
Deficiencies of vitamins E and A in the rat

Retinal damage and lipofuscin accumulation

W. Gerald Robison, Jr., Toichiro Kuwabara, and John G. Bieri

The interrelationships of vitamins E and A in maintaining various structural components of the retina were studied in four groups of weanling female rats fed purified diets adequate or deficient in each vitamin: +E, +A; -E, +A; +E, -A; and -E, -A. Groups deficient in retinol (-A) were supplemented with retinoic acid. After 14, 21, and 35 weeks, the retinas were examined histologically and ultrastructurally. At 35 weeks, the doubly deficient rats (-E, -A) had lost 92% of their rod nuclei, whereas rats deficient in vitamins A (+E, -A) or E (-E, +A) alone had lost only 34% and 20%, respectively. Vitamin E deficiency resulted in extensive lipofuscin deposits in the retinal pigment epithelium as early as 21 weeks, but the presence of vitamin A doubled the number of lipofuscin granules (-E, +A vs. -E, -A) and induced an even greater increase in their autofluorescence. Another clear influence of vitamin A was seen when +E, +A retinas autofluoresced not only much more than +E, -A retinas, which had similar numbers of granules, but also more than -E, -A retinas, which had about twice as many lipofuscin granules. In the retina, unlike the uterus, the lipofuscin-specific autofluorescence and lipofuscin granule number were not proportional. Moreover, the numbers of granules were influenced by both vitamins E and A, whereas the intensity of lipofuscin-specific autofluorescence was determined almost exclusively by vitamin A. Probably the accelerated loss of photoreceptor cells in -E, -A retinas resulted from both oxidation of membranes and oxidation of retinal vitamin A stores in the absence of vitamin E protection.

Key words: vitamin E, vitamin A, lipofuscin, retina, photoreceptor membranes, retinal pigment epithelium, lipid peroxidation, phagocytosis

Damage to the retina by vitamin A deficiency is well documented in the classic studies by Dowling and Wald^{1, 2} and has been examined in great detail by subsequent workers.^{3, 4} Retinal damage resulting from vitamin E deficiency is less well understood. However, vitamin E-deprived rat retinas show massive accumulations of lipofuscin in the pigment epithelium,⁵ disorganization of

rod outer segment membranes,⁶ and loss of photoreceptor cells.⁶

An unresolved question arising from the vitamin E deficiency studies was why the degree of photoreceptor damage in vitamin E-deprived rats varied according to the amount of vitamin A provided in the diet.⁶ In order to clarify this apparent interrelationship of vitamins E and A in the maintenance of retinal structure, a comparison was made of the histology and ultrastructure of retinas from rats deficient in both vitamins E and A, rats deficient in vitamin E alone, rats deficient in vitamin A alone, and rats with a complete diet. Unexpected findings resulted which suggest a retina-specific involvement of dietary retinol in the formation and fluorescent characteristics of lipofuscin.

From the Laboratory of Vision Research, National Eye Institute, and the Laboratory of Nutrition and Endocrinology, (J. G. B.), National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Md.

Submitted for publication Aug. 6, 1979.

Reprint requests: W. Gerald Robison, Jr., Ph.D., Building 6, Room 201, National Institutes of Health, Bethesda, Md. 20205.

Materials and methods

Female Sprague-Dawley rats were maintained as described previously⁶ and fed from weaning a basal diet* free of vitamins E and A. Within a week of weaning the rats were divided into four diet groups. The +E, +A group received a supplement of 250 mg of α -tocopherol and 2 mg of retinol per kilogram of diet. The -E, +A rats received only the retinol. The +E, -A rats received 250 mg of α -tocopherol plus 4 mg of retinoic acid per kilogram of diet, which maintains most tissues in a healthy state but results in a vitamin A-deficient retina.^{2, 3} The -E, -A rats received only the retinoic acid. The retinol was in the form of stabilized, microencapsulated retinyl palmitate (Nopco Chemical Co., Louisville, Ky.). Rats from each group were sacrificed after 14, 21, and 35 weeks on the diets. Retinol levels were determined for the plasma⁸ and liver.⁹

