The Chemistry of Rod Vision

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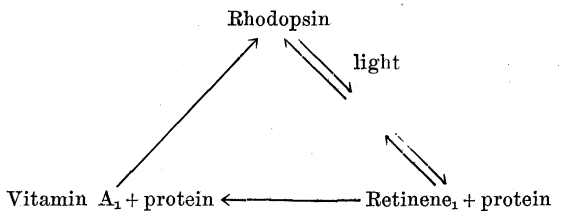
SOME YEARS AGO it was shown that rhodopsin, the red light-sensitive pigment of rod vision, takes part in a cycle of reactions of the following skeletal form (*1*):



Much of the physiology of rod vision reflects the composition of this cycle. The role of vitamin A_1 in rhodopsin synthesis is associated with the rise of night-blindness in vitamin A deficiency. The spectral sensitivity of rod vision has its source in the absorption spectrum of rhodopsin. In the light, rhodopsin bleaches to a lower steady-state concentration; the corresponding fall of visual sensitivity to a constant, depressed level is light adaptation. In the dark, rhodopsin is restored to its maximum concentration; the associated rise of visual sensitivity to a maximum is dark adaptation. Rhodopsin is synthesized in two ways—rapidly from retinene$_1$, and much more slowly from vitamin A_1; correspondingly rapid and slow modes of dark adaptation have been demonstrated in man and other animals (*2*).

Rhodopsin is found in the rods of land and marine vertebrates. In the rods of fresh-water vertebrates—lampreys, fresh-water fishes, and certain larval amphibia—it is replaced by the purple light-sensitive pigment, porphyropsin. This takes part in a cycle of precisely the same form as rhodopsin, but involving new carotenoids:

$$\text{Porphyropsin} \underset{}{\overset{\text{light}}{\rightleftharpoons}} \text{Retinene}_2 + \text{protein} \longrightarrow$$
$$\text{Vitamin } A_2 + \text{protein} \longrightarrow \text{Porphyropsin}$$ (*3*, *4*).

Recent work with these substances and processes has brought the chemistry of rod vision to a new level. I should like briefly to summarize this development.

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Rhodopsin and Porphyropsin

Rhodopsin and porphyropsin are carotenoid-proteins—proteins bearing carotenoid prosthetic groups to which they owe their color and sensitivity to light. Each of these pigments possesses a single type of prosthetic group. The protein probably varies from one animal to another; it may be called *opsin*, and named for the animal of origin.

The absorption spectrum of rhodopsin consists of three bands (Fig. 1): a broad α-band, maximal near 500 mμ, which is principally responsible for the spectral sensitivity of rod vision; a small β-band in the near ultraviolet, at about 350 mμ; and a narrow γ-band at about 278 mμ. The α- and β-bands go with the carotenoid prosthetic group, the γ-band with opsin (*2*). The α-band is displaced in position in rhodopsins from various sources; it lies at about 498 mμ in cattle, rats, and dogfish; and at 502 mμ in the bullfrog (*1*, *3*, *5*). The first rhodopsin to be isolated from an invertebrate retina, that of the squid, displays a similar trio of absorption bands: an α-band at about 490 mμ, a β-band at about 360 mμ, and the opsin band in its usual position (*6*). In porphyropsin, the chromophore bands are shifted considerably toward the red, the α-band to about 522 mμ, and the β-band to about 370 mμ (Fig. 1).

In evaluating rhodopsin spectra, one can use the ratio of the extinctions at 400 and 500 mμ (400/500

