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THE BIOLOGICAL FUNCTION OF VITAMIN A ACID

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The first symptom of vitamin A deficiency in man and other animals is the rise of visual threshold known as night-blindness. This is also the only symptom the cause of which is well understood. The photosensitive pigments of the rods and cones (rhodopsin, iodopsin) upon which the visual threshold depends are formed from vitamin A ($C_{19}H_{27}CH_2OH$), through the combination of its aldehyde, retinene ($C_{19}H_{27}CHO$), with specific proteins of the rods and cones called opsins.[1] In rats maintained on a vitamin A-deficient diet, after initial stores of vitamin A in the liver and blood have been exhausted, the level of rhodopsin in the retina begins to fall, the logarithm of the visual threshold reciprocally rising, marking the beginning of night-blindness.[2]

Several weeks later, the level of opsin in the retina also begins to decline, and with this the rod outer segments deteriorate anatomically.[2] An explanation has been suggested for this effect also. Opsin in aqueous solution is a much less stable protein than rhodopsin. It is rapidly denatured by exposures to heat,[3] acids and alkalies[4] that leave rhodopsin intact. When, as the result of the deficient diet, the retina comes to contain opsin that can find no vitamin A with which to combine, this intrinsic instability is probably the cause of its disintegration; and since the outer segments of the rods are largely composed of this protein, as opsin is lost their anatomical structure must suffer accordingly.

The deterioration of opsin and of the rod outer segments, however, is only a detail in a much wider complex of changes occurring throughout the animal at the same time; for at this time all the overt signs of vitamin A deficiency appear: loss of weight, postural imbalance, respiratory disturbances, corneal opacities, disarrangement of coat, and red secretions about the eyes. Tissues, perhaps particularly epithelia, have begun to disintegrate in many parts of the body, and within a few days more all these animals are moribund.[2]

It has long been recognized that vitamin A plays some general role in the tissues, indispensable for their integrity and the growth and maintenance of the animal. The nature of this, by far its most important function, is as yet altogether unknown. Our experiments, having begun with well-understood processes in the retina, had at this point become involved with these wider and wholly obscure phenomena.

It seemed possible that we might be able to disentangle this situation, and perhaps learn something of the tissue function of vitamin A, with the help of vitamin

A acid. This substance ($C_{19}H_{27}COOH$), first prepared by Arens and Van Dorp,[5] was shown to maintain growth in the rat and to stave off obvious signs of deficiency, with a bipotency approaching that of vitamin A itself;[6, 7] yet no matter how large the amounts in which it was fed, no vitamin A was deposited in the liver.[8] The rat seems unable to reduce vitamin A acid to vitamin A; and this special circumstance led Moore[9] to suggest that though the tissue functions of vitamin A seem to be fulfilled by vitamin A acid, this substance might *not* be able to serve as precursor of the visual pigments, which need for their synthesis the alcohol and aldehyde. These considerations formed the starting-point of the present investigation.

Methods.—Most of our procedures were described in an earlier paper.[2] Male, weanling rats from the Harvard colony were raised on the standard U. S. P. vitamin A-deficient test diet, to which supplements were added as wanted. Techniques for evaluating liver and blood vitamin A, rhodopsin, and opsin in the eye, and recording electroretinograms were as described earlier.

Vitamin A and vitamin A acid, dissolved in cottonseed oil, were administered by mouth, through a syringe with a blunted point. Since in these experiments we were interested only in maximal effects of vitamin A acid, excessive doses were given. Three feedings a week provided a dosage level equivalent to at least 50 μgm per day. This level was chosen with the thought that if the acid possesses the lowest activity yet reported for it—10 per cent as high as vitamin A[6]—we should still be providing about twice the vitamin A-activity considered to be adequate for the rat (2–2.5 μgm vitamin A per day).

A Typical Experiment.—The course of these experiments and the general nature of the results can best be introduced with such a typical experiment as shown in Figure 1. Two animals, litter mates, were placed on the deficient diet, supplemented in one of them with vitamin A acid as described. Both animals grew at about the same rate for 5–6 weeks. Then the unsupplemented animal stopped growing, rapidly lost weight, and died on the 57th day of the diet. The other animal continued to grow regularly, and appeared to remain in prime condition, as the photograph taken on the 157th day of the diet is intended to show. On the same day this rat's electroretinograms were recorded, as shown at the right of Figure 1, compared with those of a normal animal measured at the same time.

This animal, though normal in weight and appearance, was highly night-blind. Its visual threshold—the luminance of a $^1/_{50}$-second flash needed to excite a just measurable ERG—was 3.25 log units (about 1,800 times) above normal. As the figure shows, this animal yielded about the same ERG at log luminance 4 as the normal animal did at log luminance 0. Rhodopsin could be extracted from the retinas of animals in this condition in only 1–5 per cent of normal amounts. This degree of night-blindness was higher than we had ever achieved before in rats kept on the vitamin A-deficient diet without supplementation.

On the following day these animals were sacrificed, and the retinal histology examined (Fig. 2). In the animal kept for 5 months on vitamin A acid, all the retinal tissues were normal in appearance, except for the visual cells. The pigment epithelium, bipolar layer (inner nuclei), and ganglion cell layer (not shown; see Fig. 15) did not seem in any way altered.

The nuclei of the visual cells (outer nuclei) were considerably reduced in num-

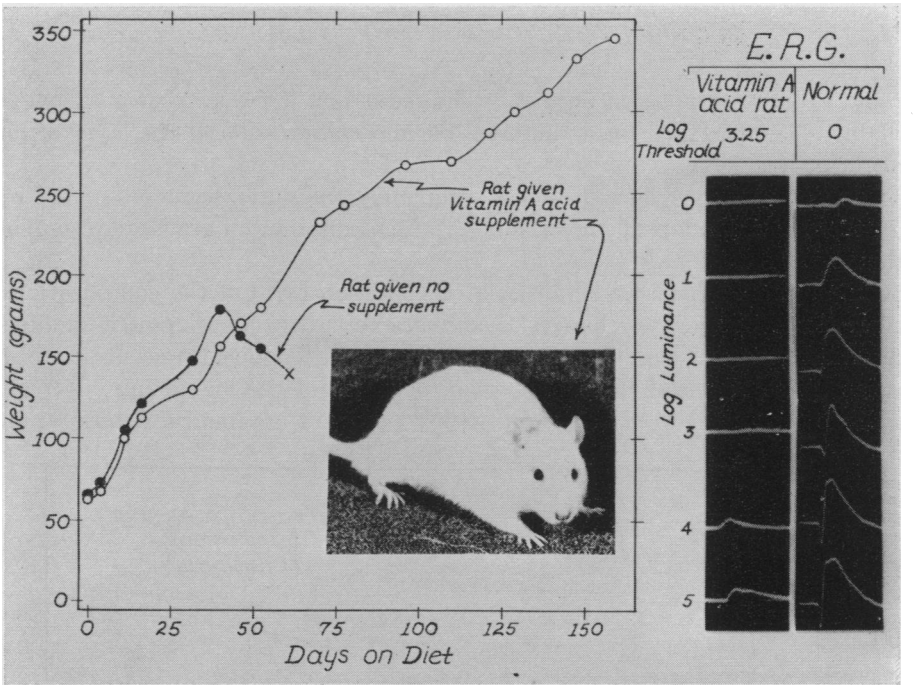


FIG. 1.—Nutritional activity of vitamin A acid. Litter mates had been placed on a vitamin A-deficient diet, and on the same diet supplemented with vitamin A acid. The rat given no supplement died on the 57th day of the diet; the animal receiving vitamin A acid continued to grow and remained in excellent condition for the duration of the experiment, a little over 5 months. The picture of this animal was taken at the end of the experiment, as were the electroretinograms shown at the right, compared with those of a normal animal. They show this rat to be highly night-blind: its visual threshold had risen 3.25 log units (about 1,800 times) above normal, and only just detectable ERG's could be evoked at even the highest luminances.

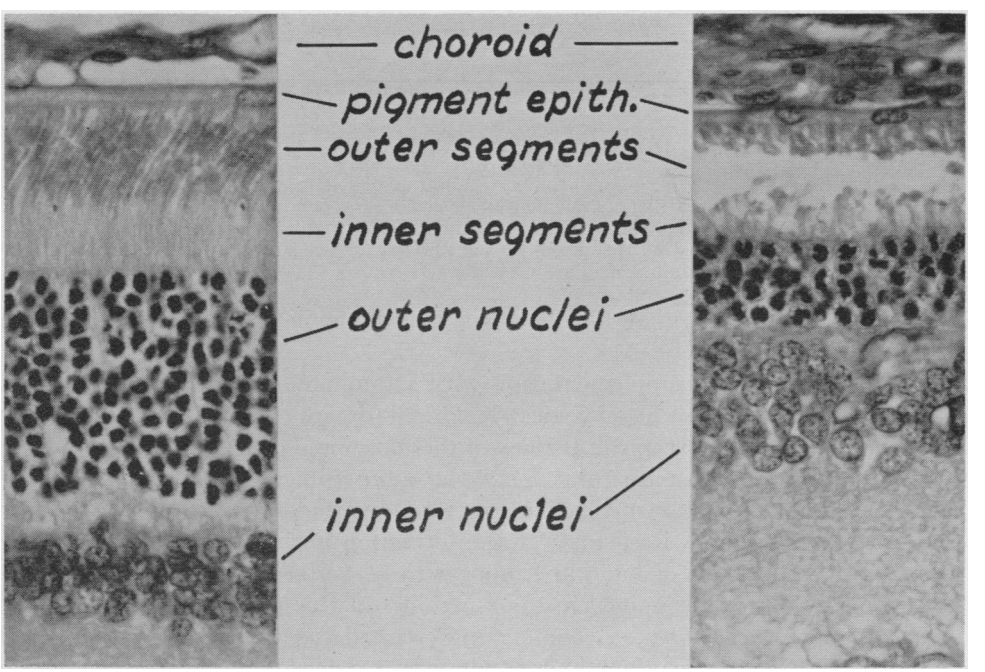


FIG. 2.—Retinal histology of the night-blind animal shown in Fig. 1, compared with that of a normal animal. All the retinal tissues are normal except the visual cells, which are reduced in number and almost completely lack outer segments.

ber, but otherwise appeared normal. The reduction in the number of visual cells probably accounted also for a thinning out and compression of the layer of inner segments.

