Production rate and removal of rod outer segment material in vitamin A deficiency*

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Normal albino rats were placed on a standardized vitamin A-deficient diet. The rate of rod outer segment renewal and removal was determined at varying levels of vitamin A deficiency by autoradiography. Increasing vitamin A deficiency causes a decreasing rate of photoreceptor outer segment renewal. The removal of rod outer segment material by the pigment epithelium normally takes ten days. The level of vitamin A deficiency did not change this time. Since rod outer segment production was slowed with deficiency, the amount of material being removed progressively became less as vitamin A deficiency increased. This finding suggests that effective phagocytic removal cannot take place until a time-related change occurs in the outer segment material.

Key words: retinal rod outer segment, retinal pigment epithelium, vitamin A deficiency, retinal dystrophy, retinitis pigmentosa, phagocytosis, rats, histopathology, autoradiography.

Vitamin A deficiency causes night blindness, diminution of the electroretinogram (ERG), and death of photoreceptor cells. With progression of deficiency, there develops blindness, absent ERG, and loss of the outer nuclear layer of the retina. Retinitis pigmentosa causes night blindness, diminution of the ERG, and death of photoreceptor cells. It progresses to blindness, absent ERG, and loss of the outer nuclear layer of the retina.

The striking similarity of the ocular manifestations of these two conditions has caused countless retinitis pigmentosa patients to be placed on massive vitamin A therapy. Gradually, however, there has been disappointment with this regimen in the treatment of familial retinitis pigmentosa because it does not work. At best, the rare clinical condition of "retinitis pigmentosa" in abetalipoproteinemia may be benefitted by vitamin A therapy. There are no other exceptions.

Actually, there is theoretical reason to believe vitamin A deficiency may be help-
Rod outer segment material

ful in retinal dystrophy. We have shown that the Royal College of Surgeons' rat with hereditary retinal dystrophy exhibits an inability of the pigment epithelium to remove its rod outer segment material which is produced initially at a normal rate.2, 3 The rod outer segments build up until their accumulation pushes the photoreceptor cells from their nutritional source. Only then does photoreceptor cell death begin. In normal4, 5 and dystrophic2 animals, rod photoreceptor outer segment material is continually produced at the inner segment and is subsequently displaced toward the pigment epithelium by newer outer segment material. The pigment epithelium removes the outer segment material by phagocytosis in the normal animals, but it is unable to do this in the dystrophic rat. We have hypothesized that if vitamin A deficiency can cause the rate of photoreceptor renewal to be slowed, a retardation of the accumulation of rod outer segment material could be effected. If it is found that some retinal dystrophies are caused by a relative inability to phagocytize the dystrophic individual's rod outer segment material, it could be possible to reach an equilibrium state between pigment epithelium removal and photoreceptor production of the outer segments. This would hold true whether the outer segment material, the pigment epithelium, or both were abnormal in the dystrophy.

It was with the above thoughts in mind that we undertook to study rod photoreceptor outer segment renewal and removal in the vitamin A-deficient normal rat.

Materials and methods

**Animals and diet.** Weanling albino Wistar rats born in our colony were placed on vitamin A test diet and supplemented with retinoic acid in a dose of 100 µg, three times a week.6 These animals did not have a retinal dystrophy. They were on the deficient diet from two to four months prior to monitoring.

The control animals were normal albino Wistar rats fed a regular laboratory diet.

**Room conditions.** Control and experimental animals were kept in the same room, which is illuminated with a cycling 60 watt light bulb which is on 12 hours per day. The only other illumination was when lights were turned on for feeding and cleaning.

**Electroretinography.** At varying intervals, electroretinograms (ERG's) were taken. Prior to ERG, the animals were allowed to dark adapt for at least 24 hours. They were then transferred in darkness to the screen room where the ERG's were taken. Anesthesia consisted of pentobarbital sodium in a dose of 3 mg. per 100 Gm. given intraperitoneally. Ophthalmic (proparacaine HCl 0.5 per cent) was used for corneal anesthesia. The ERG's were taken on a 564 Tektronix oscilloscope with a 2B67 time base unit and a 3A9 differential amplifier.

