OCULAR STRUCTURE IN VITAMIN A DEFICIENCY IN THE MONKEY.

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Introduction

The Duplicity Theory of vision is based upon the fact that the rod cells of the retina function as receptors for vision in dim light, and the cone cells for vision in bright light and for colour vision [Hecht, 1937]. The rod cells of vertebrate retina contain a red pigment, rhodopsin, and the cone cells contain a violet pigment, iodopsin. Rhodopsin and iodopsin are photosensitive, and on exposure to light undergo changes which are believed to lead to a nerve impulse. They have been characterized chemically as carotenoid proteins, which are bleached by light with the liberation of carotenoid. Both the rhodopsin and iodopsin systems have been shown to contain vitamin A and its aldehyde, retinene [Wald, 1953 *a*, *b*; Morton, 1951].

It has long been known that night blindness can be produced in experimental animals by vitamin A deficiency [Fridericia and Holm, 1925], and that the regeneration of rhodopsin is retarded in the absence of vitamin A [Tansley, 1933]. Experiments on human volunteers deprived of vitamin A for periods ranging from a few weeks to over two years have shown that a deterioration in the capacity for dark adaptation appears sooner or later in the course of the deficiency; this is corrected by the administration of vitamin A [Hecht and Mandelbaum, 1939; Wald, Jeghers and Arminio, 1938; Booher, Callison and Hewston, 1939; Steven, 1943; Hume and Krebs, 1949]. In vitamin A deficient rats, severe structural damage to the retinal rod cells has been reported [Tansley, 1933; Johnson, 1943].

The recent demonstration by Wald, Brown and Smith [1952] that the carotenoid components of rhodopsin and iodopsin systems are identical, suggests that vitamin A may be important in the metabolism of cone cells as in that of rod cells. However, the extent to which the cone cells depend upon an extrinsic source of vitamin A for normal function is not known. The biochemistry of cone function is more

complex and less well understood than that of rod function [Morton, 1951; Granit, 1950]. Most of the work on vitamin A deficiency in the past was concerned with its effects on the structure and function of rod cells. In human volunteers deprived of vitamin A, there is evidence to show that cone cell function is affected as early as rod cell function during development of the deficiency [Haig *et al.*, 1938; Hecht and Mandelbaum, 1939; Wald *et al.*, 1938; Hume and Krebs, 1949]. It seemed desirable, therefore, to investigate the structure of the cone cells in uncomplicated vitamin A deficiency. The monkey was chosen as the experimental animal since its retina closely resembles that of man and contains well-developed cones.

Experimental Technique

Seven young growing monkeys (*Macacus sinicus*) from the Nilgiri Hills were used; 5 of these were placed in the deficient group and 2 in the control group. All animals were fed on a basal diet of the following composition:—

	g.
Polished raw rice	76
Casein (alcohol extract)	15
Salt mixture [Phillips and Hart, 1935]	3
Groundnut oil	6
	mg.
Thiamine hydrochloride	0·5
Riboflavin	0·8
Nicotinic acid	4·0
Pyridoxine hydrochloride	0·5
Ascorbic acid	10·0
Calcium pantothenate	3·0
Choline chloride	50·0
Inositol	50·0
Para-aminobenzoic acid	50·0
Biotin	0·05
Pteroylglutamic	0·5

Each monkey was given 100 g. of the basal diet daily, which was completely eaten. It also received 100 I.U. of vitamin D daily. The control monkeys were given 1500 I.U. of vitamin A in oil by mouth twice a week.

The animals were weighed every 10 days and examined at intervals, special attention being paid to the changes in the cornea and conjunctiva. The vitamin A content of plasma was estimated colorimetrically by the Carr-Price reaction, following the procedure described by Dann and Evelyn [1938] and Yudkin [1941]. The photoelectric instrument used

was the Coleman Universal Spectrophotometer, Model 14. The blood sample was obtained from a posterior vein of the leg without anæsthesia. When the experiment was terminated, their eyes were enucleated under ether anæsthesia and fixed immediately in Bouin's fluid. They were embedded in low viscosity nitrocellulose and sectioned at 10 μ as described by Chesterman and Leach [1950]. The sections were stained by routine stains, including Masson's trichrome, Weigert's elastin stain and Mallory's phosphotungstic acid hæmatoxylin. Some sections were also stained by a modification of Lendrum's phloxine-tartrazine method [Leach, 1954]. By this method, basic proteins are well stained and degenerating nuclei are revealed.