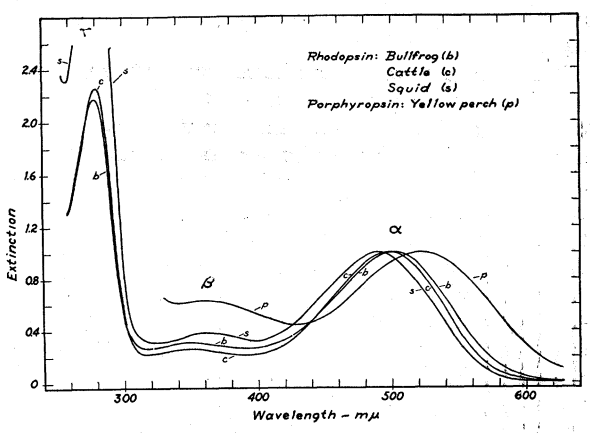

FIG. 1. Spectra of rhodopsins and porphyropsin, measured in 2 per cent aqueous digitonin. Preparations from the bullfrog (average of 3) by R. Hubbard and P. K. Brown; cattle and squid, by R. C. C. St. George; yellow perch by P. K. Brown.

ratio) as an index of "optical purity" (*1*). A similar 278/500 ratio provides a criterion of optical purity in the ultraviolet and indicates, also, the proportions of protein to chromophore in rhodopsin preparations. The spectra shown in Fig. 1 include the lowest such ratios so far recorded. They are almost equal in our best rhodopsin preparations from cattle and bullfrogs: $400/500 = 0.22 - 0.26$, and $278/500 = 2.2$. The former ratio is close to that of pure rhodopsin (*1*); the latter may be higher than that of the pure pigment.

Light absorbed by the prosthetic group of rhodopsin—i.e., in its α- and β-bands—bleaches the molecule and can be seen. Present data suggest that light absorbed in the protein band may not be available for bleaching. On bleaching rhodopsin in solution, the α- and β-bands are replaced by the spectrum of retinene$_1$, with a peak at about 385 mμ; the γ-band remains unchanged (*2*).

The spectra of rhodopsin and porphyropsin offer important clues to their chemical structure. The position of the α-band implies that, to form the chromophores of rhodopsin and porphyropsin, *two* molecules of the vitamins A or retinenes are united in conjugation. The β-band has the appearance of a cis-peak, such as is known to accompany linkages of *cis*-configuration in many carotenoids (*7*). It implies that the prosthetic groups of rhodopsin and porphyropsin contain such linkages (*2*).

The Bleaching of Rhodopsin

The bleaching of rhodopsin is initiated by a light reaction that forms unstable orange products (Lythgoe's "transient orange"); followed by ordinary chemical—i.e., "dark"—changes that end in the production of a yellow mixture of retinene$_1$ and opsin, in part loosely bound to each other (Lythgoe's "indicator yellow") (*1, 8*).

The light reaction was isolated for the first time by Broda and Goodeve, who irradiated rhodopsin at the temperature of dry ice, at which dark reactions scarcely occur (*9*). The processes involved in bleaching have recently been analyzed further (*10*).

When rhodopsin is irradiated exhaustively with white light at temperatures of $-40°$ to $-100°$ C, its α-absorption band is displaced about 5 mμ toward shorter wavelengths and rises (cattle) or falls (frog) slightly in height. The color changes very little, from red to orange-red, with little change in depth. This is the only photochemical step in the rhodopsin cycle. Its product may be called *lumi-rhodopsin.*

On warming in the dark to temperatures above $-20°$ C, lumi-rhodopsin undergoes a further shift of spectrum toward shorter wavelengths, yielding *meta-rhodopsin.* Warmed to room temperature in the dark, meta-rhodopsin yields a mixture of regenerated rhodopsin and retinene$_1$ + opsin in about equal amounts (*5, 10*).

Kühne observed many years ago that dry rhodopsin does not appear to bleach, even in sunlight. In this state also, however, rhodopsin undergoes the light reaction, though with such small change in color as to have escaped notice. Rhodopsin prepared in dry gelatin films possesses a spectrum similar to that in solution. On exposure to light it yields lumi-rhodopsin; and in the dark this is transformed, even in the dry state, to meta-rhodopsin. Meta-rhodopsin is stable indefinitely, as long as it is kept dry; but on wetting in the dark it bleaches to yield a mixture of regenerated rhodopsin and retinene$_1$ + opsin in roughly equal amounts (*10*).

