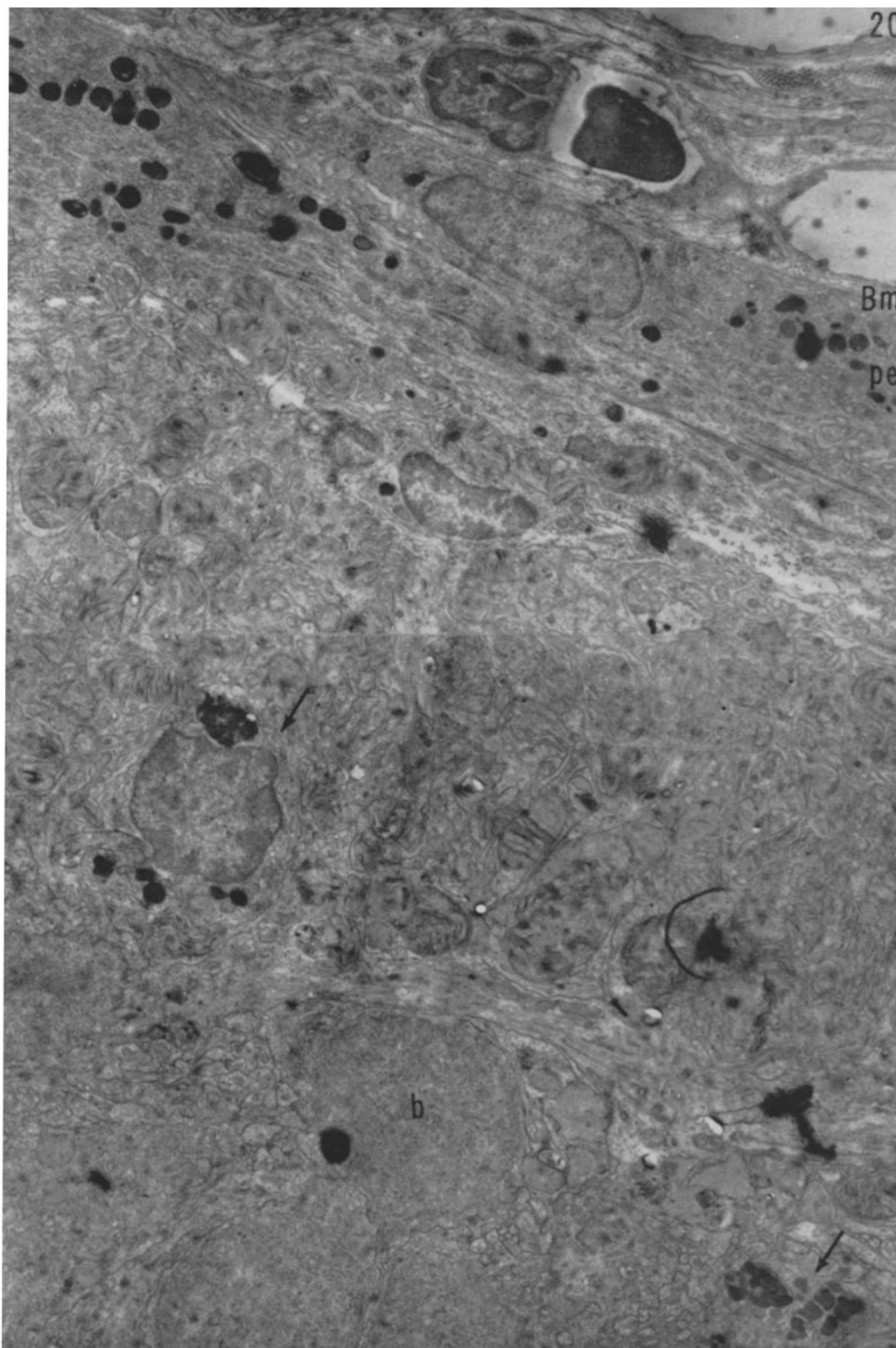


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FIGURE 20

Low power micrograph from Bruch's membrane (*Bm*) through to bipolar cells (*b*) in 60-day dystrophic retina. No receptor cell inner segments, nuclei or synapses are left by this stage. The pigment epithelial cells (*pe*) along Bruch's membrane have elongated and overlap one another. A migrated pigment cell is seen in the debris layer in the left center of the micrograph (upper arrow), and another is in the bipolar cell layer at the bottom right of the micrograph (lower arrow).  $\times 5500$ .