Results

General.—Two animals in the deficient group died during the 1st month of the experiment due to acute gastro-enteritis and are excluded. The remaining 3 deficient animals gained weight as rapidly as the controls during the first 3 months, after which their weights remained stationary, while those of the controls continued to increase. During the first 3 months the deficient animals were clinically indistinguishable from the controls. Between the 3rd and 4th months signs of xerophthalmia appeared in the eyes of two of them. The bulbar conjunctiva, which is pigmented uniformly brown in the normal animal, showed a patchy loss of pigment on the outer side of the cornea. The depigmented patches gradually increased in size, became white, opaque and slightly foamy, and closely resembled Bitôt's spots. The eyelids were swollen, and flakes of keratinized debris could be seen floating in the conjunctival sac. By the end of the 4th month of deficiency the cornea of these two deficient animals was also affected. The lesion started as a cloudy dry patch and gradually spread to involve most of the cornea, ending in the full picture of keratomalacia (fig. 1). In both these monkeys the keratomalacia was unilateral, although the bulbar conjunctiva of the "unaffected" eye showed unequivocal signs of xerophthalmia; these monkeys are later referred to as monkeys B and C. At this stage, which was reached after 4½ months of depletion, the two deficient animals and one of the controls were sacrificed. The remaining deficient monkey showed no signs of corneal involvement even at the end of 8 months, but showed clear-cut conjunctival changes; this monkey is later referred to as monkey A. It was sacrificed at this stage together with the remaining control monkey.

The two control monkeys remained free from ocular signs throughout the period of study.

Plasma Vitamin A and Dark Adaptation.—Plasma vitamin A was estimated after the animals had been on the experimental diet for

4 months when unequivocal signs of deficiency began to appear in the deficient group. The results are given below.

	I.U. of vitamin A per 100 ml. plasma
Deficient group	52
	52
	132
Control group	168
	159

A rough dark adaptation test was performed on the animals at this period. They were kept in a dimly lighted room for a period of 10 minutes, after which the eyes of each animal were exposed to light from a 500-watt bulb at a distance of 1 foot for a period of 1 minute. They were then transferred to their cages in the dimly lighted room, and the time taken by each animal to respond to threatening movements of a white stick was recorded. Care was taken to see that no sound was produced during this operation. While the control animals began to respond within a few minutes, the deficient animals remained impassive for a long period. The results are shown below.

	Time in minutes
Deficient	45
	35
	6
Control	5
	4

Two other monkeys maintained on the laboratory stock diet responded to the test under 5 minutes. The results provide an indication of the severity of loss of dark adaptation in the deficient animals. They are in accordance with the results of estimation of plasma vitamin A. It is of interest that the monkey in the deficient group which shows values for vitamin A and dark adaptation which are close to the control values was the animal in which keratomalacia could not be induced even after 8 months of the deficient diet.

Histological Results.—The fixation was found to be good. The normal eyes were in an excellent state of preservation apart from some slight distortion of the outer segments of the rods and cones. The spiral-like structure [Leach and Willmer, 1950] was well shown. The pigment cells, which are easily subject to post-mortem change, were well fixed although in places slight vacuolation was seen.