This retina however lacked almost completely the outer segments of the rods. With the loss of rhodopsin, the organelles which contain and are largely composed of this pigment had almost vanished.

Such an animal displays, as an isolated condition freed of the complications of general tissue decay, the changes that characterize the development of dietary night-blindness: on removal of vitamin A, the fall in rhodopsin concentration, with associated rise of visual threshold; then, deprived of the stabilizing effect of its prosthetic group, the decay of opsin, with consequent anatomical deterioration of

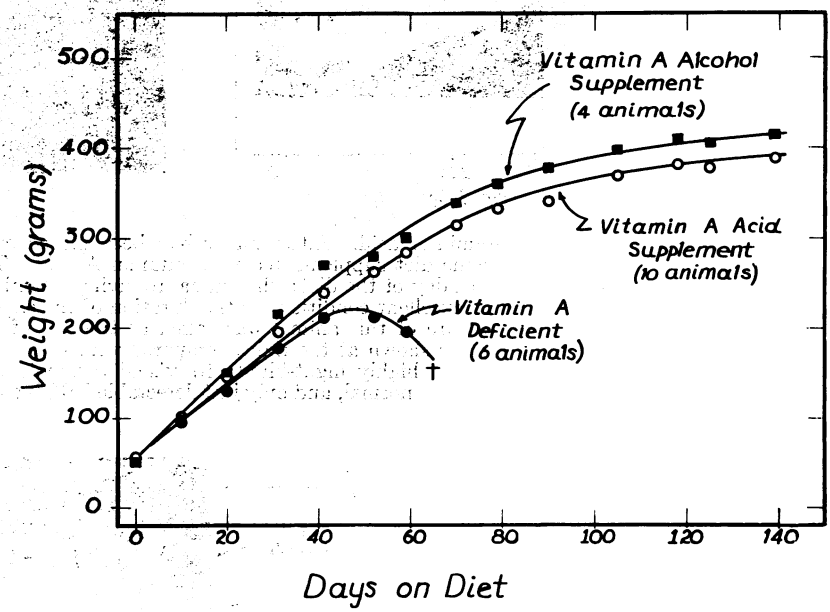


FIG. 3.—Growth of animals on a vitamin A-deficient diet, compared with growth on the same diet supplemented with 50 μgm per day of vitamin A or vitamin A acid. The unsupplemented animals lost weight after 6 weeks on the diet, and all had died by the end of the eighth week. The supplemented animals grew as well on vitamin A acid as on vitamin A.

the rod outer segments, in all likelihood the only tissue in the body of which opsin is an important component.

In this instance the supplementation with vitamin A acid had resulted in an animal which appeared physiologically normal except for its night-blindness; biochemically normal, except for its lack of rhodopsin and opsin; and anatomically normal, except for the almost total loss of the outer segments of the rods. By the same token, this animal seemed to demonstrate that the only function in the body that requires vitamin A itself may be the formation of visual pigments. All the general tissue functions of vitamin A appear to be performed by vitamin A acid. These conclusions are examined in the remainder of this paper.

Growth and Maintenance.—Weanling rats were divided into three groups, all placed simultaneously on the standard vitamin A-deficient test diet. One group

was supplemented with vitamin A, the second with vitamin A acid, both dissolved in cottonseed oil; and the third group was given the same amount of cottonseed oil alone.

The growth of these animals is shown in Figure 3. For the first 5 weeks on the diet, all three groups grew about equally. Then the unsupplemented animals

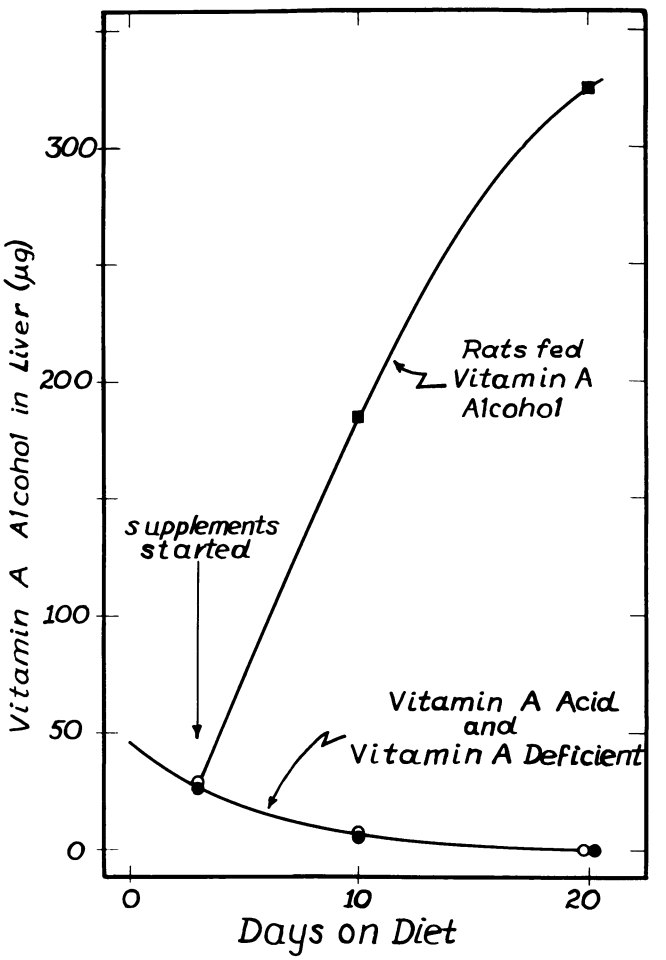


FIG. 4.—Total vitamin A content of the liver in animals placed on a vitamin A-deficient diet, and on the same diet supplemented with 50 μgm per day of vitamin A or vitamin A acid. (These animals formed part of the same experiment as in Fig. 3.) The animals supplemented with vitamin A rapidly increased their liver stores. Those receiving vitamin A acid lost their initial stores of vitamin A as rapidly as those receiving no supplementation.

stopped growing, declined rapidly in weight, and by the end of the eighth week, all had died. The animals receiving supplements of vitamin A and of vitamin A acid grew equally well throughout the experiment (140 days). The small difference in average weight shown in Figure 3 (27 grams; 6.7 per cent) does not appear to be significant. Each of the supplemented groups spread considerably in weight, and

overlapped each other widely (vitamin A: 360–450 grams; vitamin A acid: 318–512 grams). In another experiment, after 135 days of a similar regime, the average weight of the animals on vitamin A acid was slightly *greater* than that of the animals receiving vitamin A (324:314 grams). It may be concluded that the growth of the animals on vitamin A acid was entirely normal.

Both groups of animals also appeared equally sound, externally and in the gross appearance of the internal organs on autopsy. The tracheal epithelium, examined microscopically, also appeared normal in both groups.

To test further the biological effectiveness of vitamin A acid, this supplement was withheld in other experiments until the 4th and 7th week of the vitamin

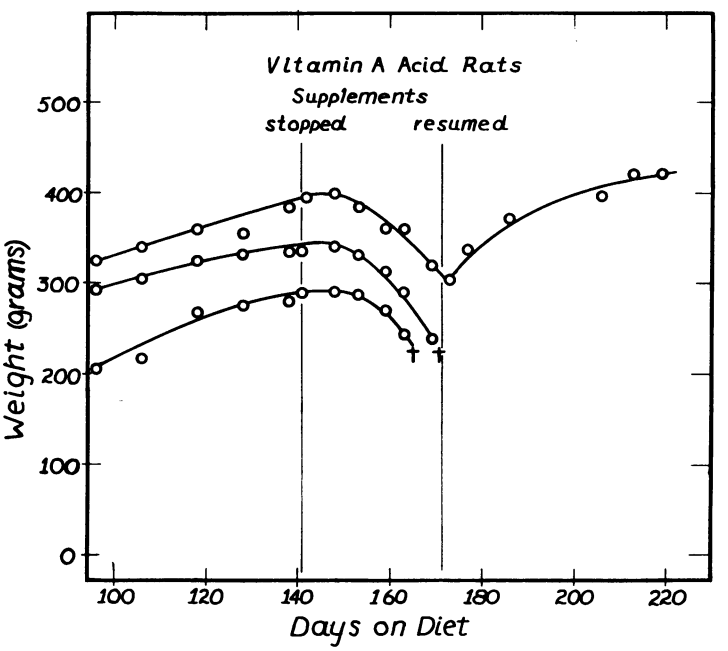


FIG. 5.—The effect of withdrawing the supplementation from animals growing normally on a vitamin A-deficient diet supplemented with excess vitamin A acid. Within a few days these animals stopped growing, and began to decline in weight. Within 3–5 weeks, two animals had died displaying the signs of severe vitamin A deficiency. The third animal, on renewed supplementation with vitamin A acid, promptly recovered from its deficiency symptoms, and regained its normal weight.