The left eye was immersed for 30 min at room temperature in a solution of 2.5% glutaraldehyde and 6% sucrose buffered to pH 7.2 with 50 mM sodium cacodylate and transferred for another 30 min to a solution of 10% phosphate-buffered formalin, pH 7.2. Then the eye was bisected, and the inferior half was frozen on Dry Ice. Frozen sections 10 μ m thick were prepared from the inferior nasal quadrant of the retina and examined for the autofluorescence typical of lipofuscin as described previously.⁶ The intensities of autofluorescence were compared among diet groups with the same exposures and development times for all the photographs analyzed. The superior half of the left eye was processed for histological sectioning and staining by embedding in either paraffin or in glycol-methacrylate. Examination of such sections confirmed the periodic acid-Schiff staining of pigment epithelial granules and corroborated the differences in retinal structure observed among diet groups, which were examined in more detail and quantitated with 1 μ m Epon sections (see below). As a verification of the effectiveness of the dietary treatments in producing lipofuscin accumulation, sections of uterus were also prepared and examined. This organ has long been known to readily form lipofuscin in vitamin E deficiency.¹⁰

The right eye was slit at the limbus and fixed in 2.5% glutaraldehyde.⁶ Portions of the retina extending from the optic disc for 2 mm radially were taken from the inferior nasal quadrant and pro-

*This basal diet⁶ did not contain taurine, but the rat can readily synthesize taurine from the methionine and cysteine⁷ present in the casein.

Table I. Plasma and liver vitamin A

Diet group	Time (weeks)	n	Plasma* (μ g/dl)	Liver* (μ g/gm)
-E, +A	14	8	27 $\pm$ 8	154 $\pm$ 8
	21	2	22 $\pm$ 4	133 $\pm$ 26
	35	1	15	97
-E, -A	14	8	0	0
	21	2	0	0
	35	3	0	0
+E, -A	14	5	3 $\pm$ 2	9 $\pm$ 5
	21	2	0	0
	35	1	0	0
+E, +A	14	6	40 $\pm$ 6	186 $\pm$ 19
	21	2	35 $\pm$ 5	240 $\pm$ 53
	35	1	20	422

*Values are means $\pm$ S.E.

cessed for light and electron microscopy by dehydration in ethanol and embedding in Epon. Sections 1 μ m thick were stained with toluidine blue and analyzed for alterations in structure and number of retinal elements by means of photomicrographs printed to obtain a total magnification of 1000 times the specimen size. Counts were taken from at least 10 separate locations within the same 2 mm portion of retina processed for each rat. Two to five rats for each diet and age group were utilized except as noted. The numbers of rod nuclei and lipofuscin granules were determined for 200 μ m lengths of retina. The lipofuscin granules were distinguished from lipid droplets and mitochondria by their greater density following staining with toluidine blue. Undoubtedly, some counts of phagosomes were included in the granule data. However, this error was minimized by randomizing the fixation times between 3 and 6 hr after the onset of light, when phagosomes in all diet groups were low in number as determined by electron microscopy of the same material. The data were analyzed for significance with the Student's t test, assuming a single-tail t distribution. Ultrathin sections were taken of representative regions and were examined with a JEM 100B electron microscope following staining with uranyl acetate and lead citrate.

Results

Vitamin status. Rats in all diet groups gained weight at about the same rate, 5 gm/day for the first 3 weeks, and by 14 weeks when the first lot was sacrificed, they ranged from 220 to 250 gm. By 21 weeks, when the second lot was sacrificed, rats not receiving vitamin E (-E, +A and -E, -A) were

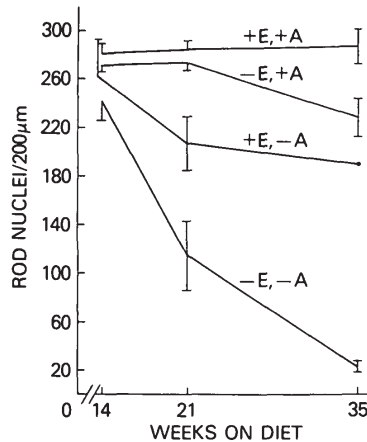


Fig. 1. Numbers of rod nuclei in the diet groups indicated per 200 μm of retinal length counted in the inferior nasal quadrant of the central retina from the optic disc to a point 2 mm radiad. S.E.M. indicated by vertical bars (see Table II for n).