The eyes of the vitamin deficient animals were also well fixed. Owing to gross damage, the more severely affected eyes of monkeys B and C were not studied in detail; the cornea were lacking, the lens must have been exposed to the air, and inflammation was intense in the region of

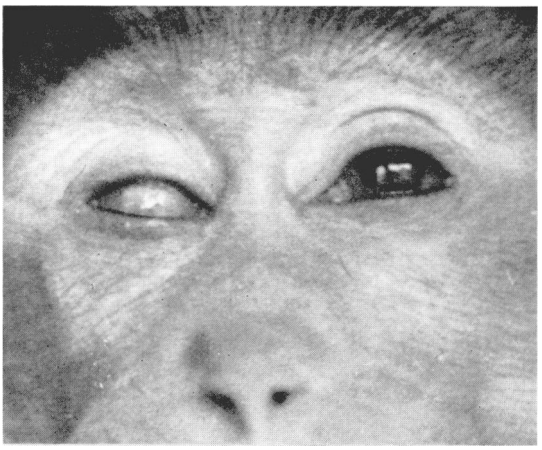

FIG. 1.—Keratomalacia of right eye in a monkey after $4\frac{1}{2}$ months of a diet deficient in vitamin A.

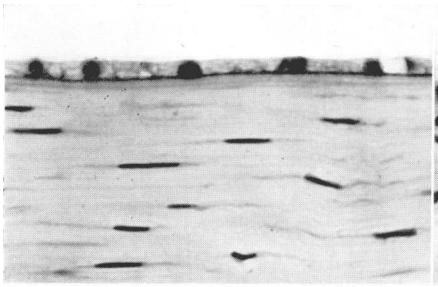

FIG. 2.—Descemet's endothelium of control monkey. Masson's trichrome stain. ×450.

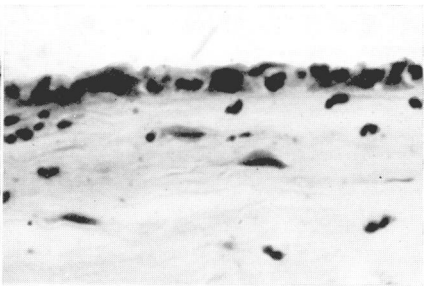

FIG. 3.—Similar but from deficient monkey C, showing pyknotic nuclei and polymorph infiltration.

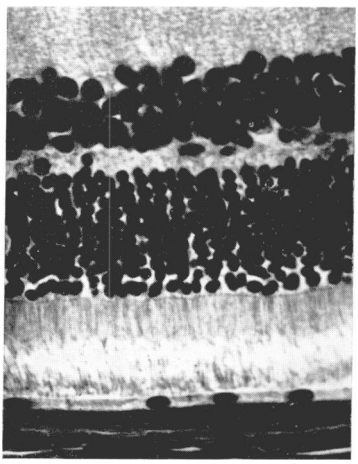

FIG. 4.—Retina of control rat. Masson's trichrome stain. ×600.

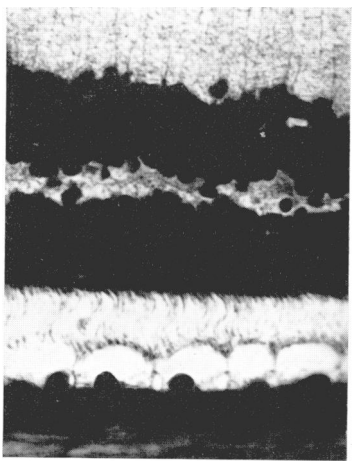

FIG. 5.—Similar but from vitamin A deficient rat, showing atrophy of the rods and cones and swelling of the pigment cells.

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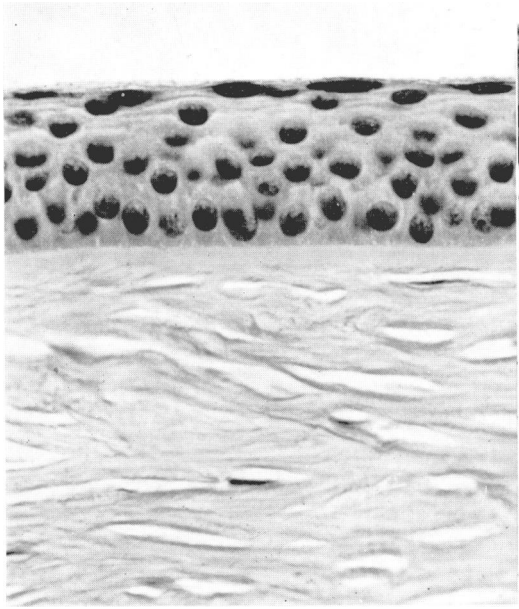

FIG. 6.—Cornea of control monkey. Masson's trichrome stain. ×520.

