

The Discovery of the Visual Function of Vitamin A

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Night blindness, a condition in which a person cannot see in dim light, has been known since ancient Egyptian times (1), although this has recently been disputed (2). Certainly, Hippocrates, who lived 460–325 BC (3) recognized night blindness and recommended eating raw liver as a cure, thus establishing a link between night blindness and nutrition. Many physicians and scientists since have observed that night blindness often accompanies malnutrition.

Another eye disease, presenting dryness of the cornea and conjunctiva (xerophthalmia) and, in severe cases, ulceration of the cornea (keratomalacia), was described as being caused by “defective nutriment” (4). It had been known as “hikan” in Japan since antiquity (5). It remained for Bitot (6) to conclude that night blindness and xerophthalmia were manifestations of the same condition. Mori (5) discovered that both could be cured by cod-liver oil. It was not until 1917, that Bloch (7) carried out a nutritional experiment with malnourished children and realized that both xerophthalmia and night blindness could be reversed by a diet including whole milk or butter. One essential nutritional factor in these foods had been discovered by the experimental work of Osborne and Mendel (8) and of McCollum and Davis (9), who named it “fat-soluble accessory factor” (10), later called “fat-soluble vitamin A” (11). Meanwhile, discoveries were made, quite independently of the clinical observations of night blindness, that ultimately led to a biochemical explanation of the condition.

The anatomical structure of the retina of the eye has been known since the microscopist van Leeuwenhoek in 1742 described the rods and cones (12), which are in close contact with the retinal pigment epithelium (RPE), backed by the black choroid. The pigmentation of the rods was first observed by H. Müller (13) in 1851 as being red, and thought to be probably due to hemoglobin. It was Franz Boll (14) who described the red pigment as unique to the rods of the retina. Marmor and Martin (15), in an article on the occasion of the 100th anniversary of the discovery of the visual cycle, commented that even today “few ophthalmologists have seen the vivid color of living unbleached retina, as the living fundus is dominated by hemoglobin and melanin.”

Working with frogs, Boll found that the red color of the retina faded to yellow within 20 seconds when the retina was removed from the eyes and appeared colorless after 60 seconds. At first he thought the bleaching of the pigment was due to the death of the animal. He observed that frogs kept in darkness showed the red pigmentation of the retinas, whereas those exposed to sunlight had colorless retinas, both in vivo and with isolated retinas in vitro. He came to the crucial conclusion that it was light that had caused the bleaching of the red pigment. In a modest and engagingly written paper

(14), he says that this was so obvious that he cannot claim any particular merit for hitting upon this thought. If frogs were first exposed to sunlight and then kept 2 h in darkness, their retinas had regenerated their red pigment. Frogs that had been killed by decapitation after being kept in the dark retained their red retinas for 24 h, provided the heads were kept in the dark, thus showing that light and not the death of the animal caused bleaching. By selective exposure to light, he found a bleached portion of the retina corresponding to those parts of the retina exposed to light.

Boll wrote (14): “The basic color of the retina is constantly consumed in vivo by the light falling on the eye. Longer action of sunlight bleaches the retina completely. In the dark, in vivo, the color is regenerated. This change in the outer segments of the rods forms indisputably a part of the process of vision.” He suggested that the outer segments of the rods contained a special substance that, by a photochemical process, conveyed the impression of light to the brain. He was not, however, able to extract and isolate the red substance with solvents. Using anesthetized frogs exposed to pure spectral colors generated by a prism, he showed that the red color of the retinas was maintained in red light, faded in yellow light and faded quickly in green light.

Marmor and Martin (15) briefly describe Boll’s tragic life story. He worked as an assistant to the physiologist Dubois-Reymond in Berlin, but in 1873 had to move to Italy because ill health forced him to live in a warmer climate. He was appointed to the chair of physiology at the university in Rome in 1877, but because his health worsened, he had to give up his experiments in 1878. He died in Davos, Switzerland, presumably of tuberculosis, in 1879, at age 30.

Within 2 mo of the publication of Boll’s first paper in 1877 (14), Willy Kühne, professor of physiology at the university of Heidelberg and a powerful figure in the field of physiology, took up Boll’s discoveries (duly acknowledged) with “fiery zeal” (16). He elaborated the biochemistry of vision to a point at which it was not further expanded until the 1920s. “Professor Kühne was tall and portly, with a commanding presence, a magnificent large head and bright eyes” (16) (Fig. 1).