A-deficient diet, that is, until the animals had stopped gaining or were rapidly losing weight. On administration of vitamin A acid, such animals began at once to grow, and soon appeared identical with those supplemented from the start of the experiment. Also any signs of vitamin A deficiency that had developed on the deficient diet rapidly healed, except for occasional scarring of the cornea, owing to earlier xerophthalmia, which remained permanently.

Vitamin A Storage and Depletion.—We had noticed that night-blindness develops equally rapidly in rats on a vitamin A-deficient diet, whether or not supplemented with vitamin A acid. During the first weeks on the diet such animals use up the supplies of vitamin A stored in the liver; and it seemed from this observation that

the rate of depletion of vitamin A in the liver is probably equally rapid, whether or not vitamin A acid is available.

Figure 4 shows this to be the case. The vitamin A content of the liver was measured in animals divided into the three dietary groups already described; indeed these animals formed part of the same experiment shown in Figure 3. Those receiving supplements of vitamin A (50 μgm per day) rapidly increased their stores

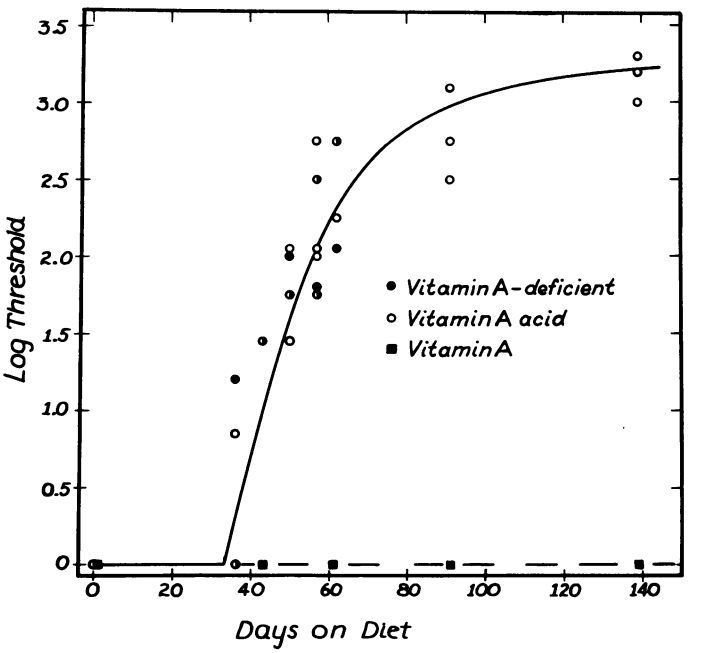


FIG. 6.—Visual thresholds of animals kept on a vitamin A-deficient diet, and on the same diet supplemented with vitamin A or vitamin A acid (same experiment as in Fig. 3). The threshold is the smallest luminance of a $^1/_{50}$ second flash of light needed to evoke a just-perceptible ERG. The vitamin A-supplemented rats formed the control group, whose thresholds are arbitrarily given the value 1 (log threshold = 0). All other thresholds are expressed relative to these, and represent therefore increments of log threshold above the control level. In the animals supplemented with vitamin A acid, the threshold rises as soon and as rapidly as in those receiving no supplementation. All the latter group have died, however, by the end of the ninth week; whereas the vitamin A acid animals survive to grow more night-blind. The thresholds level off after 12–15 weeks on the diet, at about 3.25 log units (about 1,800 times) above normal. At this time the retinas contain only 1–5 per cent of the normal amounts of rhodopsin.

in the liver. Those supplemented with vitamin A acid, however, lost their initial stores of liver vitamin A as rapidly as the rats receiving no supplements. Both groups also developed night-blindness at the same time. Vitamin A acid not only fails to contribute vitamin A to the liver, but seems to have no sparing action on the vitamin A already there. Since, however, the animals receiving the acid supplement are adequately maintained by it, except for their vision, the loss of vitamin A from the liver seems to be independent of demand. We shall have more to say of this relationship below.

The animals supplemented with vitamin A acid fail also to store this substance. Vitamin A acid is readily identified by the sharp absorption band at 573 mμ which it yields in the antimony chloride test. We have never by this means been able to detect the acid in extracts of liver, kidney, or blood of animals receiving large amounts of this substance in the diet (cf. also Sharman[7]).

One consequence of the failure to store either vitamin A or the acid is that these animals, though normal in weight and in excellent health, respond almost immediately to interruption of the supplementation (Fig. 5). Animals growing well on vitamin A acid, on removing the supplement stop growing within a few days,

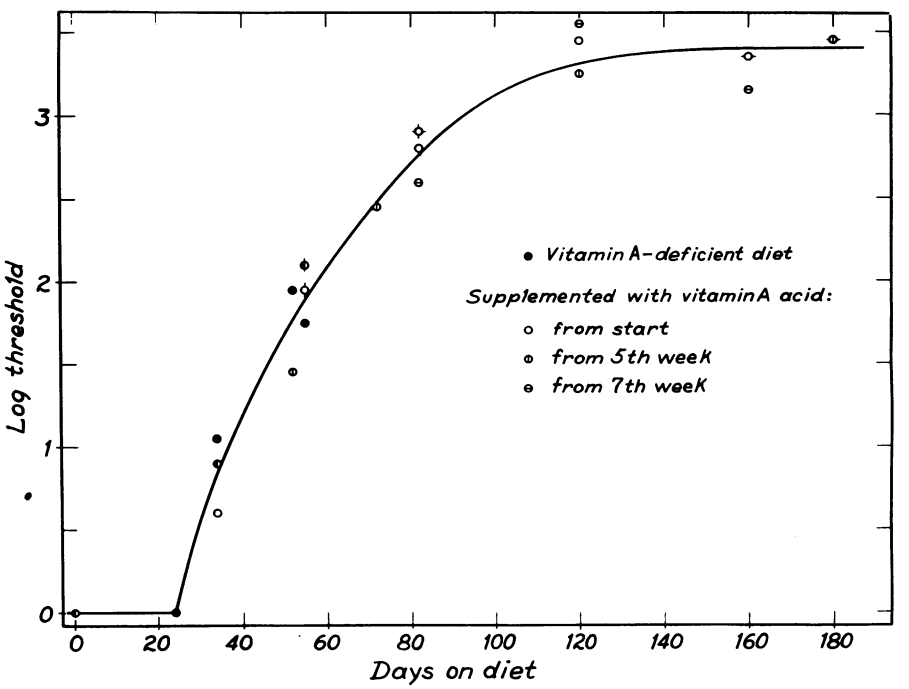


FIG. 7.—Development of night-blindness in animals on a vitamin A-deficient diet, and on the same diet supplemented with vitamin A acid from the start, or from the 5th week—when the animals had stopped growing—or from the 7th week, when they were declining rapidly in weight. In all these animals the visual threshold begins to rise at the same time, and rises equally rapidly. By the end of the 8th week, the unsupplemented animals have died; whereas those receiving vitamin A acid grow increasingly night-blind. After about 18 weeks on the diet the visual threshold of the latter group becomes constant at about 3.3 log units (about 2,000 times) above the normal level.

decline rapidly in weight, and within 3–5 weeks die displaying all signs of severe vitamin A deficiency. That is, these animals respond as do young animals on a vitamin A-deficient diet after all internal stores of vitamin A have been exhausted. If the supplementation with vitamin A acid is renewed, even toward terminal stages of the deficiency, such animals rapidly recover (Fig. 5).

Night-Blindness.—We have already remarked that animals supplemented with vitamin A acid, though otherwise sound, become night-blind as rapidly as those receiving no supplementation. This is shown in Figures 6 and 7.

Figure 6 shows the visual thresholds—the brightness of a $^1/_{50}$ second flash of light required to excite a just perceptible electroretinogram—of the animals whose weights are shown in Figure 3. Those receiving supplements of vitamin A served as controls; their thresholds are arbitrarily given the value 1 (log threshold = 0), and all other thresholds are expressed as increments of log threshold above these control values. It is clear that the threshold rises at the same time and at about the same rate in the animals receiving no supplementation, and those receiving vitamin A acid; but whereas the former have all died by the 60th day of the diet, the latter survive to become increasingly night-blind.

Figure 7 shows the results of a similar experiment, conducted with more animals and hence yielding more accurate averages. Again the unsupplemented animals and those given vitamin A acid from the start of the diet developed night-blindness at the same rate. Again the unsupplemented animals had all died by the 60th day; whereas the others, whether supplemented with vitamin A acid from the start, or from the 5th week—when they had stopped growing—or from the 7th week—when the weight was declining rapidly—continued to grow and to become more night-blind.

In both these experiments the visual thresholds do not increase indefinitely, but level off after 12–15 weeks at 3.3–3.5 log units—about 2,000–3,000 times—above normal. Direct extraction of the retinas of such animals showed that they contain about 1–5 per cent of the normal amounts of rhodopsin.

The electroretinograms of animals maintained on vitamin A acid are shown in Figure 8. The stimulus was a $^1/_{50}$ second flash of light, the luminance of which was varied in steps over a range of 7 logarithmic units (1 to 10 million). After 28 days on the diet, the ERG is still normal. At 56 days, the animal is quite night-blind. The threshold has risen about 2.8 log units (about 500 times); at luminances above the threshold the response—both a- and b-waves—is greatly diminished; and a small inflection normally found on the downward sweep of the b-wave has separated off as a second positive wave. All these effects are characteristic of this stage of night-blindness in unsupplemented, vitamin A-deficient animals.[2]

As the animals continue on vitamin A acid supplementation, the electroretinograms undergo a second type of change. The threshold, as already described, becomes relatively constant by about the 120th day of the diet, at about 3.2–3.5 log units above normal. Increasing the intensity of the stimulus above the threshold level, however, now begins to have less and less effect. At 139 days, stimuli even 4 log units above threshold yield only a slow and diffuse b-wave of low amplitude; indeed by that time the intensity of the stimulus hardly affects the form or height of the response (Fig. 8). It is as though, following upon the rise of threshold that marked the first stage of night-blindness, these animals now lose the capacity to generate an ERG. As Figure 8 shows, after 10 months on the regime, no response can be elicited at all.