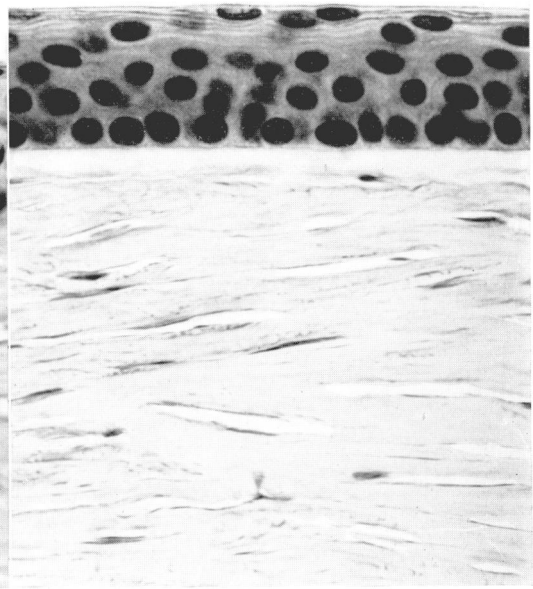

FIG. 7.—Similar but from monkey A.

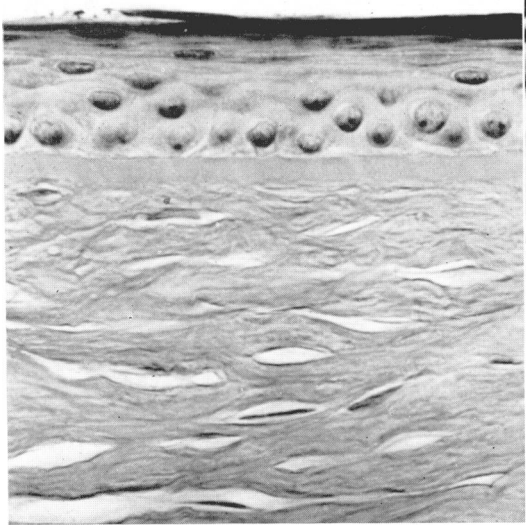

FIG. 8.—Similar but from deficient monkey B, showing slight keratinization of the superficial layers.

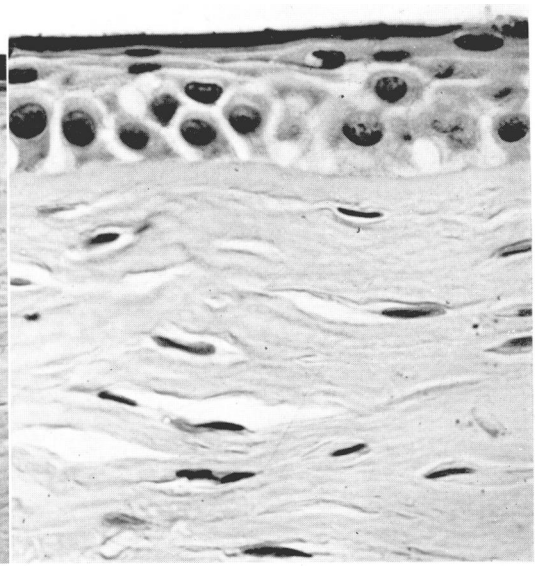

FIG. 9.—Similar but from deficient monkey C, showing marked keratinization of the superficial layers and degeneration of the basal layer.

the angle. The eye described is the less severely affected one. In monkey A also only the less severely affected eye was examined.

Changes in the Cornea. (a) *Corneal Epithelium.*—In monkey A, the classical symptoms of xerophthalmia were lacking. There was slight indication of incipient keratinization, but no more than that which can be seen in eyes of some normal monkeys (see fig. 7).

In the intact eye of monkey B there were small patches of keratinization and the epithelium was thinned (see fig. 8). In monkey C, keratinization and thinning of the epithelium were marked (see fig. 9).

(b) *Substantia propria.*—In monkeys A and B, the collagen fibres were less regular and less obviously fibrillar. In monkey C, polymorph infiltration had occurred.

