The color purple: milestones in photochemistry

Harris Ripps[1]

Department of Ophthalmology and Visual Sciences, University of Illinois College of Medicine, Chicago, Illinois, USA, and The Marine Biological Laboratory, Woods Hole, Massachusetts, USA

Earlier this year, the Illinois Eye and Ear Infirmary, a venerable institution that was later incorporated into the University of Illinois at Chicago College of Medicine, celebrated its 150th anniversary. Asked to deliver a lecture to the distinguished group of physicians and scientists that had assembled to celebrate this historic event, I was struck by the fact that in 1858, when the Infirmary opened its doors to the public, absolutely nothing was known about any aspect of the visual process. There were, to be sure, significant advances in ocular surgery, a growing number of medical treatments for eye disease, and vastly improved recognition of retinal disorders due in no small measure to the arrival two years earlier of Helmholtz's ophthalmoscope. But knowledge of how the visual signal was generated had not progressed much further than that shown in Descartes' fanciful cartoon (**Fig. 1**).

The situation changed dramatically some twenty years later, when Franz Böll, a German physiologist working in Rome, removed the retina from the eye of a dark-adapted frog, and reported the color changes he observed as the tissue was exposed to light. Böll had witnessed the light-

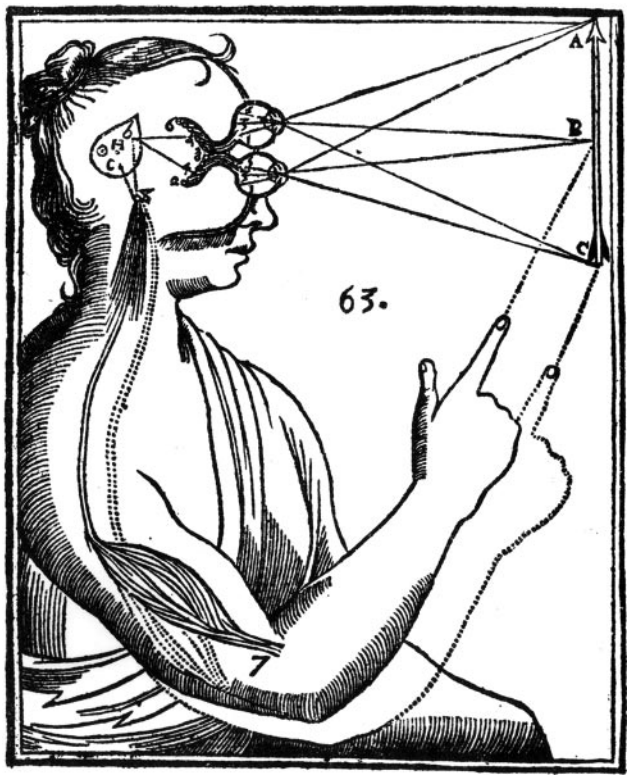

Figure 1. The Descartes cartoon (circa 1644) depicts the formation of an inverted image of the outside world on the retina. The image is somehow transmitted to the pineal, which in turn sends a message to the peripheral musculature to react. Image courtesy Art Resource.

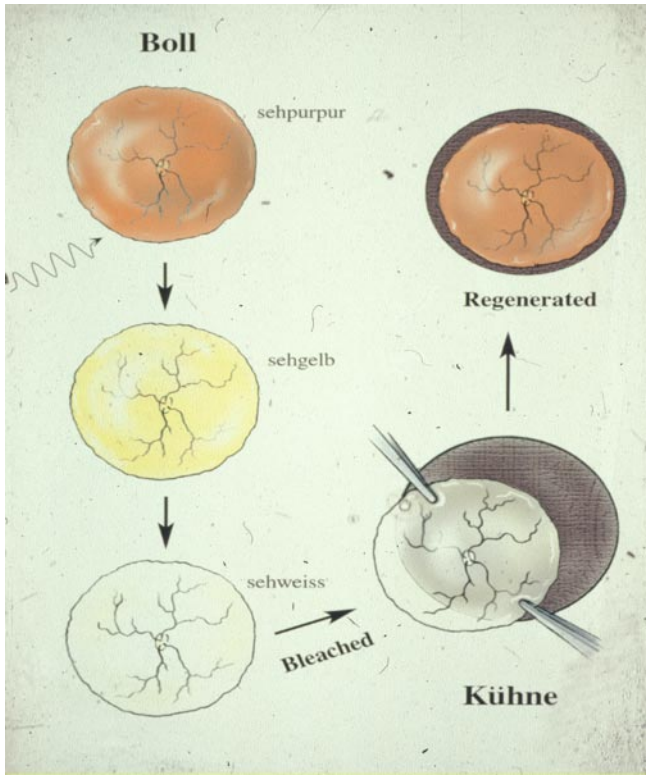

Figure 2. Bleaching and regeneration of rhodopsin in the isolated frog retina; the observations of Böll (top to bottom) and Kühne (bottom to top).