Figure 9 shows the relation between the visual threshold and the quantity of rhodopsin extracted from the retina. We had found earlier that in vitamin A-deficient animals receiving no supplementation, the *logarithm* of the threshold rises linearly as the rhodopsin content falls.[2] In animals supplemented with vitamin A acid, this relationship can be extended further. As Figure 9 shows, it is maintained over the whole range of the measurements.

Figure 9 involves a further comparison. The rhodopsin content of the retina falls in vitamin A deficiency; it falls also on exposure of the animals to bright light (light adaptation), rising again when the light is extinguished. That is, vitamin A deficiency and light adaptation are two ways of inducing night-blindness. How does the relationship between rhodopsin content and log threshold compare in both conditions? To answer this question a group of normal rats was highly light-

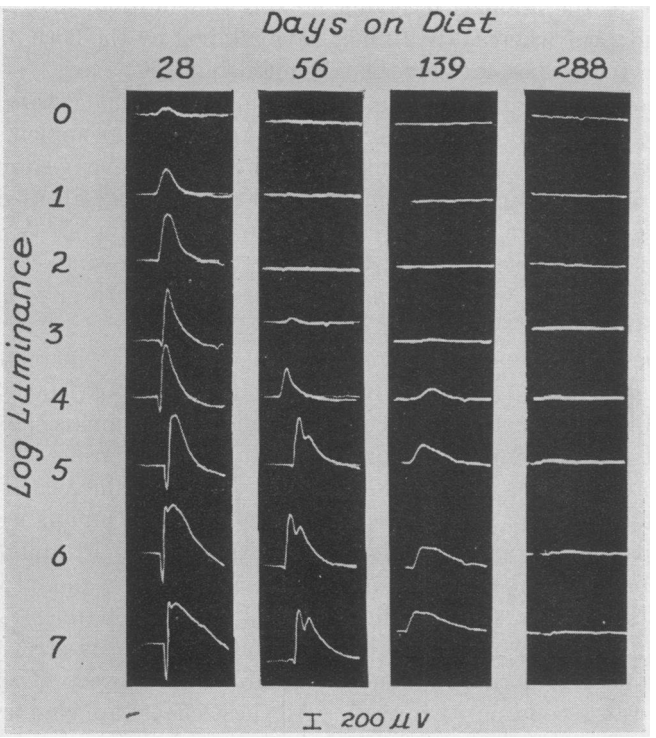


FIG. 8.—Electroretinograms of animals on a vitamin A-deficient diet supplemented with vitamin A acid. Responses to a $^1/_{50}$ second flash of light, at luminances ranging over 7 log units (1 to 10 million). The initial deflection downward is the negative a-wave; the later sweep upward is the positive b-wave. The first strip of records was made on the 28th day of the diet, when the ERG is still normal. On the 56th day the animal is typically night-blind: its threshold has risen about 500 times; the a- and b-waves have decreased greatly in amplitude; and a small inflection on the downward sweep of the b-wave has separated off as a second positive wave. By the 139th day, the threshold has risen only moderately further; but now increasing the brightness of stimulus above the threshold level has little effect. The retina is losing the capacity to generate an ERG. By the 288th day (10 months on the diet), no response can be elicited at the highest available brightness. The animal is now blind.

adapted; then the light was shut off, and periodically an animal was anaesthetized and its ERG threshold determined. The same, and also other groups of animals were light-adapted similarly, and after the same intervals in darkness animals were killed, and their retinas extracted for rhodopsin. In this way the relationship between visual threshold and the retinal content of rhodopsin was measured, this time however in normal animals in the ordinary course of dark adaptation.[10, 11]

Figure 9 shows that a change of rhodopsin concentration in the retina, whether induced by visual adaptation or by vitamin A deficiency, has the same effect on the threshold. In both conditions the logarithm of the threshold rises linearly as the rhodopsin content falls.[12] The identity of these effects implies that up to this point in the development of vitamin A deficiency, the night-blindness is accounted for completely by the loss of rhodopsin from the retina.

Anatomical Changes.—We have already seen that by the 24th week on vitamin A acid, the visual cells are considerably reduced in number, and most of the rods lack outer segments (Fig. 2). The disintegration of the rod outer segments seems

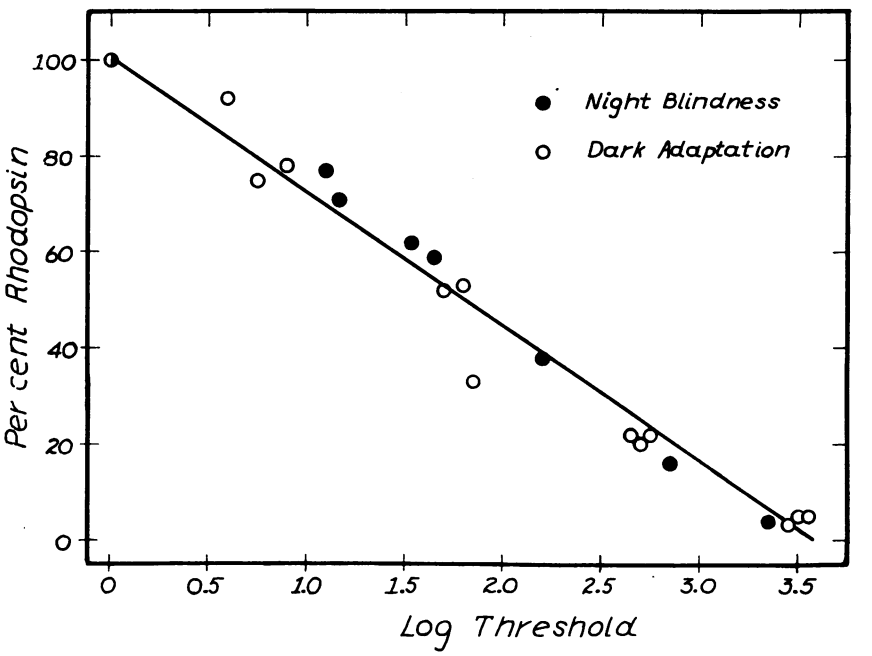


FIG. 9.—Relation between the rhodopsin content of the rat retina and the visual threshold, in animals night-blind owing to vitamin A deficiency, and in normal animals dark adapting after exposure to bright light. In both instances the same relationship is observed: the log threshold rises linearly (i. e., the log sensitivity—log 1/threshold—falls linearly) with fall in rhodopsin concentration. This relationship is described by the equation, $\log (I_t/I_o) = 3.6(R_o - R_t)/R_o$, in which I_o and R_o are respectively the threshold and rhodopsin concentration in dark adapted control animals, and I_t and R_t are respectively the thresholds and rhodopsir concentrations in vitamin A-deficient or partly dark-adapted animals.

to accompany the loss of opsin. For example, in one experiment, in the 18th week of the deficiency (with vitamin A acid supplementation), when the threshold had risen over 3 log units, and the rhodopsin level was only a few per cent of normal, the opsin had declined to half the normal level.

As the retina loses opsin, examination in the electron microscope shows progressive deterioration of the microstructure of the rod outer segments. The electron microscopy was performed in collaboration with Dr. I. R. Gibbons, and will be reported in detail elsewhere.[13] After removal of the cornea and lens, the whole fundus of the eye was fixed for one hour in buffered osmium tetroxide, washed, de-

hydrated with acetone, and embedded in araldite resin. Ultrathin sections were cut on a Porter-Blum microtome, and examined in the R. C. A. EMU-3D electron microscope.

Figure 10 shows a longitudinal section through portions of the rod outer segments of a normal rat. As in other animals, the outer segment consists of a stack of flattened sacs, wholly enclosed within an outer membrane. The outer segment is about 1.5 μ wide and about 15 μ long. It contains about 35 disks per μ, or about 525 disks in all.