(c) *Descemet's Membrane.*—In monkey A, Descemet's membrane stained less deeply with Weigert's elastin stain than did the membrane of the normal animals. In monkeys B and C, the membrane was poorly developed and stained lightly with Weigert's elastin stain.

(d) *Descemet's Endothelium.*—A brief study of this epithelium in normal eyes gave an indication that it participates in the formation of Descemet's membrane. The nuclei were of variable appearance, which can best be interpreted as part of a cyclical change. In the active phase the nucleus comes to lie on the side of the cell in apposition to Descemet's membrane. The free basic protein of the chromatin increases, and the plasmosome comes to lie on the side of the nucleus nearest Descemet's membrane. The nuclear sap may then show a positive reaction for basic protein. A cleft may appear in the nuclear membrane opposite the plasmosome. Then apparently some of the substance (both basic and acidic) of the nucleus passes into Descemet's membrane; the nucleus then collapses.

In monkey A, the cytoplasm of the cells had a more vacuolated and less granular appearance; the nuclei were more irregular in shape instead of being round or oval, and some of them showed the early sign of degeneration characterized by diffuse acidophility. In those parts of the cornea where this damage was most marked, the overlying corneal epithelium showed most clearly signs of incipient keratinization.

In monkey B, the cytoplasm of Descemet's endothelial cells was highly vacuolated. The nuclear and nucleolar changes described above were nearly absent; the nuclei are shrunken and irregular in shape.

In monkey C (fig. 3), Descemet's endothelium was heavily infiltrated with polymorphs. The nuclei were all densely basiphil and showed no variability of structure such as is seen in normal cells.

Retina. (a) *Ganglion and Bipolar Cells.*—No obvious sign of damage was seen in these cells even in the markedly deficient animals.

(b) *Rods.*—In monkey A no obvious sign of damage to the rods could be detected even in the macular region.

In the markedly deficient animals, rods were absent in the macular

region. In monkey B, sections were obtained through the fovea; rods were found to be absent up to a distance of 1 mm. from the centre of the fovea, whereas in a normal monkey they can be seen at a distance of 0·2 mm. or less. From a distance of 1 mm. to a distance of 2 mm. from the fovea, the rod nuclei showed damage—pyknosis or excess basic protein. Further out from the fovea only occasional rod nuclei showed any sign of damage.

(c) *Cones.*—Even in monkey A, damage to the cones was discernible in the macular region. The nucleoli were larger and more irregular than normal, and the neck segment was swollen and the outer segments showed more damage than that attributable to post-mortem change. The spiral-like structure on the outer segment [Leach and Willmer, 1950] was well shown in the normal eyes but absent in the eyes of all the vitamin deficient animals.

In the eyes of monkeys B and C the damage to the cones was more obvious. Their nuclei are pyknotic and contain excess basic protein, as do their inner segments (see figs. 12 and 13). The damage was mainly limited to the macular region up to a distance of about 2 mm. from the fovea.

(d) *Pigment Cells.*—In monkey A, the appearance of the pigment cells showed a definite change. The superficial pigment granules were less regularly arranged than in a normal animal and occupied a larger part of the cytoplasm. Although variation of this type is found in the peripheral region of the normal monkey retina, comparison of equivalent regions showed a consistent change throughout. In the macular region of one eye of the mildly vitamin deficient animal (A), abnormal pigment cells, which are not seen in normal monkey eyes, could be detected; in these cells the position of the pigment had been reversed and it now lay in the basal region of the cell. Near such abnormal pigment cells degenerate cones were more frequent (see fig. 11).

In monkeys B and C, the irregularity of disposition of the pigment granules was more marked, and it filled not only the apical region where it normally occurs, but also the basal region which is usually pigment-free (see figs. 12 and 13). The nuclei were pyknotic, and also showed an excess content of basic protein.

In each eye the area of pigment cell damage was greater than the area of discernible damage to rods or cones. During earlier studies on vitamin A deficiency in the rat made in Dr. Sinclair's laboratory in Oxford, severe degenerative changes in the pigment epithelium were found (see figs. 4 and 5) [Ramalingaswami, Leach and Sinclair, 1951, unpublished].