Kühne was well known for his work on the physiology of muscle and nerve, in particular the transmittance of the nerve impulse to muscle. He contributed extensively to protein chemistry and to the biochemistry of the digestion of proteins. He was the first to isolate the protease from pancreas and gave it the name “trypsin” (cleaver) (17), and was the first to use the term “enzyme.” He was author of a famous *Lehrbuch der physiologischen Chemie* (Textbook of Physiological Chemistry), much in demand at that time by physicians and students. It was so well written that it was said to read like a novel (16).



FIGURE 1 Willy Kühne (1837–1900). Reprinted from Ref. 20 with permission from Elsevier Science.

The prodigious experimental work of Kühne on the retina, begun in 1877, is contained in 22 articles published in *Untersuchungen aus dem physiologischen Institute der Universität Heidelberg* from 1878 to 1882 (18). A summary appeared in the *Handbuch der Physiologie* (19). A superb translation into English of this paper was published by Ruth Hubbard (20), together with a commentary on Boll's work. A biography of Kühne appeared on the 100th anniversary of the discovery of the visual cycle (17).

Kühne first corrected Boll's impression that the rod pigment was red; he considered it to be purple and called it visual purple (rhodopsin). Experimenting with the frog as Boll had done, he confirmed that the rod pigment was bleached by light, but that it maintained its color in the dark, even after the death of the animal. Working with the simplest equipment in a darkroom, illuminated by red light, Kühne developed a method for isolating frog retinas, i.e., the retinas together with the RPE were dissected out and hardened by immersion in alum solution; the intact retinas were then lifted off the RPE. The retinas thus isolated could then be maintained intact for long periods.

Kühne found that the purple color was located in the outer segments of the rods and that the inner segments contained protoplasm and nuclei. A key discovery was that bile (or bile salts) dissolved the rods and brought rhodopsin into solution. He vividly described how the outer segments of rods "explode like rockets full of coins" when treated with bile. He described the rods as surrounded by a protein sheath, and observed that they contained "platelets" (now known as "disks") in an interstitial substance.

The dissected retinas remained purple in the dark and became colorless when illuminated by sunlight. This bleaching process took place in distinct stages as follows: first, to orange (a mixture of yellow and purple), then to yellow, then to buff and finally to colorless. Kühne realized that the yellow color must be due to a substance differing from rhodopsin, because it

showed greater light absorption at a shorter wavelength compared with rhodopsin. The colorless substance derived from the yellow must again be different because, using a quartz prism and sunlight, Kühne observed a blue fluorescence of the dark-adapted rods in ultraviolet light, changing to green fluorescence of the bleached (colorless) rods. We now know that these were correct conclusions, i.e., the 11-*cis*-retinal combined with the protein opsin is purple with blue fluorescence; the all-*trans*-retinal-opsin is yellow, whereas the free all-*trans*-retinol, the end product of the bleaching process, is colorless with green fluorescence.

Kühne studied the rate of bleaching and found it to be dependent on the intensity of the light, on temperature and on the wavelength of the light. He determined both the action spectrum of bleaching and the absorption spectrum of solutions of rhodopsin, using a prism spectroscope. Monochromatic light gave decreasing rates of bleaching from yellow to green to blue to violet to red. In parallel sequence, light absorption was highest in the yellow and lowest in the violet region of the spectrum. Comparing retinas spread on glass plates, he found the same absorption spectrum as for rhodopsin in solution. He commented on the fact that bleaching must be a photochemical and not a thermal reaction, because infrared light did not bleach, nor can it be seen.

Upon chemical analysis of rhodopsin, Kühne found that acetic acid turned it to yellow, which could not be further bleached by light. The color was lost above 76°C. He correctly surmised that rhodopsin must be a protein because it did not diffuse through a semipermeable membrane and could be salted out with ammonium sulfate.

When Kühne caused a frog to stare into a flame for 14 h, then isolated its retina in the darkroom, he could see a bleached area on the retina in the upside-down shape of the flame. He compared the retina to a photographic plate and found that he could imprint the shape of a window onto the retinas of rabbits or frogs after making them look at the window for no longer than 3 min. He called this image an optogram. When this phenomenon became known to the general public, it was suggested that perhaps the image of a murderer, who would be seen by his victim just before death, might be found imprinted on the victim's retina and thus would identify the perpetrator (16)!