Figure 11 shows an early stage in the deterioration of this structure, typical of animals kept 3–4 months on the vitamin A-deficient diet supplemented with vitamin

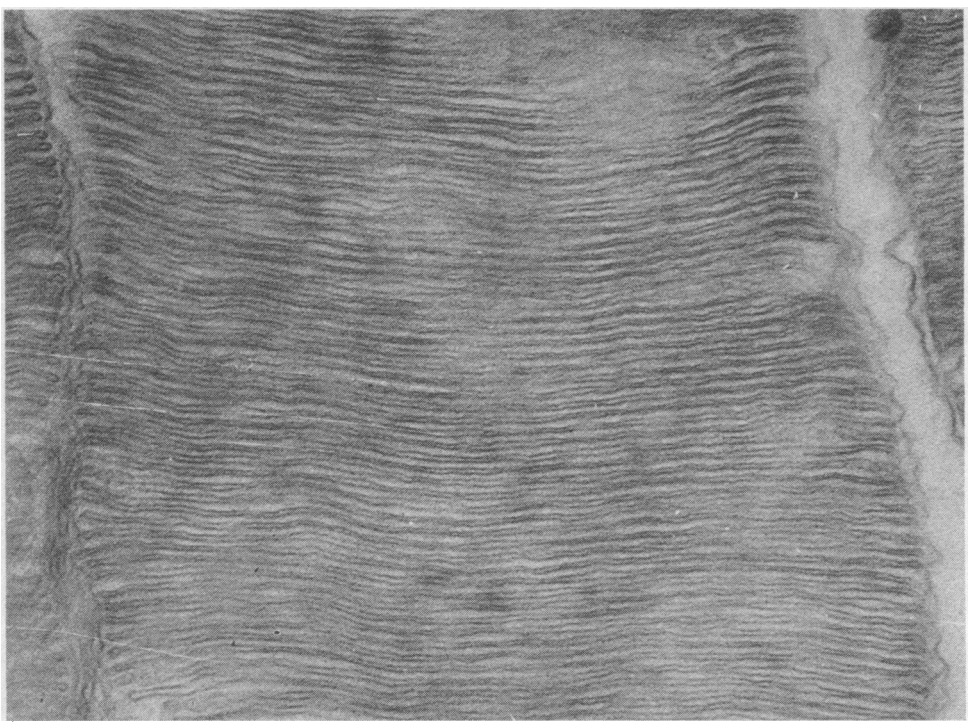


FIG. 10.—Electron micrograph of a longitudinal section through a rod outer segment, and portions of two neighboring outer segments, in the normal rat. As in other animals, the outer segment consists of a stack of transverse, flattened sacs, entirely enclosed in an outer membrane. The rat outer segment is about 1.5 μ wide and 15 μ long, and contains about 525 disks. Magnification 73,000.

A acid. Many of the retinal disks are still intact; but many others have segmented, or are in process of doing so, into groups of distended vesicles and tubules.

By 6 months on the regime, these changes have progressed further (Fig. 12). Few outer segments remain, and their internal structure is highly disorganized. They have lost also their characteristic cylindrical shape, becoming distorted and tending to collapse, often into roughly spherical shapes. By this time also the visual cells are considerably reduced in number, and their inner segments, formerly long and slender, have shortened and broadened (Fig. 2). The ultrastructure of the nuclei and inner segments of the visual cells, however, has not visibly changed.

As animals continue longer on this regime, the visual cells undergo further altera-

tions. Figure 13 shows sections of the retinas of two litter mates, one supplemented with vitamin A, the other with vitamin A acid, for 10 months on the otherwise A-deficient diet. The animal receiving vitamin A acid no longer yielded an ERG in response to the brightest lights available in our apparatus (Fig. 8). In this retina, the pigment epithelium and the layers of bipolar and ganglion cells still look normal. The visual cells, however, are reduced to a single row of nuclei, and the individual cells have contracted to a cuboidal shape, almost wholly occupied by the nucleus, with no distinguishable inner or outer segment. In this extraordinary state the pigment epithelium lies in direct contact with the layer of visual cell nuclei.

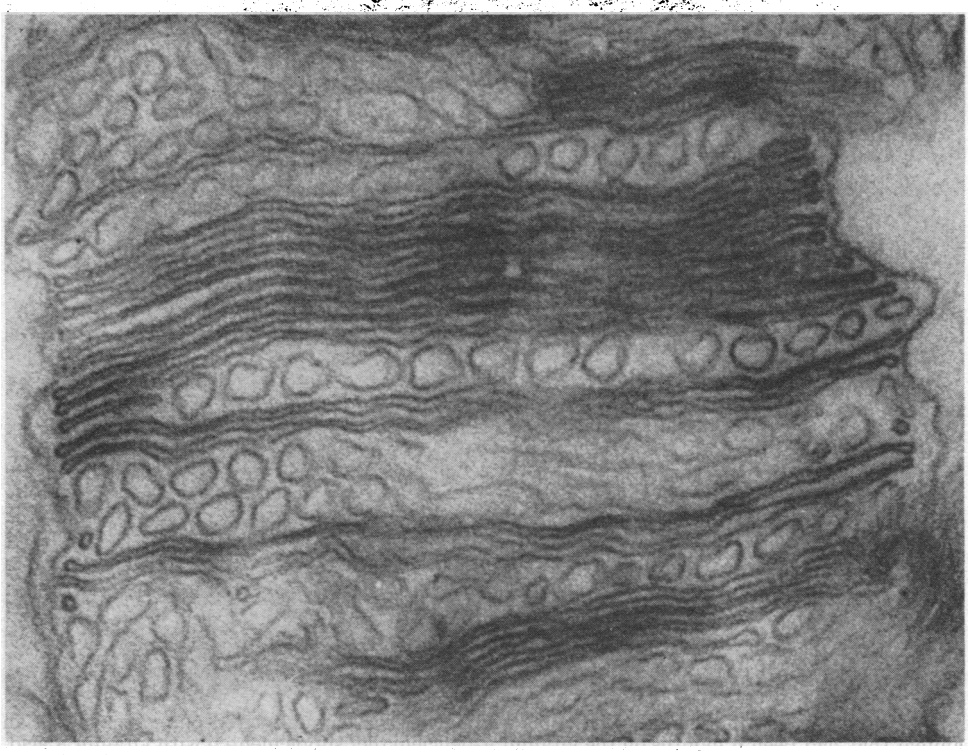


FIG. 11.—Electron micrograph of a longitudinal section through the rod outer segment in a highly night-blind rat, typical of animals that had been 3–4 months on a vitamin A-deficiency diet supplemented with vitamin A acid. Some of the transverse disks appear intact; others have segmented into groups of distended vesicles and tubules. Magnification 113,000.

Recovery.—On administration of vitamin A, animals which had become night-blind on the vitamin A-deficient diet supplemented with vitamin A acid recover in varying degree. When such animals have been on the diet up to 10 weeks, recovery ordinarily is rapid and complete. Within several hours after feeding a large dose of vitamin A in cottonseed oil, the visual threshold—which may by then have risen about 2 log units above normal—begins to fall, and within 2–3 days has reached the normal level.

After longer periods on the diet, recovery follows a more complex course. Figure 14 shows the effect of one intraperitoneal injection of 1 mgm of vitamin A on two animals that had been on the diet for 25 weeks. These animals recover in two

stages: the visual threshold first falls rapidly for 40–50 hours following the injection, reaching a level about 1 log unit above normal; thereafter it continues to fall very slowly over a period of many weeks of further vitamin A supplementation.

In animals kept still longer on the diet, these two phases of recovery change in relative importance, the fast phase contributing less, and the slow phase accounting for more and more of the total change.

It seems probable that these two stages of recovery are concerned with reversing the two phases we have described in the development of night-blindness: first, the rise of visual threshold associated with the decline of rhodopsin concentration owing

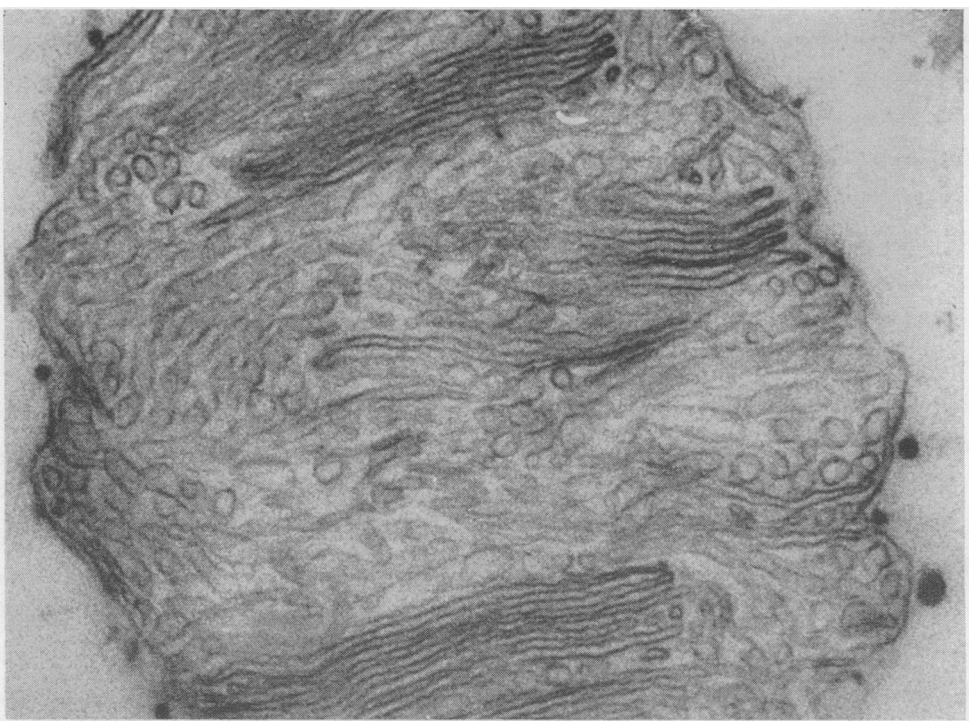


FIG. 12.—Electron micrograph of the longitudinal section through a rod outer segment in an animal that had been 6 months on the vitamin A-deficient diet supplemented with vitamin A acid. Few rod outer segments remain in such a retina; and, as in this example, they are highly distorted in shape and microstructure. A few isolated groups of disks still appear, but mainly the internal structure has degenerated into distended tubules and vesicles. The outer segments have also lost their normal cylindrical shape, and collapsed to irregular ellipsoids or spheres. Magnification 76,000.

to simple lack of vitamin A; later, the loss of opsin and anatomical deterioration of the rods. It seems reasonable to suppose that the fast phase of recovery involves the combination of whatever opsin remains with the vitamin A that is administered; whereas the synthesis of opsin and structural repair of the visual cells occupy the slow phase. Certain instances in which the recovery from experimental human night-blindness has exhibited similar fast and slow phases may perhaps be explained in the same way.[14]

In animals kept longer than 6 months on the diet, the visual threshold no longer recovers completely, even after months of vitamin A supplementation. This ir-

reversible aspect of night-blindness is probably associated with the actual loss of visual cells. Such cells, once lost, are never regained.