The spectrum of rhodopsin regenerated under these conditions lies at slightly shorter wavelengths than that of the pigment extracted from the retina. For this reason Collins and Morton have suggested that it be called "iso-rhodopsin" (*5*). Preliminary experiments in our laboratory indicate that "iso-rhodopsin" is a mixture of native and slightly altered rhodopsins. The altered forms not only have spectra displaced a few mμ toward the blue, but also are more susceptible to attack by such protein denaturants as methyl alcohol (*11*). All our experiments in which rhodopsin has been regenerated or synthesized *in solution* have yielded such products. It should be noted that the resynthesis of hemoglobin in solution from heme and globin raises similar problems (*12*).

The rhodopsin of the squid undergoes a pattern of changes on bleaching comparable with that of vertebrate rhodopsins. Bliss has described a pigment obtained from the squid retina ("cephalopsin"), which resembles rhodopsin in spectrum but is unaffected by light, though it decomposes in the dark. Bliss, however, made his preparations *in daylight* (*13*). What he has described is in fact meta-rhodopsin. Squid rhodopsin, like that of vertebrates, is relatively stable in the dark. On exposure to light it yields lumi-rhodopsin, and this decomposes in the dark by way of meta-rhodopsin to a mixture of regenerated rhodopsin and retinene$_1$ (*6*).

These experiments disclose a striking similarity between the bleaching of rhodopsin and the photographic process. In both cases light forms a "latent image" involving little visible change. Gross changes in color are the result of subsequent "dark" reactions—i.e., of "development." The parallel is particularly close in the case of gelatin films of rhodopsin, in which exposure to light yields a stable latent image composed of meta-rhodopsin, which can be developed at any later time simply by wetting (*14*). The rapidity with which the exposure of a retina to light leads to electrical responses in the optic nerve makes it probable that the excitation of the rods depends upon the light reaction itself and does not await the relatively slow bleaching of rhodopsin to retinene$_1$.

Reduction of the Retinenes to the Vitamins A

The demonstration by Morton and his co-workers that retinene is vitamin A aldehyde has given a singular impetus to recent work in visual biochemistry. Vitamin A$_1$ is the alcohol $C_{19}H_{27}CH_2OH$; retinene$_1$

is its primary oxidation product, $C_{19}H_{27}CHO$ (*15*). The structure of Vitamin A_2 is still uncertain; but it also is known to be an alcohol, and retinene$_2$ is its aldehyde (*16*). To prepare retinene one need only pour a solution of vitamin A_1 or A_2 in petroleum ether onto a short column of dry manganese dioxide; a solution of the corresponding retinene runs off as the filtrate (*17*).

In the outer segments of the rods, retinene$_1$ is reduced to vitamin A_1 by an enzyme system in which cozymase (*DPN*) acts as coenzyme (*18*):

$$C_{19}H_{27}CHO + DPN{-}H_2 \underset{\leftarrow}{\xrightarrow{\text{retinene reductase}}} C_{19}H_{27}CH_2OH + DPN$$

retinene$_1$ — vitamin A_1

This system can be assembled in solution from the following components: the coenzyme, *DPN*—H_2; as substrate, synthetic retinene$_1$, prepared by the oxidation of crystalline vitamin A_1 on manganese dioxide; and the apoenzyme, contained in a water extract of the homogenized retinas or the isolated rods of frogs or cattle (*19*).

In the retinas of fresh-water fishes, retinene$_2$ is reduced to vitamin A_2 by a similar enzyme system. The fish apoenzyme, however, like that of frogs or cattle, works equally well upon either retinene$_2$ or retinene$_1$. One has to consider, therefore, only a single apoenzyme, retinene reductase, which with one coenzyme, *DPN*—H_2 reduces either retinene$_1$ or retinene$_2$ to the corresponding vitamin A (*19*).

This enzyme system introduces a second vitamin into the chemistry of rod vision; for the key component of *DPN* is nicotinamide, the antipellagra factor of the vitamin B complex. It appears here, strangely enough, in the position of regenerating the vitamins A.