Kühne had the great gift of asking the right questions and answering them by means of simple experiments, including separate experiments that served as controls, something not often done in that era. Thus, when he excised a frog's eyeball and kept it in sunlight for 30 min, the retina was bleached; when subsequently kept in the dark, the purple color reappeared, clearly independently of the blood circulation, and appearing exactly like a retina from a dark-adapted control frog. A retina alone, lifted off the RPE, could be bleached but did not regenerate the purple. Kühne's critical experiment was to take an isolated bleached retina and to lay it onto an isolated RPE: the rhodopsin was regenerated. If a piece of porcelain was placed between the retina and RPE, there was no regeneration. He concluded that the RPE was necessary for regeneration. When a bleached retina was placed on an isolated RPE <1 h after the death of the frog, rhodopsin was regenerated; when the RPE had been "dead" for longer, no regeneration occurred. We now know that 11-*cis*-retinal, the prosthetic group of rhodopsin, is formed enzymatically in the RPE from all-*trans*-retinol. Kühne remarked that bleaching was purely photochemical, but that regeneration, which ceased above 45°C, required a "ferment" (enzyme). Under constant mild illumination in vivo, there exists, he thought, an equilibrium between bleaching and regeneration of rhodop-

sin. He accurately observed that regeneration, in contrast to bleaching, does not go through the yellow stage; we now know that the yellow all-trans-retinal-opsin is formed only upon bleaching, not upon regeneration.

Kühne extracted rhodopsin from frog retinas into solution with bile salts and separately prepared an RPE extract. The rhodopsin solution alone, after bleaching, showed no regeneration, whereas a combined bleached rhodopsin-RPE solution regenerated the purple color. He concluded that there was a substance in the RPE that interacted with bleached rhodopsin to convert it back into the purple form.

Kühne observed that retinas of birds and reptiles lacked rods and therefore rhodopsin, but contained pigmented globules in their cones, which were clearly their photoreceptors. These were not, however, bleached by light. He called this process "seeing without visual purple" and postulated a second visual system pertaining to those species. Interestingly, he detected exceptionally long and intensely purple rods in the retinas of night-hunting birds such as owls. As we now know, rhodopsin of the rods, as distinct from the visual pigment of the cones, serves vision in dim light (black and white vision). Kühne could not detect rhodopsin in the cones of the fovea centralis of the human retina. We now know that cones contain retinal bound to proteins similar to, but distinct from opsin, forming light-sensitive pigments that serve color vision in strong light. Kühne was able to detect the electrical impulses emitted by isolated retinas upon illumination and correlated them with the bleaching process.

Kühne summarized his findings as follows: 1) it is the function of rhodopsin to be decomposed by light; 2) the products of this photochemical reaction then stimulate the nerve impulse to the brain. Thus, he recognized a local, direct and unique chemical change giving rise to a nerve impulse.

Shortly after publication of the gigantic work of Kühne and his collaborators (A. Ewald, W.C. Ayres, J. Steiner), Parinaud (21) proposed that night blindness was caused by excessive

illumination that caused decomposition of rhodopsin. This would make the rods less sensitive to light, whereas the cones of the fovea conserved their vision. He wrote: "There are two kinds of ocular sensitivities to light. The first gives us a sensation in diffuse light, independent of color. It is due to the rods, depending on visual purple. The second, which is that of the cones, gives us delicate differences of color and illumination."

In light of the work of Mori (5) and Bloch (7) that in humans a deficiency of a lipid dietary factor present in milk and butter was the cause of xerophthalmia and night blindness, and building on the results of McCollum and Davis (9) that a dietary deficiency of a "fat-soluble vitamin A" produced xerophthalmia in rats, Fridericia and Holm (22) investigated the influence of dietary vitamin A on the rhodopsin of the retina. They gave one group of rats a diet with butter fat, another group a diet with lard. The latter developed signs of xerophthalmia within 4-7 wk. Both groups were kept in bright light for several days, to bleach their rhodopsin. They were then placed in darkness. The eyes of the anesthetized rats were removed sequentially over 3 h and the regeneration of rhodopsin measured by comparing the reappearance of the purple color of the isolated retinas on an arbitrary scale. The investigators found that the lard-fed rats showed a regeneration rate about one third that of those fed butter. Clearly, the rats lacking the "fat-soluble vitamin A" had a "defect in the function of visual purple."

Yudkin (23) achieved one of the earliest identifications of vitamin A as a component of the retina. He isolated retinas together with RPE from 100 pigs' eyes obtained from the abattoir, carefully separated them from the choroid membrane and freeze-dried them in vacuo. He made seven rats vitamin A-deficient by feeding them a lard-based diet until they developed xerophthalmia and lost weight. Five control rats were fed a supplement of cod-liver oil and grew normally. Three deficient rats served as negative controls. When four of the