A decline in the density of visual cells must raise the visual threshold, just as a decrease in the area of the visual field normally raises the threshold; and this should be a source of various degrees of permanent night-blindness. If indeed it is true that a decrease in the density of visual cells has about the same effect as a decrease in the area of the visual field, it should raise the threshold by a factor corresponding to the reciprocal of this decrease (Riccò's Law) or the square root of the reciprocal (Piper's Law). That is, in such a condition as shown in Figure 2,

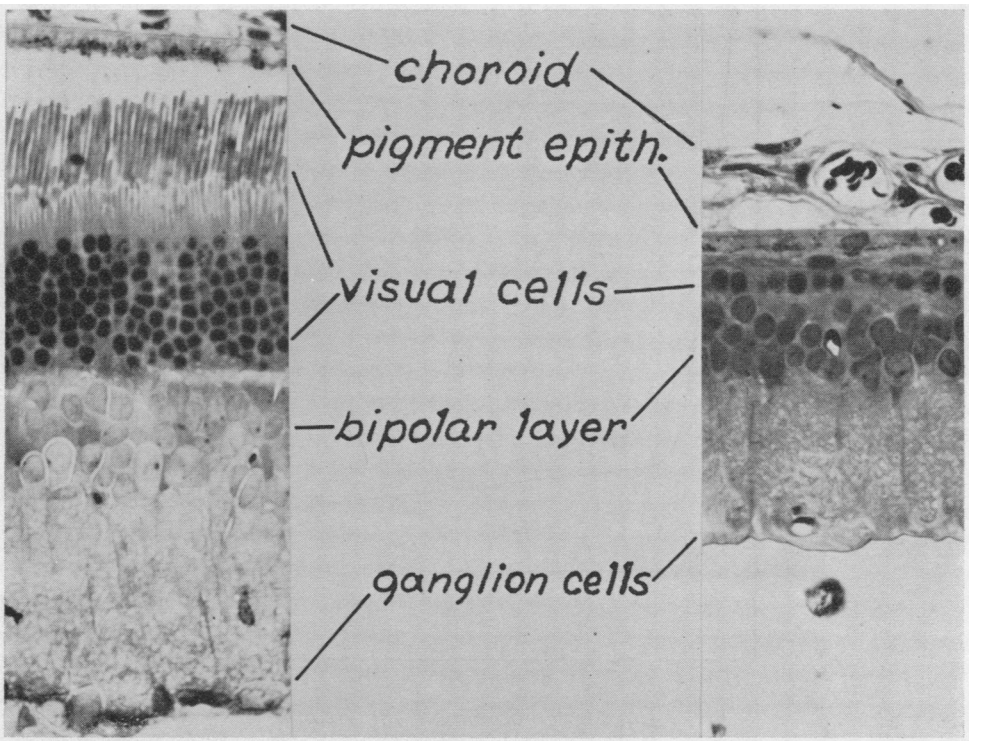


FIG. 13.—Sections through the retinas of two animals—litter mates—both of which had been 10 months on the vitamin A-deficient diet, supplemented with vitamin A (left), or with vitamin A acid (right). The retina on the left is normal; that on the right is also normal, except for its visual cells. The visual cells, only about $^1/_{10}$ of which remain, form a single, almost complete row of nuclei, directly apposed to the pigment epithelium. No inner or outer segments are visible. Such an animal is not only night-blind, but blind (cf. Fig. 8, ERG's at 288 days); and does not regain its vision after months of vitamin A supplementation.

in which the number of visual cell nuclei has been reduced to about $^4/_9$, if as a result of vitamin A administration all the remaining cells again became fully functional, without increasing in number, the final threshold should be raised permanently by $^9/_4$ or $\sqrt{^9/_4}$ (i.e., 2.25 or 1.50 times) over the normal threshold. Such changes do not loom large on a logarithmic scale: they correspond to raising the threshold 0.35 or 0.18 log unit.

A typical example of such an experiment is shown in Figure 15. Three litter mates were kept on the vitamin A-deficient diet for $6^1/_2$ months, one supplemented with vitamin A to serve as control, the other two supplemented with vitamin A

acid. Then the ERG's of the control and one vitamin A acid animal were measured; and on the following day the control and the other vitamin A acid animal were killed and their retinas sectioned.

The retina and ERG's of the control animal were normal (Fig. 15, left). The vitamin A acid animals, however, displayed the usual anatomical and physiological signs (Fig. 15, center). In the retina, only remnants of outer segments re-

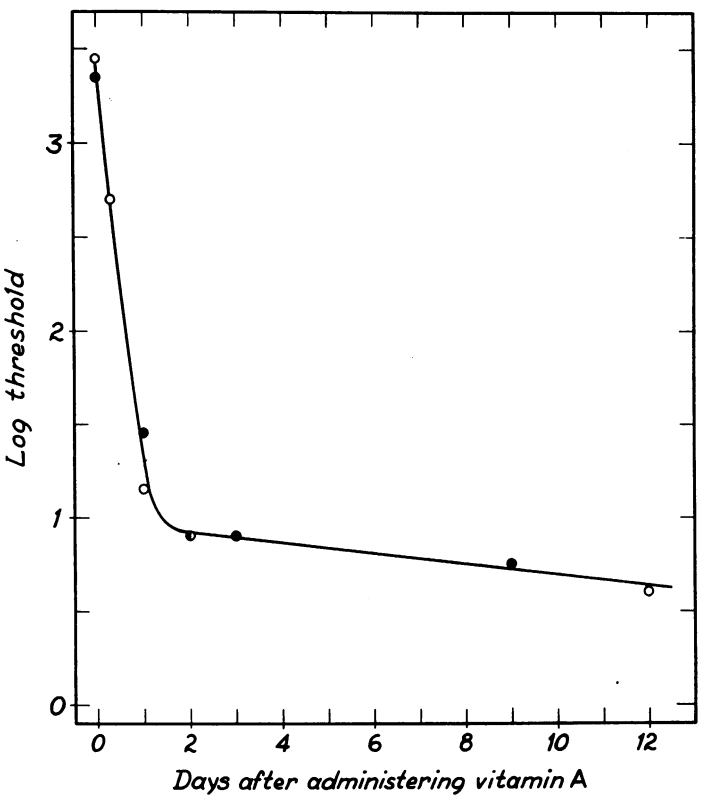


FIG. 14.—Recovery from night-blindness on administration of vitamin A. Two animals that had been kept for 25 weeks on the vitamin A-deficient diet supplemented with vitamin A acid received an intraperitoneal injection of 1 mgm vitamin A in cottonseed oil, followed by regular dietary supplementation with vitamin A. The visual threshold, starting from levels 3.3–3.4 log units (about 2,000 times) above normal, first falls rapidly to a level about 1 log unit above normal. Thereafter it continues to fall slowly for several weeks, returning almost to the normal level (cf. Fig. 15).

mained, and the inner segments were few and stumpy in appearance. The layer of visual cell nuclei was reduced from the normal 9 to about 4 rows. The ERG record shows the mate of this animal to have been highly night-blind; the visual threshold had risen more than 3 log units, and the ERG's at higher luminances displayed all the usual changes that go with this condition.

The surviving vitamin A acid animal was supplemented for 16 days with vitamin A. Then his ERG's were re-measured, and the retinas sectioned immediately