A modified "pledget" was used, consisting of silver-silver chloride wire placed in a Pasteur...
Fig. 2. Autoradiograph of rats 15 minutes after injection of tritiated methionine. All photomicrographs show the pigment epithelium of the retina at the top and the rods and nuclear layers beneath it. Uptake of radioactivity is seen in the pigment epithelium and the inner segments of the photoreceptor cells. a = Control, b = 2 log units less sensitive by ERG, c = 5 log units less sensitive by ERG. Note: All photomicrographs were chosen to exhibit the band position and comparable areas of the retina are not necessarily exhibited, so thickness of the layer of rods is not significant. x400.

Fig. 3. Autoradiograph of rats five days after injection of tritiated methionine. Band of maximal labeling is seen about midway in the outer segment material. a = Control, b = 2 log units less sensitive by ERG, c = 3 log units less sensitive by ERG. Note: Decreased thickness of the layer of rods is due to the photo being taken in the extreme anterior retina near the ora serrata as the rest of the histologic specimen was detached and folded. x400.
Fig. 4. Autoradiograph of rats 10 days after injection of tritiated methio- 
nine. Band of maximal labeling is in distal outer segment material near the 
pigment epithelium. Only random background radiation persists. a = Control, b = 3 log units less sensitive by ERG, c = More than 5 log units less sensitive by ERG. Note: No ERG was obtained on the low strobe setting used in all the animals in this study. Response could be seen at high strobe setting. x400.

Fig. 5. Autoradiograph of rats 15 days after injection of tritiated methio- 
nine. Band of maximal labeling has already been removed by phagocytosis 
of the pigment epithelium. Only random background radiation persists. a = Control, b = 3 log units less sensitive by ERG, c = More than 5 log units less sensitive by ERG. Note: No ERG was obtained at the low strobe setting used in all the animals in this study. Response could be seen at high strobe setting. x400.
Table I. Vitamin A deficiency levels and time of sampling of animals reported

<table>
<thead>
<tr>
<th>Time of sampling after injection of tracer</th>
<th>15 minutes</th>
<th>5 days</th>
<th>10 days</th>
<th>15 days</th>
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<tr>
<td>Number of log units of neutral-density filter needed to extinguish B wave of ERG in dark-adapted rats at low intensity strobe</td>
<td>6 &lt;i&gt;o&lt;/i&gt;*, †</td>
<td>6 &lt;i&gt;o&lt;/i&gt;*, †</td>
<td>6 &lt;i&gt;o&lt;/i&gt;*, †</td>
<td>6 &lt;i&gt;o&lt;/i&gt;*, †</td>
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*Photomicrographs of these animals are shown in this publication. Number beneath asterisk is that of shown figure.
†Denotes nondeficient control animals.

pipette filled with Beckman Offner paste which was adjustable by a tuberculin syringe plunger placed in the top of the pipette. The Offner paste is jelly-like, sticks to the cornea, and will move with mild animal movements without losing contact with the eye. A cheek electrode and an indifferent electrode in the tail completed the circuit. The lids were held open with sutures. Dilation was by 1 per cent Mydriacil (tropicamide HCl). Log threshold was determined for our strobe at low intensity by calibrating against normal rats, which required six neutral density filters to extinguish the B wave of the ERG (Fig. 1). Animals were studied from control to more than 5 log units less sensitive than the control, as determined by their dark-adapted ERG.

Autoradiography. Rate of photoreceptor renewal was followed by injecting the animals with 20 µCi. per gram of generally tritiated D-L-methionine, and enucleating eyes under anesthesia at 15 minutes, 5 days, 10 days, and 15 days after injection. The autoradiographic technique was identical to that of a previous publication. The essential amino acid methionine served as a label of the protein production of the outer segment material. It is incorporated into the outer segment protein during the formation of this protein which takes place in the inner segment of the rod photoreceptor cells.

Results

Analysis of the findings are divided into two parts.

Part I. The relative position of the radio-label to both the pigment epithelium and the outer limiting membrane at the same time interval after injection of the tracer was compared at the varying levels of vitamin A deficiency. At each time interval studied, no difference in the relative position of the radiolabel could be found among the varying levels of vitamin A deficiency studied and/or the control.