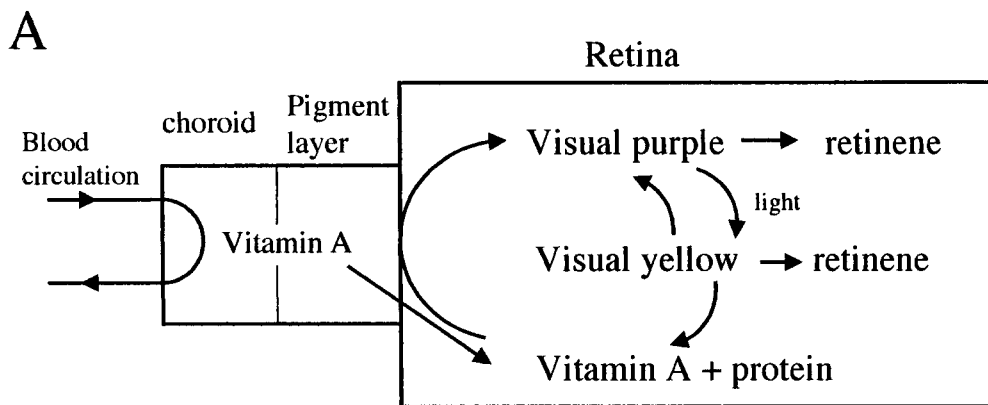
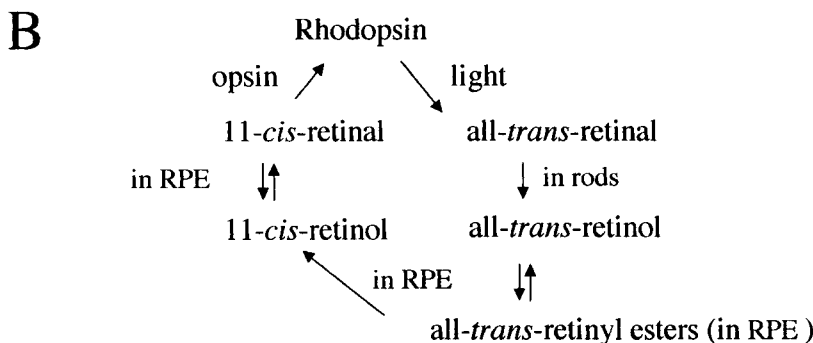


FIGURE 2 (A) Diagram of the visual cycle adapted from Wald (27). Retinene, now known as retinal; visual purple, also known as rhodopsin, is opsin combined with 11-cis-retinal; visual yellow is opsin combined with all-trans-retinal. Wald comments that the left-hand curved arrow, representing the reaction of vitamin A + protein to visual purple, "occurs only in an eye in which the relation of the retina to the pigment epithelium has remained undisturbed." (B) Diagram of the visual cycle as we know it today. RPE, retinal pigment epithelium.



deficient rats began to lose weight, they were given 50 mg/d of the dried retinal tissue. This restored their growth rate to normal. Yudkin concluded that the retinas must have contained vitamin A. Importantly, the newly discovered color test of Rosenheim and Drummond (24) of an ether extract of dry retinas showed the violet color with arsenic or antimony trichloride characteristic of the fat-soluble accessory factor of McCollum and Davis (10). Subsequently, Wald (25) determined the amount of vitamin A present in pig retinas (including the RPE) spectrophotometrically, as 24 $\mu\text{g/g}$ dry tissue. Therefore, the dry tissue Yudkin gave the rats daily must have contained $\sim 1.2 \mu\text{g}$ vitamin A, an amount certainly sufficient to reverse vitamin A deficiency (26).

G. Wald (27), who in 1933 had identified vitamin A (retinol) spectroscopically in intact retinas of frogs, sheep and cattle, established the complete visual cycle in a grand sweep of breathtaking scope and originality, principally by spectroscopic analysis. He determined that rhodopsin consisted of "retinene" (now known as retinal), bound to a protein (later called opsin) as a prosthetic group. He showed that light decomposed rhodopsin to retinal and opsin. Retinal could either recombine with opsin to reform rhodopsin or it could be converted to free retinol. Retinol could reform rhodopsin, but only, as Kühne had found, in the presence of the RPE. Wald argued that "vitamin A is the precursor of visual purple [rhodopsin] as well as the product of its decomposition; the visual processes therefore constitute a cycle" (Fig. 2).

Wald recognized that retinol was derived from carotenoids and that "the visual system expends vitamin A and is dependent upon diet for its replacement." Many details have been filled in since, but in outline, the visual cycle is still that for which Wald was rewarded with the Nobel prize in 1964.

The discovery of vitamin A's visual function presents a good example in the history of science of the step-wise nature of discovery. The story started with the crucial observation that light bleaches the color of the retina. The properties of this process in relation to vision were then exhaustively explored. The connection was made to dietary vitamin A deficiency and night blindness; vitamin A was found to be present in the retina. The final step was the discovery of the involvement of vitamin A in a cyclic visual process and its explication at the biochemical and finally the molecular level.

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