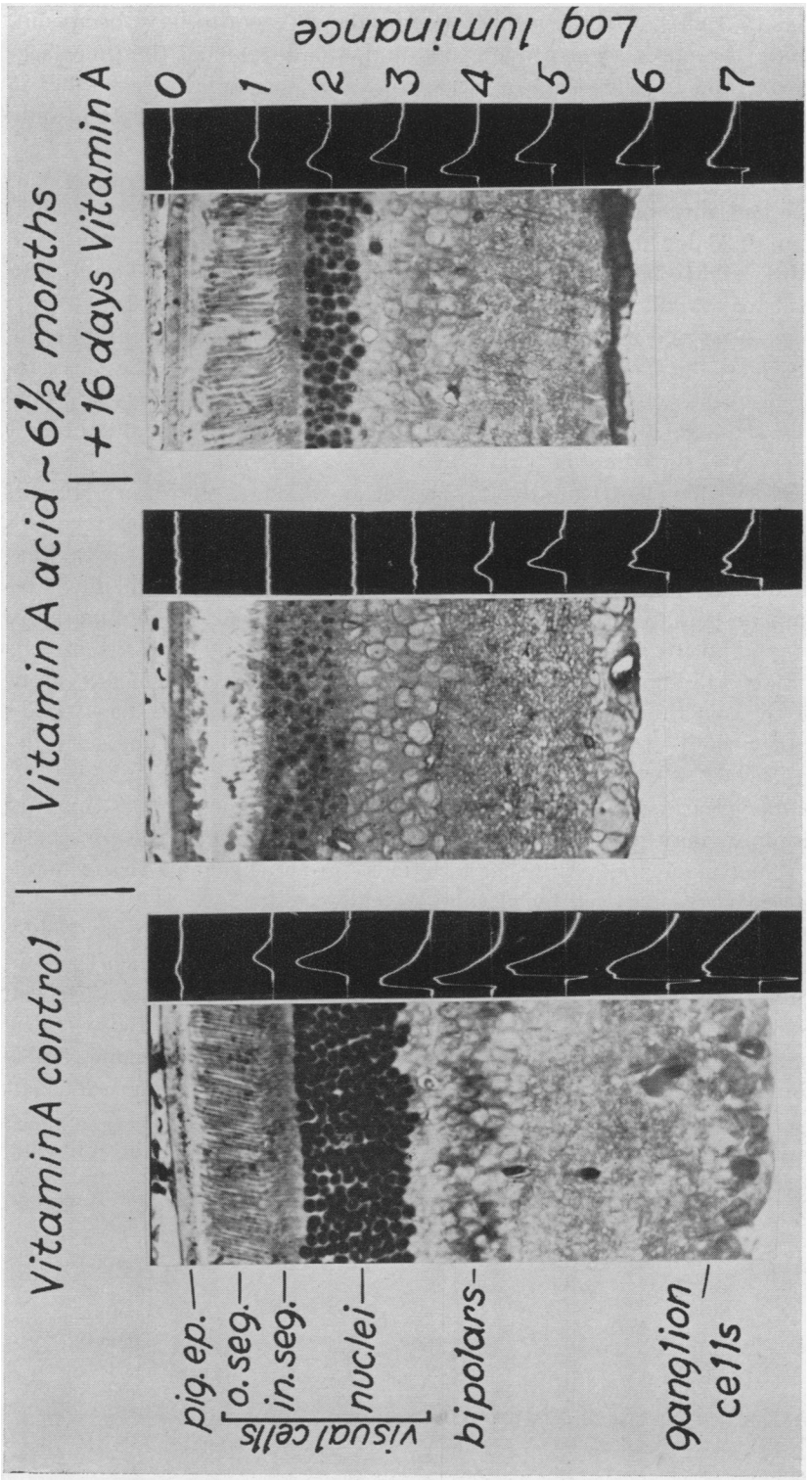


FIG. 15.—Development of and recovery from night-blindness. Three litter mates had been kept for 6½ months on a vitamin A-deficient diet, supplemented in one with vitamin A to serve as control, and in the others with vitamin A acid. The ERG responses and retinal histology of the control animal are shown at the left. At this time the ERG's of one of the vitamin A acid animals were also measured; the other one was killed and its retinas sectioned (middle). The ERG's show a high degree of night-blindness, and the retina the almost complete loss of outer segments and reduction of the number of visual cells to about 4/9 the normal population (compare Figs. 1 and 2). The surviving vitamin A acid animal was now given vitamin A supplementation for 16 days. Then its ERG's were re-measured, and its retinas sectioned immediately afterward (right). The rods are still reduced to about 4/9 the normal number, but seem to have recovered their normal structures. The visual threshold has returned to about 0.25 log unit above the normal level; but the ERG's at higher luminances are much reduced in amplitude compared with the control. This small elevation of visual threshold above normal and decrease in the size of the ERG are apparently permanent effects, owing to the reduction in number and density of visual cells.

afterward (Fig. 15, right). In the retina, the visual cells seem to have been completely repaired: the outer segments are long and well-developed, the inner segments are shorter and broader than in a control retina, but otherwise normal in appearance. The visual cells, however, had not increased in number, and only about $^4/_9$ the normal population remained.

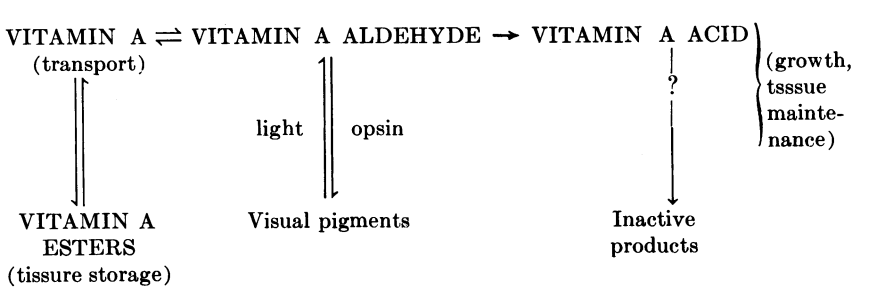
Under these circumstances the electroretinograms present an interesting condition. As we had anticipated, the visual threshold, though again nearly normal, remained about 0.25 log unit above that of the control litter mate—about the elevation of threshold to be expected with this reduction in number of visual cells. At higher levels of stimulation, the electroretinograms displayed a further effect: though once again normal in shape, they were considerably reduced in amplitude, presumably because the ERG's in such an animal are generated by a greatly reduced density of visual cells. Probably one could mimic all these effects in a normal animal by reducing the area of the visual field, and hence the numbers of visual cells affected by the stimulus.

An animal kept as long as 10 months on the diet, in which, as shown in Figure 8, even our brightest stimuli fail to evoke an ERG, displays no perceptible recovery even after months of high supplementation with vitamin A. Such an animal may be permanently blind. Yet it still possesses visual cells, though much reduced in number—perhaps still such a row of visual cell nuclei as in Figure 13. Why then does it fail to recover measurable visual responses? Such visual cells as shown in Figure 13 apparently can no longer regenerate outer segments, probably because by this time they have lost the flagellar structures—basal body and rudimentary shaft—from which the outer segments are embryonically derived.[15]

Discussion and Conclusion.—It appears from these experiments that the only function vitamin A may perform directly in the rat is to supply the prosthetic group of its visual pigments. All other functions—growth, general tissue maintenance—are served equally well by vitamin A acid.

The rat, however, seems unable to reduce the acid to the aldehyde or alcohol, as shown by the observation that no matter how high the level at which the acid is fed, no vitamin A is deposited in the liver, nor is any visual pigment synthesized. For this reason animals kept on a vitamin A-deficient diet supplemented with the acid, though they grow normally and remain in excellent health, become night-blind, indeed more night-blind than they survive to become on a vitamin A-deficient diet alone.

The general metabolism of vitamin A seems therefore to involve the following relationships (cf. ref. 9):



Vitamin A is commonly stored in the tissues, principally in the liver, as esters. It is transported in the blood mainly as the free alcohol.[16] The equilibrium of the alcohol dehydrogenase system which oxidizes it to retinene lies far over toward reduction;[17] no appreciable oxidation to retinene occurs unless the latter is removed as fast as formed. In the retina this is accomplished through trapping of the retinene by opsin to form the visual pigments.[18] The irreversible removal of retinene through oxidation to vitamin A acid may provide a second such mechanism.

The general growth and tissue functions of vitamin A appear to be fulfilled either by vitamin A acid itself (presumably in some combined or activated form) or by some product of its further metabolism. Experiments are in progress to determine what the active principle may be. It is already clear, however, that neither vitamin A acid itself, nor any derivative of it that may possess biological activity, is stored by the animal. Shortly after feeding the acid, none of it can be found in the blood, liver, or kidney; and if active derivatives were stored, we should find that animals which had been plentifully supplied with vitamin A acid over long periods could withstand removal of this supplementation longer than they do. Our experiments show that such animals stop growing within a few days of deprivation, and develop severe symptoms of vitamin A deficiency within 1–2 weeks (cf. Fig. 5).

Vitamin A acid, however effective immediately, does not "buffer" the animal against deprivation. Having been formed irreversibly, it is apparently rapidly degraded; and it or its derivatives can exercise therefore only a transient function.[19] Ordinarily the rat must rely for vitamin A acid on the continuous oxidation of its stores of vitamin A. It is as the storable precursor of vitamin A acid that vitamin A probably fulfills its most important function.

This relationship may explain our observation that vitamin A is depleted from the liver at the same rate, whether or not the rat is supplied with vitamin A acid, as though this depletion proceeded independently of demand by the tissues. Such a condition might result automatically from the irreversible oxidation of vitamin A to the acid. A given amount—in young rats 2–2.5 μgm per day—may be oxidized to the acid in the liver or elsewhere, whether needed or not. Alternatively it is possible that the rat uses vitamin A preferentially as long as it remains available, perhaps because better equipped to transport it among the tissues, or for other reasons.

The animals maintained on vitamin A acid exhibit a sequence of changes, that seem to remain wholly restricted to the visual cells for as long as our experiments have continued: first, the decline in rhodopsin concentration, accounting completely for the rise of visual threshold; then the loss of opsin, and attendant disintegration of the outer segments of the rods; by this time also the retraction of the inner segments and irreversible decrease in number of the visual cells. Finally only a single, incomplete row of visual cell nuclei remains, devoid of inner segments. The animal is now blind, and apparently cannot recover its vision.

The retina in this final state (cf. Fig. 13) resembles closely that described in so-called "rodless" mice, a genetic condition associated probably with several distinct recessive mutations.[20, 21] In one such mutant strain (C3H), the retina develops normally until the mouse is 10 days old, outer and inner segments of the visual cells by then being clearly distinguishable. Thereafter, visual cells begin to die,

and by the age of about 25 days the retina has reached a state almost identical with that shown in Figure 13. A single row of stripped visual cell nuclei remains, the retina and pigment epithelium appearing otherwise normal, as they continue to do in the adult.[21]

The same histological pattern appears also in Johnson's description of a rat which, having been severely vitamin A-deficient, was allowed to "recover" through 12 weeks of vitamin A supplementation (cf. Johnson's Fig. 2D[22]). Again a single row of visual cell nuclei remains, devoid of inner or outer segments, in an otherwise relatively normal retina.