At 15 minutes after injection, each animal monitored showed uptake of the labeled amino acid in the pigment epithelium and the inner segments of the rod photoreceptor cells (Fig. 2). Vitamin A-deficient animals studied at 15 minutes after injection produced ERG's that were up to 5 log units lower (less sensitive) than the controls (Table I).

At 5 days after injection, each animal monitored showed a reaction band of labeled rod outer segment material about half way between the inner segments and the pigment epithelium (Fig. 3). This is an indication that the protein produced five days earlier in the inner segments has been displaced about halfway toward the pigment epithelium by more recently produced rod outer segment material. Vitamin A-deficient animals monitored at 5 days after injection produced ERG's that were up to 3 log units lower (less sensitive) than the controls (Table I).

At 10 days after injection, each animal monitored showed labeling in the distal rod outer segment material adjacent to the pigment epithelium (Fig. 4). The reaction band of labeled amino acid produced 10 days before was being removed by the pigment epithelium. Animals monitored at 10 days after injection varied to more than 5 log units less sensitive than the controls (Table I).

At 15 days after injection, only diffuse labeling is seen in the rod outer segment material (Fig. 5). This diffuse labeling indicates that the band of labeled outer segment material has been removed by the pigment epithelium. Animals monitored 15 days after injection produced ERG's up to more than 5 log units lower (less sensitive) than the controls (Table I).

Additionally, it is to be noted that in each animal studied, the relative position
of the reaction band between inner segment and pigment epithelium was the same no matter what area of the retina was being examined. This is of significance since the thickness of the layer of rods between the inner segment and the pigment epithelium varies with different locations in the retina. For example, this distance is shortest in the normal retina in the anterior areas as the pars plana is approached.

**Part II.** Comparative measurements of the thickness of the layer of rods between the external limiting membrane and the pigment epithelium were carried out in similar, comparable areas of the retina in control animals and at varying levels of vitamin A deficiency. Intrinsic variables in this type of measurement include the area of the retina studied because the thickness of the layer of rods diminishes as one moves into the extremely anterior retina and adjacent to the optic nerve. Also, a tangential section through the layer of rods causes the distance to seem artificially increased. Fixation artifacts must also be considered. Several animals were excluded as the histologic specimens available were inappropriate for the measurements desired. The aforementioned variables were considered when the measurements were carried out, and only approximate trends, not absolute ones, are indicated. Measurements of the thickness of the layer between the external limiting membrane and the pigment epithelium were observed at 400 μ intervals starting 1,000 μ from the ora serrata. Twelve measurements were taken from each specimen studied at each deficiency level, and each deficiency level was compared to the control which was arbitrarily taken to be 100 per cent.

Compared to the normal animals, the deficient animals showed a decrease in the amount of rod outer segment material and inner segments, and thinning of the outer nuclear layer (Fig. 6). The general picture was that the more diminished the ERG, the greater the loss of rod outer segment thickness and the fewer the cells of the outer nuclear layer. In the extremely deficient animals, there was hardly any remaining rod outer segment material. The thickness of the measured layer varied from 77 per cent of normal at 2 log units deficient to 29 per cent of normal at more than 5 log units of deficiency. The extent of decrease of retinal thickness at low levels of vitamin A deficiency will be further documented in a future publication.

**Discussion**

The movement with time of the reaction band of labeled protein in the rod outer segment material followed the same time course in the control and experimental animals. In both deficient and control animals, it took essentially 10 days for the labeled rod outer segment material to be displaced from the inner segment to the pigment epithelium by subsequently produced rod outer segment lamellae.

However, the absolute distance traversed by the labeled rod outer segment material...
as it was displaced from the proximal outer segment location toward the pigment epithelium decreased as vitamin A deficiency increased. This distance was determined by comparable measurements of the thickness of the layer of rods. In the most deficient animals this distance diminished to 3/10 of normal.

Since both normal and experimental animals took the same time interval from production to removal of rod outer segment material, the thickness of the layer of rod outer segment gives a direct indication of the production rate of rod outer segment material. The thickness of the control retina can be considered “normal thickness.” This “normal thickness” represents 10 days production of rod outer segment material because it takes 10 days for the labeled amino acid to be displaced to the pigment epithelium. One-tenth this thickness is the amount of rod outer segment material produced per day in the normal rat.