[1] Correspondence: Department of Ophthalmology and Visual Sciences, University of Illinois College of Medicine, 1855 W. Taylor Street M/C 648, Chicago, IL 60612, USA; E-mail: harrripp@uic.edu

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induced bleaching of a reddish purple substance (later called *sehpurpur* or visual purple) to the colorless *sehweiss* (visual white), having passed transiently through yellowish *sehgelb* (**Fig. 2**). Histological examination of flat mounts convinced Böll that these changes were confined to the rods of the bacillary (photoreceptor) layer of the retina, and that after bleaching, the red color returned-when the eye of a *living* animal was kept in darkness. The paper he delivered in 1876 to the Berlin Academy describing these phenomena (1, 2) marked the beginnings of retinal photochemistry.

The significance of this landmark discovery did not escape the attention of Friederich Wilhelm (Willy) Kühne, Professor of Physiology at the University of Heidelberg, whose expertise in physiological chemistry, and the availability of superior resources, enabled him to isolate the substance underlying these chromatic changes. To the insoluble protein he had extracted with bile salts he gave the name "rhodopsin;" it was the first membrane protein to be brought into solution. And by demonstrating that the absorption spectrum of rhodopsin paralleled the spectral sensitivity of the dark-adapted retina, Kühne provided incontrovertible evidence that this substance was the photosensitive pigment subserving rod vision (3–5). Moreover, Kühne showed that the original color of the retina could be restored in darkness by simply replacing the "bleached" tissue on the surface of the retinal pigment epithelium (RPE). Thus, although nothing was known of the chemical reactions involved, or the nature of the exchange that was taking place between the RPE and the photoreceptors, there was already an indication that the bleaching and regeneration of rhodopsin was a cyclical process. In the opening paragraphs of his lengthy monograph on "The Photochemistry of the Retina and on Visual Purple," Kühne wrote:

> *In a recent communication to the Berlin Academy, Herr Fr. Böll announced the beautiful, and beyond doubt, important discovery, that the bacillary layer of the retina of all animals is in the living condition not colorless, as has been hitherto supposed, but of a purple red color. During life, says Böll, the proper color of the retina of all animals is continually being destroyed by the light which falls into the eye; it is restored in the dark, and after death only remains a few moments.*

Kühne goes on to state:

> *Whoever has busied himself with the retina will be reminded by Böll's discovery (and thereby receive a wholesome admonition of the limits of his own ability), that he has already seen something of the kind before. He will perhaps remember that puzzling blood clot—which at one moment he saw under the retina, and which the next moment disappeared. What he then passed over so lightly was nothing less than the key of the secret, how a nerve can be excited by light. In other words, it was the first fact disclosing the existence of photo-chemical processes in the retina* (5).

Despite this clear statement of attribution, a controversy, not unlike many that occur today, arose regarding priorities. It originated neither with Böll nor Kühne, who apparently bore no malice toward one another, but rather with a scathing note written by Dr. E. Warlomont, editor of the journal Annales d'Oculistique:

> *All of the discovery of the proper color of the retina belongs to Professor Böll, with all of the consequences that Kuhne seems to be taking prematurely for himself. Boll had evidently foreseen all of these consequences and it seems to us that it would have been in good taste to leave him the time to unravel them at his leisure* (6).