This condition also is characteristic of the human disease, *retinitis pigmentosa.*[23] Here again, though not invariably, the visual cell layer may come to contain finally a single row of nuclei, lacking inner and outer segments, though the bipolar and ganglion cell layers appear to remain intact. It has been suggested that this disease may involve some form of localized vitamin A deficiency.[23] One difficulty with this notion, however, is that in true vitamin A deficiency, degenerative changes are not confined to the visual cells, but may involve eventually all the retinal layers.[22, 24] One can, however, as the present experiments show, induce retinal changes closely comparable with those of *retinitis pigmentosa* by keeping animals on a vitamin A-deficient diet supplemented with vitamin A acid. The invasion of the retina by pigment is a secondary aspect of this disease, not to be looked for of course in our albino animals.

A characteristic feature of human *retinitis pigmentosa* is that the rods deteriorate before the cones.[23, 25] We have reason to anticipate the same sequence in vitamin A deficiency, whether or not supplemented with vitamin A acid. Cone visual pigments are synthesized very much more rapidly than rod pigments,[11] so that when vitamin A is in short supply, the cones may capture all they require, while the rods go hungry. (Cf. ref. 25). We have not yet succeeded in testing this point adequately, a difficult business in any case in the rat, in which cone vision seems to play only a minor role.

We have suggested mechanisms for the rise of visual threshold and the loss of rod outer segments in animals kept on the vitamin A-deficient diet supplemented with vitamin A acid. Why, however, when their other tissues remain intact, do such animals lose entire visual cells? This process seems to bear some relationship to certain types of traumatic degeneration known to occur in the retina and brain.[26] So, for example, when mammalian optic nerve fibers are cut, not only do their cells of origin, the retinal ganglion cells, degenerate ("retrograde degeneration"), but so also do cells of the lateral geniculate nucleus with which they make synaptic connection ("trans-synaptic degeneration"). Injury commonly has the effect of causing the degeneration of cells in the central nervous system; and it has been suggested that the subsequent degeneration of other cells with which they communicate may be caused by lack of excitation. Either explanation might be applied to the visual cells. They may go because the disintegration of their outer segments constitutes an "injury", even though this does not seem to deprive them of any vital part. On the other hand, the loss of their outer segments does deprive these cells of stimulation, and this may be a more powerful factor in their decay.

The loss of visual cells, so far as our experiments have gone, ends with the tissue

reduced to a single layer, in which the cells have lost all the special structures that marked them formerly as rods and cones; a condition shared, as already noted, with genetically "rodless" mice, and certain instances of human *retinitis pigmentosa*. At this point what remains of the layer of visual cells has the appearance of a simple, cuboidal epithelium (cf. Fig. 13). It faces another such simple epithelium, the pigment epithelium. These two tissues have the same embryonic origin, arising as adjoining patches lining the inner surface of the primary optic vesicle, composed of such potentially ciliated ependymal cells as ordinarily line the ventricles of the brain. Having come far apart in their subsequent development, they have been brought by the regression of the visual cells to resemble each other again to a remarkable degree.

Summary.—The general tissue functions of vitamin A that support growth and maintenance in the rat are served also by vitamin A acid; but since this substance is not reduced, it forms neither the alcohol, the form in which vitamin A is stored, nor the aldehyde (retinene) needed for the synthesis of visual pigments. For this reason, rats maintained on vitamin A acid, though growing normally and otherwise in good condition, become extremely night-blind, and eventually blind. The failure to form visual pigments also has specific anatomical consequences: the outer segments of the visual cells deteriorate, followed by the loss of almost all the cells themselves, in an otherwise normal retina. These anatomical changes resemble those observed in certain hereditary forms of blindness and in human *retinitis pigmentosa*.

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We should also like to thank Dr. Aaron Ladman of the Harvard Medical School for preparing the retinal sections shown in Fig. 2.

[1] Wald, G., *Amer. J. Ophth.*, **40**, 18 (1955); *Exp. Cell Research*, Suppl. **5**, 389 (1958); Wald, G. and P. K. Brown, *Nature*, **177**, 174 (1956); Wald, G., P. K. Brown, and P. H. Smith, *J. Gen. Physiol.*, **38**, 623 (1954–1955).

[2] Dowling, J. E., and G. Wald, these *Proceedings*, **44**, 648 (1958); *Ann. N. Y. Acad. Sci.*, **74**, 256 (1958).

[3] Hubbard, R., *J. Gen. Physiol.*, **42**, 259 (1958–1959).

[4] Radding, C. M., and G. Wald, *J. Gen. Physiol.*, **39**, 923 (1955–1956).

[5] Arens, J. F., and D. A. Van Dorp, *Nature*, **57**, 190 (1946); Van Dorp, D. A., and J. F. Arens, *Rec. Trav. chim. Pays-Bas*, **65**, 338 (1946).

[6] Van Dorp, D. A., and J. F. Arens, *Nature*, **158**, 60 (1946).

[7] Sharman, I. M., *Brit. J. Nutr.*, **3**, viii (1949).

[8] Arens, J. F. and D. A. Van Dorp, *Nature*, **158**, 622 (1946).

[9] Moore, T., in *Symposium on Nutrition*, ed. R. M. Herriott, Johns Hopkins Press, (Baltimore: 1953), p. 28. Dr. Moore's words were: "Perhaps I may be allowed to suggest to Dr. Wald that it would be very interesting to examine the effect of vitamin A acid, if any, in curing defective dark adaptation in animals deficient in vitamin A." He had already explained that he thought it probably would have no effect. See also the discussion of these relationships in Moore's book, *Vitamin A* (Amsterdam: Elsevier Publ. Co., 1957), pp. 282–283.

[10] Dowling, J. E., unpublished experiments, cited in Dowling, J. E., and G. Wald, *Symposium in Vitamin Metabolism, Proc. IV Internat. Congress, Biochemistry, Vienna, 1958* (London: Pergamon Press, 1959); also in Wald, G., in *The Squibb Centennial Lectures* (New York: G. P. Putnam's Sons, 1959), p. 49.

[11] The first such measurements were performed in normal and vitamin A-deficient rats by K. Tansley, *J. Physiol.*, **71**, 442 (1931). She assumed, as the simplest relation, that the sensitivity (1/threshold) is proportional to the concentration of rhodopsin. We find the *logarithm* of the sensitivity to exhibit this proportionality. Compare also ophthalmoscopic measurements of the bleaching and regeneration of rhodopsin in the rat retina during light and dark adaptation, by Lewis, D. M., *J. Physiol.*, **136**; 615, 624 (1957).

[12] Wald, G., P. K. Brown, and P. H. Smith, *J. Gen. Physiol.*, **38**, 623 (1954–1955).

[13] Dowling, J. E., and I. R. Gibbons, in *Symposium on Eye Structure, Proc. Internat. Congress Anatomists, New York, April 11–16, 1960* (to be published by Academic Press, New York).

[14] Hecht, S., and J. Mandelbaum, *Science*, **88**, 219 (1938); *J. Am. Med. Assoc.*, **112**, 1910 (1939); *Amer. J. Physiol.*, **130**, 651 (1940); McDonald, R., and F. H. Adler, *Arch. Ophth.*, **24**, 447 (1940).

[15] de Robertis, E., *J. Biophys. Biochem. Cytol.*, **2**, 319 (1956); 2 (Suppl.), 209 (1956).

[16] Glover, J., T. W. Goodwin, and R. A. Morton, *Biochem. J.*, **41**, 97 (1947); Krinsky, N. I., D. G. Cornwell, and J. L. Oncley, *Arch. Biochem. Biophys.*, **73**, 233 (1958).

[17] Bliss, A. F., *Arch. Biochem. Biophys.*, **31**, 197 (1951).

[18] Wald, G., and R. Hubbard, these PROCEEDINGS, **36**, 92 (1950). The enzyme, retinene reductase, mentioned in this paper appears to be identical with liver alcohol dehydrogenase [Bliss, A. F., *Biol. Bull.*, **97**, 221 (1949)].

[19] The degradation of vitamin A in the rat has recently been examined (Wolf, G., S. G. Kahn, and B. C. Johnson, *J. Amer. Chem. Soc.*, **79**, 1208 (1957). Large amounts of vitamin A (3–4 mgm) labeled with penultimate C^{14}, were injected intraperitoneally. Surprisingly large fractions were excreted in the form of water-soluble, apparently aliphatic, compounds in the urine.

[20] Keeler, C. E., these PROCEEDINGS, **12**, 255 (1926); *J. Exp. Zool.*, **46**, 355 (1937).

[21] Noell, W., *Arch. Ophthal.*, **60**, 725 (1958); *Ann. N. Y. Acad. Sci.*, **74**, 355 (1958).

[22] Johnson, M., *Arch. Ophthal.*, **29**, 793 (1943).

[23] Cogan, D. G., *Trans. Amer. Acad. Ophthal. and Otolar.*, July-August, 1950, p. 629

[24] Tansley, K., *Proc. Roy. Soc. London*, **114**, 79 (1933); Johnson, M. L., *J. Exp. Zool.*, **81**, 67 (1939).

[25] Zeavin, B. H., and G. Wald, *Amer. J. Ophthal.*, **42**, 253 (1956).

[26] Cf. discussion of axonal (retrograde) and transneuronal degeneration in Polyak, S. L., *The Retina* (University of Chicago Press, 1941), pp. 68–72.

EVIDENCE FOR A VICINAL DITHIOL IN OXIDATIVE PHOSPHORYLATION

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The phosphorylation associated with electron transport in the respiratory chain can be uncoupled by physical damage to the structure of mitochondria or by a variety of special compounds. Several investigators have studied the mechanism of action of these compounds, 2,4-dinitrophenol (DNP) in particular, in an attempt to elucidate the coupling process. One more compound was added to the