Similarly, in each deficient animal, the thickness of the layer of rod outer segments represented 10 days production of rod outer segment material. Thus, one-tenth of this thickness is the amount of rod outer segment material produced per day in each deficient rat. Since the 10 day interval from production to removal was constant in both normal and all ranges of deficiency, one can simply compare the rate of production of rod outer segment material by comparing the thickness of the layer of rod outer segments observed in each animal. Thus, it becomes apparent that vitamin A deficiency slows the production rate of rod outer segment material, and the greater the level of vitamin A deficiency, the greater the slowing of this production rate. Vitamin A deficiency is, therefore, a possible technique which could be employed to help restore an equilibrium between production rate of rod outer segment material and its removal by the pigment epithelium. If human pathologic retinal conditions similar to those in the dystrophic rat are found to be the result of a similar but relative imbalance between production and removal, perhaps clinical vitamin A deficiency would be helpful to prolong retinal life.

Perhaps the most significant finding is the fact that in all animals studied, both deficient and control animals, there was always an elapsed 10 day period before the labeled rod outer segment material was removed. The pigment epithelium appeared to vary its removal rate precisely to that required to keep up with the production rate which varied over a range of more than three times. At the lower production rates, the rod outer segment material was physically adjacent to the pigment epithelium processes and thus located in a position favorable for phagocytic removal for a three times greater time interval before it was removed. It did not seem likely to us that the pigment epithelium would independently slow down its phagocytic capability just so it could exactly keep up with the rate of rod outer segment production. Since all animals were exposed to the same lighting conditions, it becomes apparent that all the rod outer segment material had been exposed to the same “time-light interval” before it was removed by the pigment epithelium. Other workers have shown that increasing light exposure causes breakdown in rod outer segment material. Kuwabara and Gorn have shown the quantitative disruption of rod outer segment lamellae to be related to duration of exposure to relatively low-intensity light. Noell, Delmelle, and Albrecht have shown that the ERG and rhodopsin content of the retina in vitamin A deficiency is illumination dependent. Young has described a slight increase in the speed of progression of the reaction band of labeled rod outer segment material by increasing the light and temperature of the environment.

The curious finding of the constant time-light interval prior to phagocytosis regardless of large variations of the time of pigment epithelium outer segment contact, coupled with the other works on light dosage to the retina, has led us to a hypoth-
esis of the pigment-epithelium and rod outer segment interrelationships.

It seems possible to us that the pigment epithelium is constantly mobilized and available for phagocytosis. However, it is unable to remove rod outer segment material which is adjacent to it until a change has occurred in that rod outer segment material. This hypothetical change in the rod outer segment material occurs after a certain time-light exposure. (In the lighting condition in our laboratory, rat rod outer segment material took about 10 days before it became phagocytizable to the pigment epithelium.) Thus, the rod outer segment material itself appears, possibly, to hold the key to when the "normal" pigment epithelium can remove it.

From our findings it seems that vitamin A must be available for rod outer segment production as lack of it slows the production rate. Whether this or other factors are needed in the normal state to govern production is not yet known. But it does seem likely that the removal of rod outer segment material by the pigment epithelium is a step governed by the condition of the outer segment material and not the pigment epithelium. Thus, in breakdowns in the normal rod outer segment production, pigment epithelium phagocytosis equilibrium, a defect in rod outer segment structure might just as effectively destroy the balance between production and removal as could a defect in the phagocytic properties of the pigment epithelium itself.

Summary

Vitamin A deficiency slows the production rate of rod outer segment material. The greater the level of deficiency, the less the production of rod outer segments.

Rod outer segment material required a constant time-light interval before it was removed from the pigment epithelium. In the conditions of this study the interval was 10 days.

Vitamin A deficiency is a means of slowing the production rate of rod outer segment material and could be of value in re-establishing equilibrium where there is a relative imbalance between rate of rod outer segment production and pigment epithelium phagocytosis.

Based on the findings of this study, a hypothesis is presented which suggests that the normal removal of rod outer segment material by the pigment epithelium is dependent on a change in the rod outer segment material which occurs after a constant time-light interval. This further suggests that in pathologic conditions, where this normal equilibrium between production rate of outer segment material and phagocytic removal by the pigment epithelium breaks down, the breakdown could be caused by either the outer segment material or the pigment epithelial cell.

REFERENCES