5% to 10% lighter than those getting vitamin E. All animals appeared healthy and gave no outward evidence of either retinol or tocopherol deficiency. Their vitamin A status was monitored by analyzing both plasma and liver for retinol (Table I). Plasma and liver vitamin A were undetectable in $-E, -A$ rats by 14 weeks and barely detectable in $+E, -A$ rats (plasma = 3 $\mu\text{g}/\text{dl}$, liver = 9 $\mu\text{g}/\text{gm}$). In the groups receiving retinol, those rats fed vitamin E ($+E, +A$) tended to have higher plasma and liver values (40 $\mu\text{g}/\text{dl}$ and 186 $\mu\text{g}/\text{gm}$, respectively) than those not getting vitamin E ($-E, +A$ rats: 27 $\mu\text{g}/\text{dl}$ and 154 $\mu\text{g}/\text{gm}$, respectively). Similar relationships held at later periods.

Histology. After the rats had been 14 weeks on the diets, only a few changes were noted (Fig. 1, Table II). The retinas of the $-E, +A$ group showed slightly increased numbers of lipofuscin granules in their pigment epithelium (approximately 1.6 times normal) and misaligned disk membranes near the distal ends of their rod outer segments. Otherwise, they were similar to retinas of the $+E, +A$ group. The $+E, -A$ retinas did not differ significantly from the $+E, +A$ retinas. The $-E, -A$ retinas showed some disorgani-

zation of outer segment membranes and a 14% decrease in rod nuclei which was not significant ($p = 0.1$).

At 21 weeks, the retinas of $-E, +A$ rats had extensive accumulations of lipofuscin in the pigment epithelium (Table II), greater misalignment of disk membranes, and some swelling of outer segment tips compared to $+E, +A$ control retinas. The $+E, -A$ retinas showed no significant accumulations of lipofuscin or shortening of outer segments; however, they had some areas of vesicle formation intercalated between normally aligned outer segment disk membranes and had a 27% loss of rod nuclei (Fig. 1). The $-E, -A$ retinas exhibited a twofold increase in lipofuscin over the $+E, +A$ group and showed much vesiculation and swelling of outer segments as well as a 60% loss of rod nuclei (Fig. 1, Table II).

By 35 weeks on the diets, the $-E, +A$ retinas, compared to $+E, +A$ controls, exhibited an obvious swelling of the pigment epithelium, about a fivefold increase in the number of lipofuscin granules (Fig. 2, Table II), and a dramatic increase in lipofuscin-specific autofluorescence (to be described in detail below). The outer segments were swollen, slightly shorter, and had disorganized disk membranes in their distal halves. Approximately 20% of the rod nuclei were lost. The $+E, -A$ retinas had no more lipofuscin granules than the $+E, +A$ controls but had greatly shortened rod outer segments and showed a 34% loss of rod nuclei (Figs. 1 and 2, Table II). The $-E, -A$ retinas after 35 weeks had about a twofold increase in lipofuscin granules over $+E, +A$ retinas but were left with only small remnants of the outer segment membranes and less than 10% of the control number of rod nuclei (Figs. 1 and 2, Table II). Dark brown pigmentation of the uteri from both vitamin E-deficient groups compared to no pigmentation in the $+E$ groups verified the effectiveness of the $-E$ diets for inducing lipofuscin accumulation. There was no difference in uterine pigmentation or autofluorescence whether the $-E$ rats were fed retinol ($-E, +A$) or retinoic acid ($-E, -A$).¹¹