Not content with such churlish comments, he added further insult with this allegorical tale:

> *Two children walked along a path. The first whistled a tune, but when he was only halfway through, the second took it over. "Another time," said the first with some annoyance, you would do well to begin your own."*

This unwarranted attack angered Kühne as well as his students, one of whom wrote that Warlomont's statements are:

> *as much opposed to truth as they are to the interests of science, or as they are repugnant to good taste. When a scientific man has published a discovery it is to the interest of the scientific world that all who will or can should be at liberty to repeat the experiments or observations which led to it.*

Kühne also felt obliged to respond:

> *Since Herr W. apparently skipped reading the originals, some points escaped him. First, his complaints could not be more misguided than to be directed towards me, since I gave material credit to Herr Böll and as much as anyone can give. . . . If now Herr W. is of the opinion that everything also could have been found by Böll, he will find to his surprise that Böll worked "at his leisure" for at least 4 months and overlooked what I found in 4 days.*

There is little more to add to events of the late 19th century. Although the foundation of visual photochemistry had been laid, more than 50 years elapsed before George Wald undertook the formidable task of identifying the substances that were responsible for these chromatic changes. In the course of this research, Wald, together with his wife, Ruth Hubbard, and a cadre of outstanding students and co-workers, produced a remarkable series of papers elucidating the role of vitamin A in vision, and the physico-chemical processes that propel the "rhodopsin cycle"[2] (7–9). Among their many discoveries, perhaps the most startling was the revelation that rhodopsin consists of the (thermodynamically unlikely, but remarkably stable) bent and twisted form of the aldehyde of vitamin A (11-*cis* retinaldehyde) covalently bound to a the protein opsin via a protonated Schiff's base linkage (10), and that the sole action of light in vision is to instantaneously unbend and untwist the chromophore into the all-trans configuration of retinal (**Fig. 3**).

[2] The "rhodopsin cycle" is, with some justification, frequently referred to as the "visual cycle." However, it is not vision that is being recycled. Indeed, the bleaching/regeneration of rhodopsin proceeds at normal rates despite total visual loss through optic nerve damage or cranial injury, as well as in various forms of congenital night blindness (16).

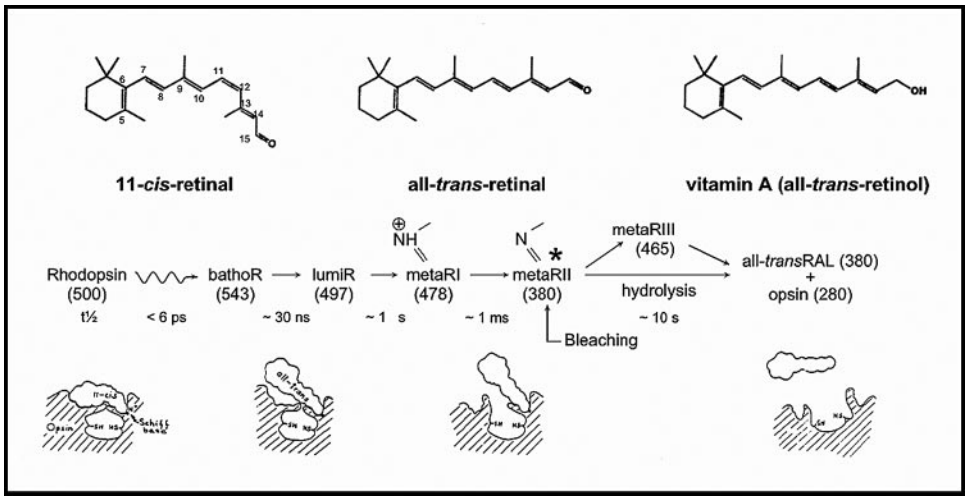


Figure 3. The molecular forms of vitamin A are shown in the upper part of the figure, and immediately below, a schematic illustration of some of the key intermediates that form after photic exposure as rhodopsin, held in liquid nitrogen, is exposed to progressively warmer temperatures. Numbers in parentheses indicate the λ_{max} of each intermediate's absorption spectrum. The lower set of drawings show Wald's concept of the molecular changes in rhodopsin as 11-cis retinal is isomerized to its all-trans configuration, the Schiff's base linkage to opsin is disrupted, and the chromphore is released from its binding site to be reduced ultimately to vitamin A (all-trans retinol). The asterisk at metarhodopsin II, indicates the stage that Wald correctly predicted was the active intermediate that triggers the electrical response. Image ©Nobel Foundation.

We know now that the *cis-trans* isomerization occurs in less than 200 femtoseconds (2×10^{-13} sec) following the absorption of a single quantum of photic energy. And as Wald went on to show, all subsequent events, including the formation of an active intermediary that triggers the electrical response of the visual cell, the conformational changes in opsin that free the chromophore from its binding pocket in opsin, and the reduction of all-*trans* retinal to form vitamin A (all-*trans* retinol), are essentially thermal events no longer requiring the presence of light. Soon thereafter, one of Wald's students, John Dowling, demonstrated the exchange of retinoids between the neural retina and the RPE in the course of light- and dark-adaptation (11).