Discussion

The clinical appearance, results of estimation of plasma vitamin A and dark adaptation, and the histological changes in epithelial tissues,

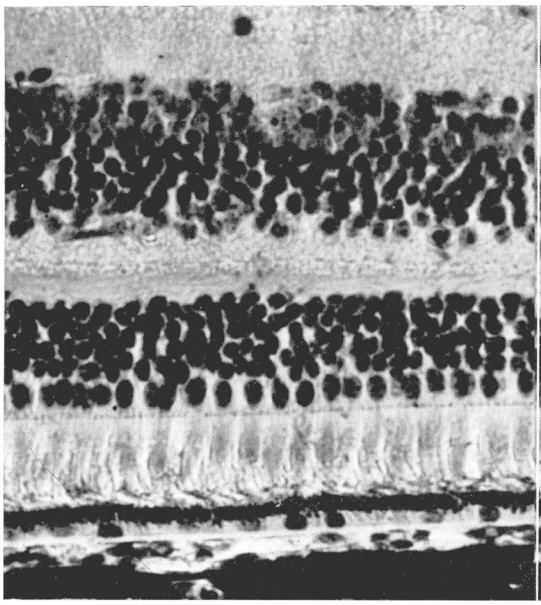

FIG. 10.—Retina of a control monkey. Mallory's phosphotungstic acid hæmatoxylin. ×450.

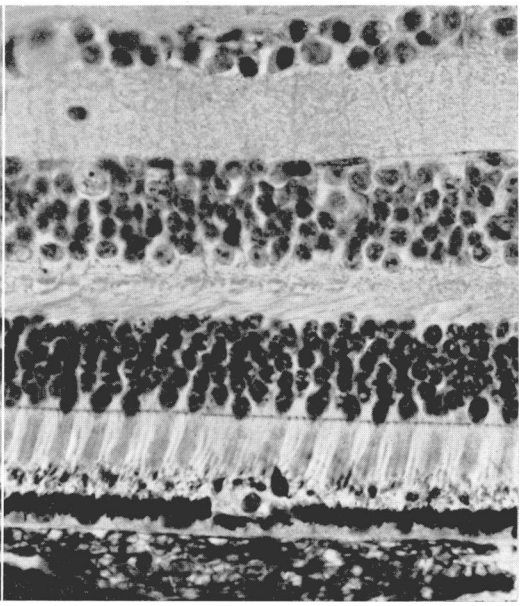

FIG. 11.—Similar but from deficient monkey A, showing reversed pigment granules in a pigment cell and nearby a damaged cone.

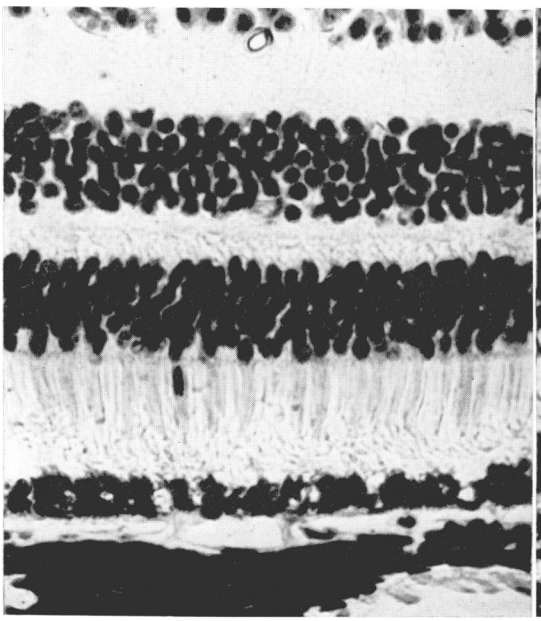

FIG. 12.—Similar but from deficient monkey B, showing invasion of pigment into basal part of pigment cells and pyknosis of rod and cone nuclei.