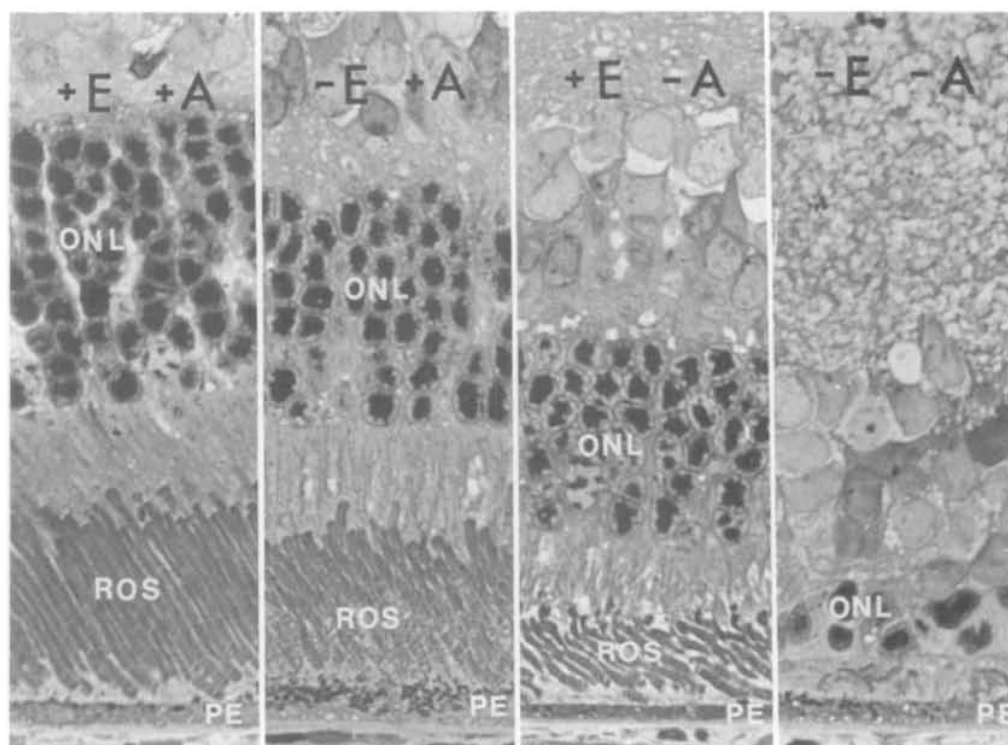


Fig. 2. Representative sections (1 μ m) of retina for each diet group at 35 weeks showing changes in the outer nuclear layer (ONL), the rod outer segments (ROS), and the pigment epithelium (PE). ($\times 1000$.)

Tissue autofluorescence. Yellow autofluorescence typical of lipofuscin was confined to the pigment epithelium of all the retinas examined. As early as 14 weeks the amount of lipofuscin autofluorescence differed in retinas from different diet groups. By 35 weeks, the differences were striking. Retinas of the -E, +A diet group exhibited by far the brightest autofluorescence, and this showed no fading. The yellow autofluorescence of all other groups faded somewhat with time under exposure to short wave length light (ca. 360 nm peak). The fast-fading green autofluorescence typical of vitamin A was not included in these measurements nor recorded on the photographs. Unexpectedly, the +E, +A retinas had more yellow autofluorescence than did the -E, -A retinas, and the +E, -A retinas rated last, at markedly lower intensities. Although the intensities in -E, +A and +E,

+A retinas increased with time on the diets (i.e., 14 weeks vs. 35), the intensities in +E, -A and -E, -A retinas remained approximately the same.

Surprisingly, this retinal autofluorescence was not always proportional to the numbers of lipofuscin granules in the pigment epithelium (Fig. 3). That is, although -E, -A retinas had many more lipofuscin granules than +E, +A retinas, they exhibited much less autofluorescence. However, the -E, +A retinas showed both an intense autofluorescence and massive accumulations of histologically countable granules. Neither the autofluorescence nor the granule counts were the same in -E, +A compared to -E, -A retinas.