It was now clear that all-*trans* retinol released on bleaching enters the RPE where it is esterified and stored, but the vitamin A congener returned to the ROS and its stereoisomeric configuration remained conjectural. In short, where and by what mechanisms are the isomerization and oxidation processes that convert all-*trans* retinol to 11-*cis* retinal taking place? These issues were resolved more recently in an elegant and highly innovative series of studies by Robert Rando and co-workers (12, 13). They convincingly demonstrated that these transformations occur conjointly in the RPE under the control of an isomerohydrolase, which processes all-*trans*-retinyl esters (mainly retinyl palmitate) directly into 11-*cis* retinol (**Fig. 4**). Indeed, the energy source used to drive the thermodynamically unfavorable isomerization is provided by the free energy of hydrolysis of the membrane phospholipids. As a result of their work, and that of many others, we now have a firmer grasp of the enzymes and biochemical reactions that enable the reconstitution of rhodopsin from the photoproducts of bleaching.

In light of such noteworthy advances in our knowledge of the visual process, it may seem surprising to learn that Study Sections of the National Eye Institute, the main source of financial support for biomedical studies, were considering curtailing much of the funding for this basic research effort. As a member of study section in the '80s, and heavily committed to the *in vivo* study of rhodopsin kinetics (14–16), I shudder to recall deliberations on the worthiness of rhodopsin research, which "quite clearly had neither *clinical relevance* nor *translational potential*." Needless to say, the ship was righted when the first report appeared of a point mutation in rhodopsin that caused autosomal dominant retinitis pigmentosa, a devastating eye disease that leads to the progressive loss of rods and cones throughout the retina (17). All that was required to produce such widespread destruction—and eventual blindness—was the transversion of a single proline residue to histidine at position 23 (P23H) in the polypeptide chain of 348 amino acids that comprise the opsin molecule. We know now that more than 200 mutations in opsin, as well as countless mutations in many of the enzymes engaged in the rhodopsin cycle, are associated with various forms of night blindness, RP, and related ocular disorders. Clearly, we would do well to heed the words of our former Surgeon General, C. Everett Koop, who cautioned: "Insufficient medical research can be hazardous to your health."

Space constraints compel me to leave for another time a description of the work that solved the basic problem in visual excitation: how the absorption of light by a visual pigment results in the generation of an electrical signal.

THE PRINCIPALS

For readers who may wish to learn a bit more about the early pioneers whose discoveries are described in the foregoing text, I have prepared a few brief notes,