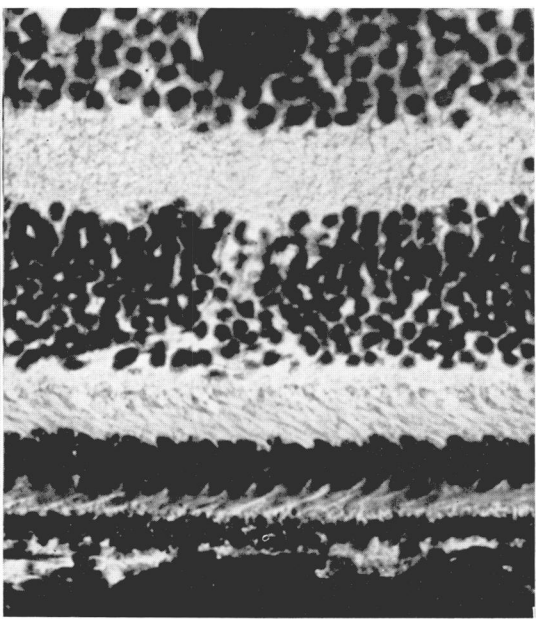

FIG. 13.—Similar but from deficient monkey C, showing degeneration of the cones.

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leave no doubt that a severe deficiency of vitamin A was produced by dietary means. The promptness with which Bitôt's spots and keratomalacia were induced in the monkey calls for comment. In a review of the literature on this subject, Day [1944] stated that only 3 out of 58 monkeys in whom vitamin A deficiency was attempted to be induced, developed "xerophthalmia". It is well known that xerophthalmic signs, which are based on morphological changes, could not be produced in human volunteers even after several months of feeding a vitamin A deficient diet, although biochemical evidence of deficiency was present [Hume and Krebs, 1949]. Obviously, a number of factors such as age of the animal, vitamin A reserves in the liver, purity of the diet, etc. are involved in determining the speed with which deficiency develops. In the present experiment, one out of the three deficient animals was resistant to keratomalacia. It showed a higher level of plasma vitamin A than the other two deficient animals, and a dark adaptation time almost identical with that of the controls. It is possible that this monkey had higher reserves of vitamin A to start with.

The changes noted here in the structure of the cornea are similar to those described elsewhere. But the changes noted in Descemet's endothelium and membrane have not been described before; they are not gross and could easily have escaped attention [Wolbach and Howe, 1925]. In Fuchs's epithelial dystrophy, it is thought [Duke Elder, 1938] that the epithelial changes are secondary to changes in Descemet's membrane. So it would seem possible that the changes described in the corneal epithelium may be attributed in part to the effect of the vitamin deficiency on Descemet's endothelium and membrane. It is possible that the changes in Descemet's endothelium are secondary to the damaging effects of the avitaminosis on the corneal epithelium; but the sequence of changes noted here would seem to indicate that the avitaminosis has a primary effect on Descemet's endothelium. As a result, the endothelial cells may become incapable of undergoing the changes necessary for the elaboration of the substance of Descemet's membrane. It is not suggested that the avitaminosis has no primary effect on the corneal epithelium. Indeed, the studies of Fell and Mellanby [1953] on tissue culture of epithelia would make such an assumption untenable. But it could be suggested that the corneal epithelium was especially vulnerable to avitaminosis because it was affected in two ways: (*a*) by direct action on the epithelium; (*b*) by faulty nutrition caused by the damage to Descemet's endothelium and membrane. Epithelia elsewhere in the body would show marked change at a later stage because they were only affected by the direct action.

The retinal changes observed in the deficient animals are clear-cut. They show that the cone cells are damaged in vitamin A deficiency in the monkey and that the damage is as severe as in the rods. The results presented here are in agreement with the biochemical observations

implicating vitamin A in the iodopsin system [Wald, 1953 *a*, *b*; Morton, 1951] and with the results of dark adaptation studies in human volunteers deprived of vitamin A [Haig *et al.*, 1938; Hecht and Mandelbaum, 1939; Wald *et al.*, 1938; Hume and Krebs, 1949]. They permit the conclusion that a continual supply of vitamin A is essential for the structural and functional integrity of the cone cells as well as of the rod cells.