In contrast, the autofluorescence of the uterus appeared to vary in intensity in direct relation to the numbers of histologically demonstrable lipofuscin granules. The -E,

Table II. Retinal changes in rats deficient in vitamins E and A

Diet group	Time (weeks)	n	Rod nuclei* (per 200 μ m)	RPE height*	RPE granules* (per 200 μ m)
-E, +A	14	5	271 $\pm$ 4	5.4 $\pm$ 0.6	111 $\pm$ 18
	21	3	273 $\pm$ 5	6.4 $\pm$ 0.6	300 $\pm$ 56†
	35	4	229 $\pm$ 15†	6.7 $\pm$ 0.4†	406 $\pm$ 31†
-E, -A	14	5	242 $\pm$ 16	5.1 $\pm$ 0.4	88 $\pm$ 7
	21	4	115 $\pm$ 28†	5.3 $\pm$ 0.3	124 $\pm$ 15†
	35	3	23 $\pm$ 3†	6.3 $\pm$ 0.7	157 $\pm$ 12†
+E, -A	14	3	263 $\pm$ 31	4.3 $\pm$ 0.7	73 $\pm$ 11
	21	2	207 $\pm$ 23†	4.7 $\pm$ 0.2	80 $\pm$ 13
	35	1	190	3.9	60
+E, +A	14	3	281 $\pm$ 9	4.8 $\pm$ 0.1	70 $\pm$ 6
	21	2	284 $\pm$ 7	4.7 $\pm$ 0.6	60 $\pm$ 2
	35	3	287 $\pm$ 14	5.0 $\pm$ 0.2	90 $\pm$ 8

RPE = retinal pigment epithelial.

*Values are means $\pm$ S.E.†Significantly different from controls (+E, +A) of the same time group at less than the 0.05 level of significance as determined by Student's *t* test and a single-tail *t* distribution.

+A and -E, -A uteri had similar numbers of lipofuscin granules and exhibited autofluorescences which were indistinguishable.¹¹

Ultrastructure. The lipofuscin granules which accumulated in the pigment epithelium of +E, -A and -E, -A retinas tended to be smaller than the granules in -E, +A retinas (Fig. 4). They were more similar to those in +E, +A retinas. However, more unusually large granules ($>5 \mu$ m) were found in -E, -A retinas than in any of the others. The pigment epithelium was shortest in the +E, -A retinas and tallest in the -E, +A retinas (Fig. 4, Table II).

The rod outer segments showed membrane alterations in all the vitamin-deficient retinas (Fig. 4). The -E, +A retinas had swollen outer segments with membrane vesiculation throughout their distal regions, as reported previously.⁶ The rod outer segments of +E, -A retinas differed in that vesiculation of their disk membranes tended to be intermittent. That is, groups of vesicles were intercalated between regions of well-aligned disks. The -E, -A retinas showed much more disorganization of their outer segment membranes than did -E, +A or +E, -A retinas. After 35 weeks of diet, only occasional remnants were left to represent the rod inner and outer segments. The retinas had thinned, so that the outer limiting

membrane came close to the extended microvilli of the pigment epithelium (Fig. 4).

Discussion

The accelerated degeneration of photoreceptor cells in rats maintained on a -E, -A diet compared to those fed a +E, -A diet indicates an important interrelationship between vitamins E and A in the retina. Vitamin E is an effective tissue antioxidant which is normally found in the retina¹² and probably is important in protecting the highly unsaturated lipids of retinal membranes from autoxidation.^{6, 13, 14} Also, vitamin E protects stores of vitamin A from oxidation in both the liver¹⁵ and the retina.^{16, 17} Vitamin A deficiency results in loss of photoreceptor cells,¹⁻⁴ which has been attributed to the dependence of photoreceptor membranes on structural support supposedly provided by rhodopsin.³ Although other body tissues are able to utilize the retinoic acid provided in the diet, the retina requires retinol, which becomes an essential structural component of outer segment membranes. The increased retinal damage in -E, -A retinas could represent simply the additive effects of increased membrane oxidation in the absence of vitamin E, superimposed on membrane instability resulting from the absence of retinol. However, it is more likely that the degree of acceleration in damage observed occurred because vitamin E de-

iciency, in addition to its effects on membranes, triggered increased oxidation of vitamin A stores in the retina and liver. This could induce an early and more acute vitamin A deficiency in the photoreceptor cells. In any case, normal dietary levels of both vitamins E and A are essential for the structural maintenance of the neural retina and for a normal pigment epithelium.