Retinene reductase may be even less specific than we have described. Bliss has stated that crude alcohol dehydrogenase preparations from mammalian liver catalyze the equilibrium between retinene$_1$ and vitamin A_1 (*20*). This observation has since been confirmed in our laboratory, with the crystalline alcohol dehydrogenase of Bonnichsen (*21*). Warren Yudkin has found also that an enzyme system from the retina oxidizes ethyl alcohol to acetaldehyde. It is possible, therefore, that retinene reductase is identical with alcohol dehydrogenase.

When the retinene reductase system has completed its action in the retina or in neutral solution, no measurable amount of retinene remains. It has been converted quantitatively to vitamin A. As will appear below, the system can be driven in the oxidative direction; but this demands special circumstances and the performance of external work.

The Synthesis of Rhodopsin from Retinene$_1$

Many years ago Kühne described two modes of synthesis of rhodopsin: a rapid "anagenesis" from yellow or orange products of bleaching, which occurs in the isolated retina and even in solution; and a much slower "neogenesis" from colorless precursors, which Kühne believed to occur only in the intact eye. These processes can now be identified with the synthesis of rhodopsin from retinene$_1$ and from vitamin A_1.

Recently Hecht *et al.* confirmed Kühne's observation that rhodopsin regenerates in solution after bleaching to retinene$_1$ and opsin. The largest regeneration recorded was about 15 per cent (*22*).

When rhodopsin is bleached in solution in the presence of added retinene$_1$, it regenerates strongly, with yields as high as 85 per cent. The concentration of retinene$_1$ that provides maximum regeneration is about 20 μg/ml (about 7×10^{-5} *M*). A mixture of rhodopsin and retinene$_1$, bleached repeatedly in the light, regenerates repeatedly in darkness (*23*).

Rhodopsin has also been synthesized in solution from its separate precursors. Opsin has been prepared from frog and cattle rods by a procedure that excludes most other molecules. Rod outer segments, isolated from completely bleached retinas, are tanned with alum to make most proteins insoluble; leached exhaustively with buffer solutions to remove all water-soluble material; and frozen-dried and extracted with petroleum ether, to remove fat-soluble substances, including carotenoids. From the solid residue of these treatments, clear, colorless opsin is extracted with the aid of the detergent digitonin. Opsin alone yields no light-sensitive material on incubation in the dark, but on mixing with synthetic retinene$_1$, it yields a lively synthesis of rhodopsin (*23*).

No other molecules seem to take part in this reaction. It is a spontaneous—i.e., an energy-yielding—process. It is the *bleaching* of rhodopsin—probably specifically the formation of lumi-rhodopsin—that requires energy, usually furnished by light.

Why does the regeneration of rhodopsin in solution require added retinene$_1$? The main reason for this is that the retinene$_1$, formed when rhodopsin bleaches, wanders away from its original sites of attachment to opsin, to couple with other groups on opsin and other molecules. One function of added retinene$_1$ is to saturate all such positions, and so make adequate retinene$_1$ available at the sites concerned with rhodopsin synthesis. Another function is simply to speed the synthesis of rhodopsin; for free opsin deteriorates relatively quickly in solution, and the faster rhodopsin is regenerated, the larger is the yield.

Frog opsin condenses with retinene$_2$ as effectively as with retinene$_1$ to yield a light-sensitive pigment. This possesses an absorption spectrum intermediate between those of rhodopsin and porphyropsin. Opsin from a porphyropsin retina (yellow perch), allowed to react with retinene$_1$ and retinene$_2$, yields much the same result: rhodopsin with retinene$_1$, the intermediate pigment with retinene$_2$ (*23*). The nature of the pigment obtained with retinene$_2$ is still uncertain. Perhaps it is "iso-porphyropsin."