In the two monkeys in which keratomalacia developed, the damage to the visual cells was quite severe, and it is of importance to determine whether it can be reversed completely by administration of vitamin A. Experiments are now being made to clarify this point. Opportunity for its study, by the dark adaptation technique in man, is denied because of the associated corneal opacity which precludes such a study. The fact that in the absence of keratomalacia one of the monkeys showed signs of damage to the retinal receptors and pigment epithelium, suggests that retinal damage might be present in persons exposed to a chronic deficiency of vitamin A who do not show corneal involvement. In Southern India where vitamin A deficiency is prevalent, retinal damage may be found to affect even more people than show symptoms of keratomalacia.

Changes in the pigment epithelium were a striking feature of the experiment. There is a widespread belief that the pigment epithelium is intimately connected with the photoreceptive function of the rods and cones. As early as 1895, Kühne believed that "neogenesis" of rhodopsin from colourless products requires the co-operation of the pigment epithelium. Whenever the rods and cones are damaged, the pigment epithelium is also damaged. Thus, in retinitis pigmentosa, degenerative changes are found both in the rods and cones and pigment epithelium [Duke Elder, 1940]. It is, however, not clear whether the damage to the pigment epithelium is primary, or secondary to damage to the visual cell layer. Unfortunately, our findings do not throw much light on this problem. The fact that in one monkey there was definite abnormality of the pigment epithelium, associated with only slight damage to the visual cells, suggests that damage to the pigment epithelium might well be the primary retinal lesion in vitamin A deficiency.

Recent work provides a clue as to the role of the pigment epithelium in the visual process. With the aid of fluorescence microscopy, Popper and Greenberg [1941] found vitamin A in the pigment epithelium, which probably delivers the vitamin from the circulation to the retinal receptors. It is now known that the *all-trans* forms of retinene and vitamin A which are liberated from the visual pigments on exposure to light must be isomerized to neoretinine b and neovitamin Ab before the pigments can regenerate [Hubbard and Wald, 1952]. Bliss [1951] and Hubbard and Wald [1951] reported that some water-soluble

factor, apparently protein in nature, can be extracted from the pigment epithelium and choroidal layer of frog retina, and this can be shown to promote synthesis of rhodopsin from retinene and vitamin A. It is implied by these studies that the pigment epithelium might normally contribute a retinene or vitamin A isomerase. Damage to pigment epithelium in vitamin A deficiency might depress this function and add to the visual impairment.

In a series of publications, Mellanby described widespread degeneration of cranial nerves, including the optic nerves, and of spinal nerves in the growing animal deprived of vitamin A [Mellanby, 1947]. According to him, nerve degeneration is due to a thickening and dysplasia of bone which injures the nerve fibres by pressure. Wolbach believes, however, that it is due to bony pressure caused by different rates of growth of the bony and nerve tissues in vitamin A deficiency [Wolbach and Bessey, 1941]. In the present experiment none of the monkeys showed any clinical evidence of involvement of cranial and peripheral nerves. At autopsy there was no evidence of obvious pressure on the optic nerves in the optic foramina, and the spinal cord did not appear to be crowded in the spinal column. No histological study was, however, made of the nerve-bone relationships in this experiment.

Summary and Conclusions

1. The role of vitamin A in the metabolism of cone cells of the retina was investigated, from the morphological angle, by studying their structure in induced deficiency of vitamin A in three monkeys.

2. Unequivocal signs of structural damage were observed in the cone and rod cells of the deficient animals, which also showed the classical signs of vitamin A deficiency in other organs.

3. In vitamin A deficiency, damage to the visual cell layer of the retina occurred in one monkey in the absence of corneal involvement. This finding suggests that chronic vitamin A deficiency in the community may lead to progressive damage to the visual cells in a much larger number of persons than the incidence figures for keratomalacia indicate.

4. Degeneration of pigment epithelium was present in retinal sections from all the deficient animals. The possible role of the pigment epithelium in the pathogenesis of the visual defect in vitamin A deficiency has been discussed.

5. Degenerative changes were noted in Descemet's endothelium. This damage may contribute to the degeneration of the corneal epithelium.

Acknowledgments

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