The loss of photoreceptor cells in +E, -A rats was less than that which has been reported in other investigations of vitamin A deficiency.⁴ However, such a finding is not unexpected, since the vitamin E levels and other dietary factors were not equivalent, and all previous studies utilized male rats which are known to become depleted of tissue vitamin A sooner than do females.¹⁸

The extraordinary numbers of lipofuscin granules which accumulated in the retinal pigment epithelium of all rats deprived of vitamin E suggest an increased oxidative damage to rod outer segment membranes.^{5, 6} Such membranes would be phagocytized by the pigment epithelium where they would become partially degraded and their unprocessed remnants concentrated into packets of highly peroxidized lipids having the characteristics of lipofuscin.^{5, 6, 14}

The fact that -E, -A rats showed only a twofold increase in lipofuscin, whereas the -E, +A rats displayed a fivefold increase indicates an effect of retinol. This influence of dietary retinol on lipofuscin formation is apparently retina-specific, since lipofuscin accumulation in the vitamin E-deficient uterus was unaffected by vitamin A status.¹¹ But whether the effects of retinol on lipofuscin formation are direct or indirect remains to be investigated. Retinol deficiency decreases phagocytosis by the pigment epithelium.³ This and other such changes in outer segment turnover could influence lipofuscin formation as indirect effects of retinol deficiency. On the other hand, retinol may be involved directly in the formation of lipofuscin through retinoyl complexes, since these are suggested components of lipofuscin in the brain of patients with Batten disease.¹⁹

The yellow autofluorescence typical of

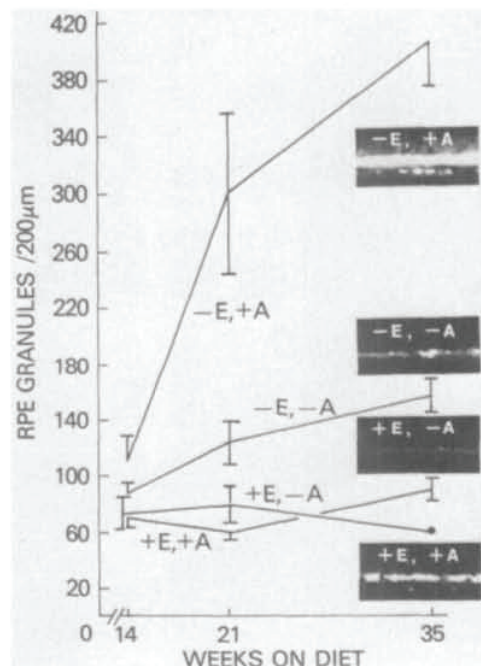


Fig. 3. Numbers of lipofuscin granules in the retinal pigment epithelium (RPE) counted for each diet group per 200 μ m of retinal length (see Fig. 1 legend for the retinal region counted). S.E.M. indicated by vertical bars (see Table II for *n*). Insets, Autofluorescence of the pigment epithelium from the inferior nasal quadrant of an eye in each diet group after 35 weeks with micrographs exposed, developed, and printed equally. ($\times 115$.)

lipofuscin pigment was more intense in retinas that received retinol (+E, +A and -E, +A) than in retinas deprived of retinol (+E, -A and -E, -A). This fluorescence appeared to be related to the dietary retinol and not simply to granule number, since -E, -A retinas had more lipofuscin granules yet exhibited less autofluorescence than +E, +A retinas. Apparently, increased numbers of lipofuscin granules were formed in response to vitamin E deficiency, but these were less fluorescent than normal or nonfluorescent in the absence of retinol. In contrast, the uterus was able to form fully fluorescent lipofuscin granules in the same rats. Indeed, very different levels of retinol had no effect on the number of granules or their fluorescence in the uterus.¹¹ Perhaps these differences are