The synthesis of rhodopsin from retinene$_1$ and opsin is blocked by hydroxylamine (0.10 *M*), which binds retinene$_1$ in the form of its oxime:

$$C_{19}H_{27}HC{=}O + NH_2OH \underset{\leftarrow}{\longrightarrow} C_{19}H_{27}HC{=}NOH + H_2O$$

retinene$_1$ + hydroxylamine — retinene$_1$ oxime + water

The synthesis of rhodopsin is blocked also by formaldehyde (2 per cent; 0.7 *M*) (*23*). We interpreted this effect originally as a competition between formaldehyde and retinene$_1$ for the amino groups of opsin with which both aldehydes readily couple; but a similar competition seems to involve sulfhydryl groups. We find that retinene$_1$—like formaldehyde (*24*)—reacts with the –SH groups of cysteine and glutathione, apparently yielding products of the type:

$$C_{19}H_{27}HC{=}O + RSH \rightleftharpoons C_{19}H_{27}CHOH{-}SR$$

(retinene$_1$ + sulfhydryl amino acid or peptide = retinene$_1$ thio complex).

Such a reaction probably plays some part in the synthesis of rhodopsin, for its regeneration after bleaching is blocked completely by the sulfhydryl poison, *p*-chloromercuribenzoate ($7 \times 10^{-5} M$). This inhibition is reversed by adding glutathione. It may be concluded that sulfhydryl groups of opsin play an essential role in rhodopsin synthesis (*25*).

We have stressed in this discussion the synthesis of photosensitive pigments from the retinenes. In reality, however, we had in these experiments synthesized rhodopsin from vitamin A$_1$, and a comparable light-sensitive pigment from vitamin A$_2$; for our retinenes were prepared from the corresponding vitamins A by oxidation on manganese dioxide. This was an unphysiological process; but its success led us to look for a physiological mechanism that might oxidize vitamin A to retinene efficiently in the retina.

The Oxidation of Vitamin A$_1$ to Retinene$_1$

In solution and in the isolated retina the equilibrium of the retinene reductase system lies far over toward the side of reduction. The same can be said of the closely comparable alcohol dehydrogenase systems of liver and yeast, in which the equilibrium in neutral solution lies far over toward the production of alcohol. To make the latter type of system produce appreciable quantities of acetaldehyde at neutral reactions, it is necessary to introduce an aldehyde-trapping reagent, which drives the system in the oxidative direction by binding aldehyde as fast as it is formed.

By the same means one can drive the retinene reductase system to oxidize vitamin A$_1$ to retinene$_1$ (*20, 26*). As retinene-trapping reagent we have introduced hydroxylamine. In the presence of this reagent, retinal homogenates have been observed to oxidize vitamin A$_1$ to retinene$_1$ with yields of about 50 per cent (*26*).

It is not commonly understood that such a trapping reaction plays an essentially *energetic* role. To drive a reaction away from its equilibrium position requires that work be done; and the essence of a trapping reaction is that it does this work. In the present instance, the energy needed to oxidize vitamin A$_1$ to retinene$_1$ is supplied by the exergonic condensation of retinene$_1$ with hydroxylamine.

For this reason it is important that the condensation of retinene$_1$ with opsin also is exergonic, and so can serve as a retinene-trapping reaction. In the rods, opsin may substitute physiologically for hydroxylamine and may drive a continuous oxidation of vitamin A$_1$ to retinene$_1$, by continuously removing retinene$_1$ to form rhodopsin. This constitutes a potential mechanism for the synthesis of rhodopsin from vitamin A$_1$ in the retina.

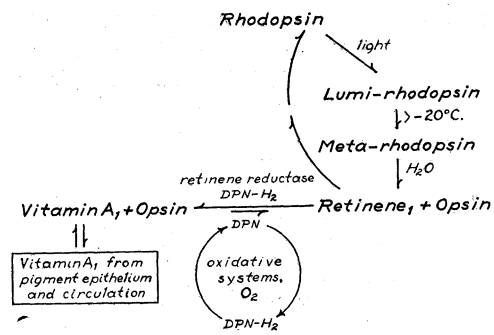

FIG. 2. Known components of the rhodopsin system. The intermediate steps in the bleaching of rhodopsin may not all be retraced when retinene$_1$ and opsin recombine to form rhodopsin. The bulk of the rhodopsin system lies within the outer segments of the retinal rods, but it is supplemented with vitamin A$_1$, respiratory factors, and oxygen itself from the pigment epithelium and the blood circulation.