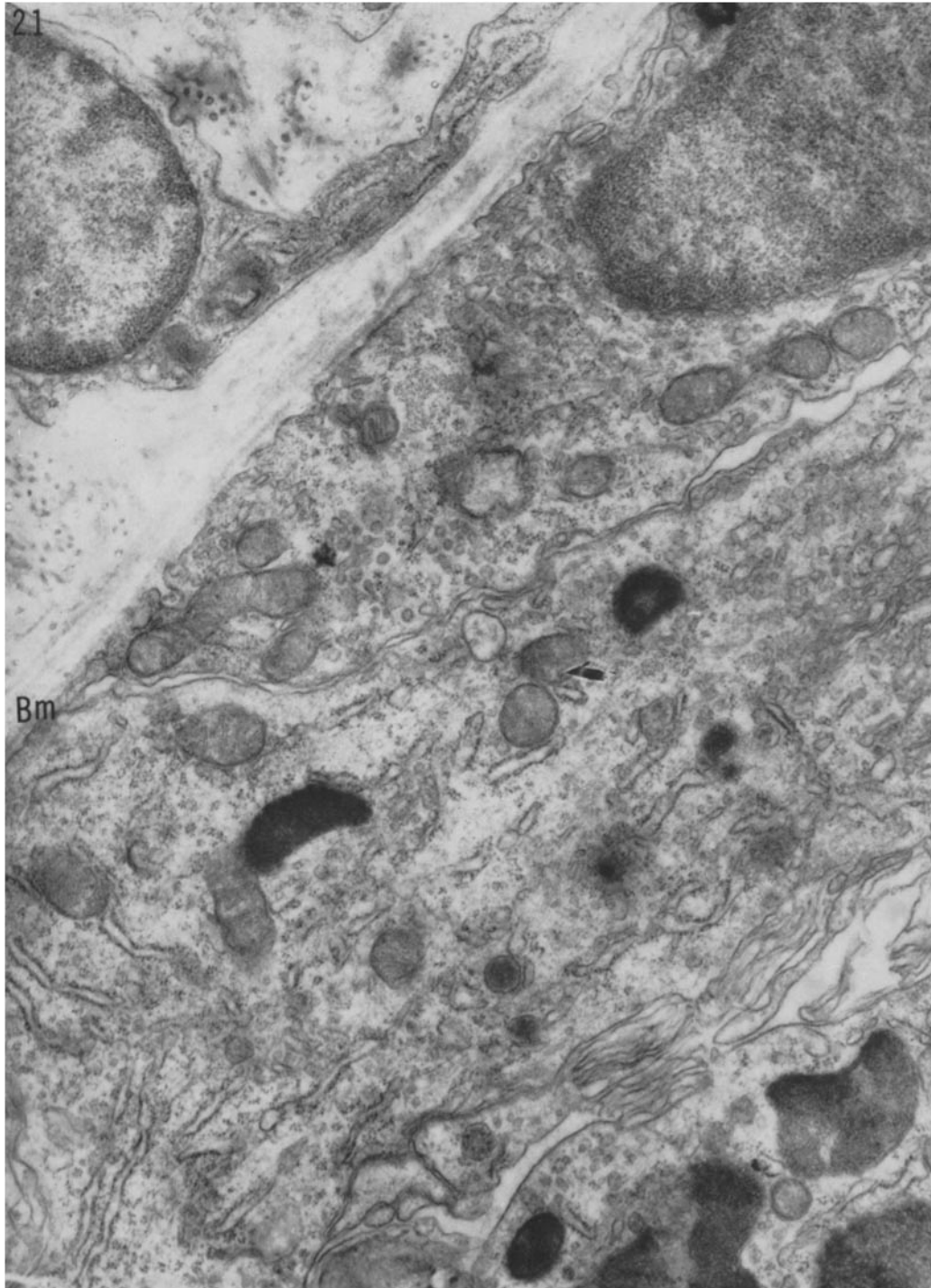


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**FIGURE 21**

A higher power micrograph of parts of three pigment epithelial cells in 60-day-old dystrophic retina. The cells appear to have dedifferentiated to some extent; the endoplasmic reticulum is less dense than normal and often tubular in form, the cell surface bordering Bruch's membrane (*Bm*) is no longer extensively infolded, and the cells have changed shape and characteristically overlap one another. The area of pigment cell cytoplasm which occupies the lower right corner of the micrograph contains typical granular inclusion particles found in rat pigment epithelial cells.  $\times 44,000$ .



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FIGURE 22

Region from Bruch's membrane (*Bm*) to bipolar cells from 1-year-old dystrophic retina. The debris has almost entirely disappeared by this time. A thin layer of pigment cell cytoplasm can be seen coating Bruch's membrane at the top of the micrograph. The pigment cells in the middle of the micrograph are apparently cells that had migrated, but have now been pushed back towards Bruch's membrane as debris has disappeared. Blood vessels (*bv*), that in the normal retina reach only ganglion and bipolar cells, now penetrate among the pigment epithelial cells. Villus- and sheet-like processes extend from pigment epithelial cells toward the retina and contact remnants of the photoreceptor cells and debris. A bipolar cell nucleus (*b*) is seen in the lower left corner of the micrograph.  $\times 10,000$ .