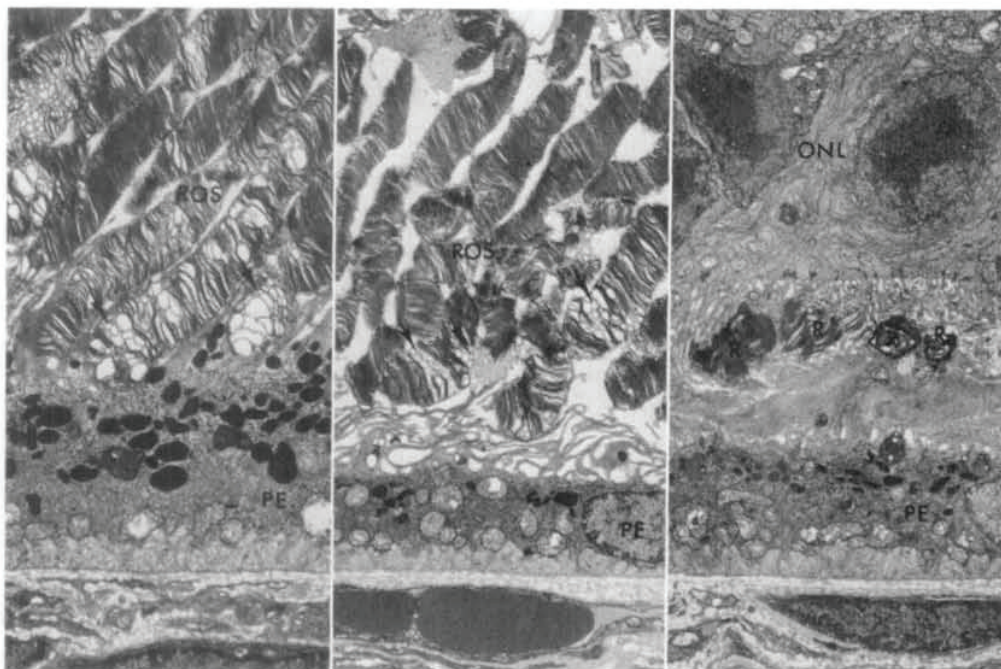


Fig. 4. Electron micrographs of retinas after 35 weeks on a -E, +A diet (left), a +E, -A diet (middle), and a -E, -A diet (right) showing changes in the pigment epithelium (PE), vesiculation (arrows) in the rod outer segments (ROS), remnants of rod inner and outer segment membranes (R), and altered position of the outer nuclear layer (ONL). ($\times 6200$.)

related to the fact that the uterus is able to utilize retinoic acid as well as retinol, whereas the retina is dependent on retinol.¹⁻³ The uterus contains binding proteins for both retinoic acid and retinol,²⁰ whereas the retinal pigment epithelium may have only a retinol binding protein.²¹ The changes in autofluorescence suggest that the composition of lipofuscin in the retina may be different depending on the vitamin A status of the animal. However if retinal lipofuscin simply contained retinyl palmitate, it should have fluoresced green and faded rapidly. Perhaps its fluorescence derives from retinoyl complexes,¹⁹ but just how retinol or its derivatives might be involved in lipofuscin formation and what similarities lipofuscins from different sources might have remained unknown yet intriguing questions.

We appreciate the technical assistance of Mrs. Anne Groome and Mrs. Teresa Tolliver.