The Synthesis of Rhodopsin from Vitamin A$_1$

The idea just expressed encounters an immediate difficulty. It has been believed since Kühne that the isolated retina, bleached to colorlessness, cannot regenerate rhodopsin. Yet it contains all the components considered above: vitamin A$_1$, retinene reductase, cozymase, and opsin. This inadequacy of the isolated retina caused Kühne to conclude that the synthesis of rhodopsin from colorless precursors requires new material, obtained in part from the pigment epithelium.

We have found that isolated frog retinas and retinal homogenates do in fact regenerate rhodopsin from vitamin A$_1$, in amounts about 10 per cent as large as are formed during dark adaptation *in vivo*. If a retinal homogenate is supplemented with cozymase, the yield of rhodopsin is approximately doubled. The addition to a retinal homogenate of a homogenate of the pigment layers of the eye—pigment epithelium and choroid—also doubles the yield. The pigment layers add to the system something other than cozymase, for the effects of these two supplements are additive, and when both are added together to a retinal homogenate, the yield rises to about 40 per cent (*26*).

We have observed also the synthesis of rhodopsin from vitamin A$_1$ in clear digitonin extracts of bleached retinas, supplemented with vitamin A$_1$ and cozymase. The maximum yield obtained in such preparations was about 25 per cent (*26, 27*).

The apparent role of cozymase in these syntheses is to supply coenzyme to the retinene reductase system. It suggests that rhodopsin synthesis may indeed proceed via the coupled oxidation of vitamin A$_1$ to retinene$_1$. We have therefore inquired systematically into other conditions that are expected to promote this

oxidation: a high concentration of vitamin A_1, and the presence of oxidative mechanisms that might keep cozymase in the oxidized state.

The addition of vitamin A_1 in oil to retinal homogenates increases their yield of rhodopsin by about 65 per cent. The addition of a so-called succinoxidase system of heart muscle (*28*) also increases the yield about 35–50 per cent. It is true, therefore, that those factors which promote the oxidation of vitamin A_1 to retinene$_1$ by the retinene reductase system also aid in the synthesis of rhodopsin (*27*).[2]

What do the pigment layers contribute to this synthesis? We can now offer a partial answer to this question, for in our homogenates the pigment tissue can be demonstrated to supply the retina with vitamin A_1. Retinal homogenates, freed from vitamin A_1 by extraction with petroleum ether, are unable to form rhodopsin. On addition of pigment layer homogenate they synthesize rhodopsin in high yield. The only vitamin A_1 available to them for this process is supplied by the pigment epithelium (*27*).

Finally, we have constructed a model system from the following components in solution: purified opsin, prepared from cattle retinas; the crystalline alcohol dehydrogenase of Bonnichsen, prepared from horse liver; vitamin A_1; and cozymase. On incubation in the dark, this mixture generates rhodopsin (*27*).

There is little doubt, therefore, that in the retina rhodopsin is synthesized at least in part by the oxidation of vitamin A_1 to retinene$_1$ by retinene reductase, coupled with the condensation of retinene$_1$ with opsin to form rhodopsin. In this system, the endergonic oxidation of vitamin A_1 to retinene$_1$ is the limiting process. It can be accomplished only to a small degree by the isolated retina; but with adequate supplementation, retinal homogenates and extracts, and our model enzyme system, perform this process efficiently.

There is as yet no evidence for an alternative mechanism of rhodopsin synthesis. Such an alternative pathway may exist; but it now appears probable that what has been interpreted since Kühne as a special mechanism for the synthesis of rhodopsin from vitamin A_1 consists in reality of the special conditions which drive the oxidation of vitamin A_1 to retinene$_1$.

The present status of the rhodopsin system, in terms of its known reactions, is summarized in Fig. 2. By all indications, a similar diagram will eventually describe also the porphyropsin system.

[2] Some of these factors must overlap in their effects, for the largest yield of rhodopsin we have obtained *in vitro*, using supplements in combination, is about 60 per cent.

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