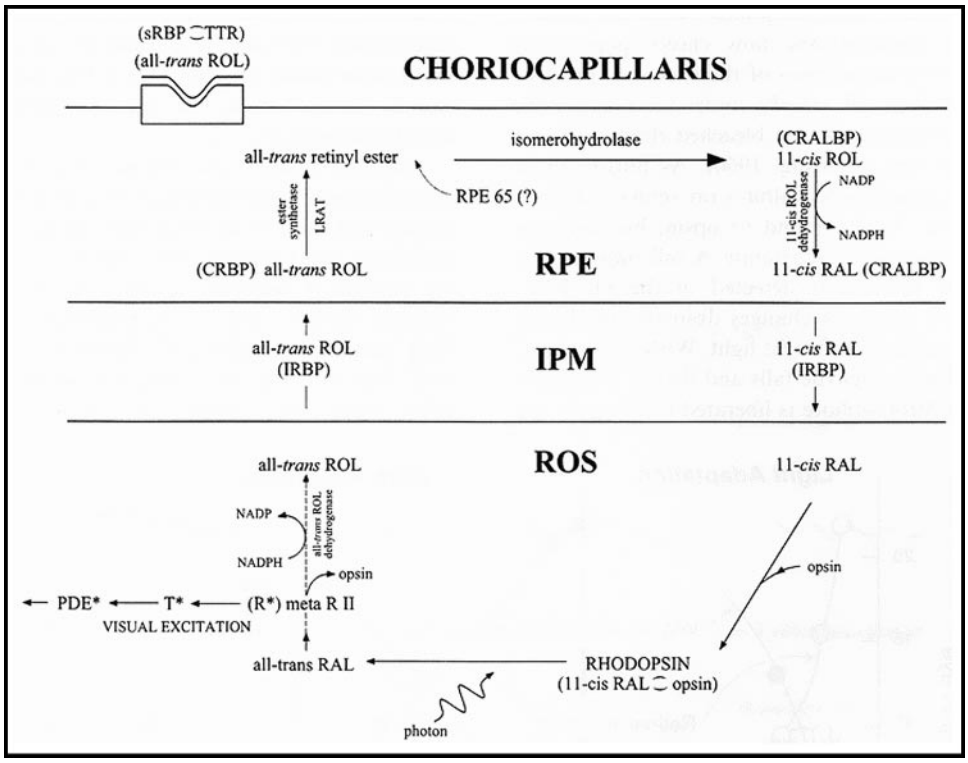


Figure 4. All-trans retinal is released from opsin after bleaching, and is then rapidly reduced to all trans-retinol (vitamin A) in the rod outer segment by one of the retinol dehydrogenases. Vitamin A is then transported to the RPE where it is esterified to long-chain fatty acids for storage. The ester provides the substrate for the subsequent isomerization and hydrolysis of all-trans retinyl ester to 11-cis retinol, which then undergoes oxidation to 11-cis retinal. In this form, the retinoid is returned to the photoreceptors, where it becomes covalently linked to its active-site lysine in opsin by means of a protonated Schiff's base to reform the rhodopsin complex and complete the "rhodopsin cycle." The retinoid binding proteins sRBP (serum retinol binding protein), IRBP (interphotoreceptor binding protein), CRBP (cellular retinol binding protein), and CRALBP cellular retinaldehyde binding protein) serve as carriers to promote the movement and delivery of the various forms of vitamin A to their intra-and extra-cellular destinations. Image courtesy Elsevier.

condensed from several more extensive biographical sketches (18–22).

Unfortunately, too little is available in English on the short life of Franz Böll. We know that he was educated in medicine at Bonn, Heidelberg, and Berlin, and there is every indication that, like many of his contemporaries, he was a Renaissance man, well versed in literature and science, and engaged in numerous cultural pursuits. He had trained in the laboratory of Emil duBois-Reymond in Berlin, and as a student of Max Schultze in Bonn, wrote important histological treatises on dental pulp and the structure of the lacrimal glands for the Journal of Microscopical Science. Difficulties finding a suitable position in Germany, together with failing health, attributable in part to time spent as a military physician caring for typhoid patients during the Franco-Prussian War, prompted Böll to move to Italy where he eventually acquired the Chair of Anatomy and Physiology at the University of Rome. It was there, at the tender age of 27, that he conducted his study of the retinal pigment, and where he died three years later, unable to pursue further his monumental discovery.

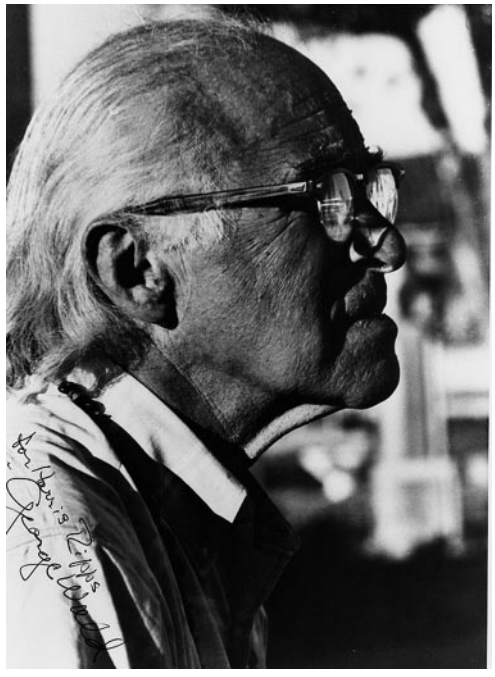
Franz Böll (1849–1879). Image courtesy Elsevier.