REFERENCES

1. Dowling JE and Wald G: Vitamin A deficiency and night blindness. *Proc Natl Acad Sci USA* 44:648, 1958.
2. Dowling JE and Wald G: The biological function of vitamin A acid. *Proc Natl Acad Sci USA* 46:587, 1960.
3. Dowling JE and Gibbons IR: The effect of vitamin A deficiency on the fine structure of the retina. In *The Structure of the Eye*, Smelser GK, editor. New York, 1961, Academic Press, Inc., p. 85.
4. Carter-Dawson L, Kuwabara T, O'Brien PJ, and Bieri JC: Structural and biochemical changes in vitamin A deficient rat retinas. *INVEST OPHTHALMOL VIS SCI* 18:437, 1979.
5. Katz ML, Stone WL, and Dratz EA: Fluorescent pigment accumulation in retinal pigment epithelium of antioxidant-deficient rats. *INVEST OPHTHALMOL VIS SCI* 17:1049, 1978.
6. Robison WC Jr, Kuwabara T, and Bieri JC: Vitamin E deficiency and the retina: photoreceptor and pigment epithelial changes. *INVEST OPHTHALMOL VIS SCI* 18:683, 1979.
7. Awapara J and Wingo WJ: Mechanism of taurine formation from cysteine in the rat. *J Biol Chem* 203:189, 1953.

8. Neeld JB Jr and Pearson WN: Macro- and micro-methods for the determination of serum vitamin A using trifluoroacetic acid. *J Nutr* 79:454, 1963.
9. Ames SR, Risley HA, and Harris PL: Simplified procedure for extraction and determination of vitamin A in liver. *Anal Chem* 26:1378, 1954.
10. Moore T and Wang YL: Formation of fluorescent pigment in vitamin E deficiency. *Br J Nutr* 1:53, 1947.
11. Bieri JC, Tolliver TJ, Robison WG Jr, and Kuwabara T: Lipofuscin in vitamin E deficiency and the possible role of retinol. *Lipids* 15:10, 1980.
12. Dilley RA and McConnell DG: Alpha-tocopherol in the retinal outer segment of bovine eyes. *J Membr Biol* 2:317, 1970.
13. Farnsworth CC and Dratz EA: Oxidative damage of retinal rod outer segment membranes and the role of vitamin E. *Biochim Biophys Acta* 433:556, 1976.
14. Tappel AI: Lipid peroxidation and fluorescent molecular damage to membranes. In *Pathobiology of Cell Membranes*, Trump BF and Arstila AU, editors. New York, 1975, Academic Press, Inc., p. 145.
15. Moore T: *Vitamin A*. Amsterdam, 1957, Elsevier Publishing Co., pp. 201 and 225.
16. Robison WG Jr, Kuwabara T, and Bieri JC: Effects of vitamins A and E on lipofuscin accumulation in retinal pigment epithelium and extraocular muscle. *INVEST OPHTHALMOL VIS SCI* 17(ARVO Suppl.):157, 1978.
17. Robison WG Jr and Kuwabara T: Vitamin A storage and peroxisomes in retinal pigment epithelium and liver. *INVEST OPHTHALMOL VIS SCI* 16:1110, 1977.
18. Moore T: *Vitamin A*. Amsterdam, 1957, Elsevier Publisher Co., p. 502.
19. Wolfe LS, Kin NMKNY, Baker RR, Carpenter S, and Andermann F: Identification of retinoyl complexes as the autofluorescent component of the neuronal storage material in Batten disease. *Science* 195:1360, 1977.
20. Chytil F, Page DL, and Ong DE: Presence of cellular retinol and retinoic acid binding proteins in human uterus. *Int J Vitam Nutr Res* 45:293, 1975.
21. Wiggert B, Masterson E, Israel P, and Chader CJ: Differential retinoid binding in chick pigment epithelium and choroid. *INVEST OPHTHALMOL VIS SCI* 18:306, 1979.

Information for authors

Most of the provisions of the Copyright Act of 1976 became effective on January 1, 1978. Therefore, all manuscripts must be accompanied by the following written statement, signed by one author: "The undersigned author transfers all copyright ownership of the manuscript (title of article) to The Association for Research in Vision and Ophthalmology, Inc., in the event the work is published. The undersigned author warrants that the article is original, is not under consideration by another journal, and has not been previously published. I sign for and accept responsibility for releasing this material on behalf of any and all co-authors." Authors will be consulted, when possible, regarding republication of their material.