Willy Kühne was a productive, greatly admired, and highly successful scientist due undoubtedly to his broad range of interests, his talents as an experimentalist, and the remarkable training he received at early stages in his career. At the age of 17 he enrolled at the University of Göttingen where he acquired a fine chemical background under the tutelage of Friederich Wohler, who had in 1828 demonstrated that an organic molecule, urea, could be synthesized without the intervention of living cells. Also in Göttingen were Rudolf Wagner (physiology), Wilhelm Weber (physics), J. B. Listing (physiological optics), and the anatomist Jacob Henle,

Friederich Wilhelm Kühne (1837–1900). Image courtesy of the Clendening History of Medicine Library, University of Kansas Medical Center.

all of whom had a profound influence on the young Kühne. After completing his doctoral thesis in 1856 on artificial diabetes in frog, he moved to Jena working in the laboratory of the physiological chemist Carl Gotthelf Lehmann, and after a brief period, left for Berlin to train in electrophysiology in the laboratory of Emil du Bois-Reymond. There, under the influence of Felix-Hoppe-Seyler, he began his studies of bile, the origin of hippuric acid, and of jaundice. Work on the properties of the pancreatic juice and digestive tract led him to coin the terms "enzyme" and "trypsin," and soon thereafter, working in Paris in the laboratory of the great Claude Bernard, he published several papers on muscle physiology, including a description of the structure of the motor end plate and the propagation of the electrical impulse. A return to Berlin to work in Rudolf Virchow's laboratory gave Kühne the opportunity for further study of frog muscle from which he successfully extracted myosin from the contractile myoplasm. Following a brief unproductive stint as Professor of Physiology in Amsterdam, Kühne received a welcome invitation from Heidelberg to replace Helmholtz, who was resigning to become Professor of Physics in Berlin. Kühne worked and lived happily in Heidelberg from 1871 until his death in 1900. In his will he requested that his remains be cremated while the first movement of Beethoven's Ninth symphony was played—"and played well." A former student, Jacob von Uexküll, wrote in Kühne's obituary "Ein Herrscher ist von uns gegangen."

As John Dowling wrote in memory of his late mentor, "Biology lost one of its towering figures of the twentieth century with the passing of George Wald (22)." Like Kühne, Wald had the benefit of being trained by some of the outstanding scientists of the day, both in the US and abroad. His graduate studies at Columbia University were in the laboratory of Selig Hecht, a superb biophysicist whose work led to the discovery that absorption of a single quantum of photic energy by only one of the approximately 10^8 molecules of rhodopsin contained in a single rod was sufficient to generate and transmit an electrical signal. However, because of his keen interest in identifying the substances that produced the photochemical changes seen by Böll and Kühne, Wald wisely chose to do his post-doctoral studies in Berlin under the guidance of Otto Warburg, a leading biochemist and recipient of the Nobel Prize. Spectroscopic evidence he obtained in Warburg's laboratory that rhodopsin was a carotenoid-linked protein led to a visit with Paul Karrer in Zurich, where they confirmed the presence of vitamin A in retinal extracts from several different mammalian species. After a stay in the laboratory of another Nobel laureate, Otto Meyerhof, Wald returned to the States where, as a member of the Harvard faculty from 1934 until his death in 1997, he completed his studies on the chemical stages intervening between light absorption and the products of rhodopsin bleaching. He and Ruth spent many summers at the Marine Biological Laboratory in Woods Hole, where Wald taught in the famous physiology course, and studied the visual system of virtually every creature he could acquire, both large and small. It should be noted that Wald was a brilliant lecturer with a unique style that entranced students and the many audiences he often addressed on public affairs. For his sterling contributions to visual science, Wald received numerous honors, including the Nobel Prize in Physiology and Medicine (1967), an award he shared with two other visionaries, H. Keffer Hartline (Rock-



George Wald (1906–1997).

efeller University) and Ragnar Granit (Karolinska Institute). On that occasion, he observed: "The Nobel Prize is an honor unique in the world in having found its way into the hearts and minds of simple people everywhere. It casts a light of peace and reason upon us all; and for that I am especially grateful." FJ

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The title of this article, taken from the Pulitzer Prize winning novel by Alice Walker, was suggested by my learned friend Thoru Pederson. Since none other seemed more appropriate, I acknowledge with pleasure his thoughtfulness, and the constructive comments on an earlier draft. I am especially grateful to Michael Marmor for permission to quote extensively from the splendid translations (French and German) that appeared in his scholarly account, written in celebration of the 100th anniversary of the discovery of rhodopsin. Work in the author's laboratory is supported by a Senior Scientific Investigator Award from Research to Prevent Blindness, Inc.

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