ON RHODOPSIN IN SOLUTION

By GEORGE WALD*

(From the Biological Laboratories of Harvard University, Cambridge)

(Accepted for publication, March 22, 1938)

I

INTRODUCTION

Three photolabile retinal pigments are now known: rhodopsin; porphyropsin, the visual purple of fresh-water fishes (Köttgen and Abelsdorff, 1896; Wald, 1937*b*); and iodopsin, a pigment of the cones (Wald, 1937*c*).

In the experiments to be described, solutions of rhodopsin have been examined from four species of marine fish—the sea robin, *Prionotus carolinus*; sea bass, *Centropristes striatus*; scup, *Stenotomus chrysops*; and killifish, *Fundulus heteroclitus*; two species of frog—*Rana pipiens* and *catesbiana*; rabbits and rats.

It has already been shown in all of these animals but *Fundulus* that rhodopsin participates in a retinal cycle which includes the carotenoids retinene and vitamin A (Wald, 1935–36; 1936–37; unpublished observations on mammals). The porphyropsin system is based upon other carotenoids; probably the same is also true of iodopsin. The association with retinene and vitamin A is therefore a specific characteristic of the rhodopsin system.

The spectrum of rhodopsin is equally characteristic. A representative collection of spectra of rhodopsin preparations from various animals, and for comparison a typical porphyropsin spectrum, are shown in Fig. 1. It is clear that the rhodopsin spectra form a distinct and homogeneous group with a single broad maximum at 500 ± 2 mμ.[1]

* This research has been aided in part by a grant from the Milton Fund of Harvard University. A short account of the experiments has appeared in *Nature* (1937 *a*).

[1] Bayliss, Lythgoe, and Tansley (1936) have reported that preparations of visual purple from a number of marine fishes possess spectra intermediate between those of rhodopsin and porphyropsin. The significance of these observations is

Rhodopsin therefore is clearly characterized chemically and spectroscopically and may be treated as a chemical individual without special reference to its source.

Kühne[2] described the bleaching of rhodopsin in solution as a wholly photochemical transformation in two stages: (1) the formation of a yellow product with increased absorption in the violet; and (2) the slow fading of this product on prolonged irradiation to a final condition "colorless, like water." The second process appears to be a common photo-oxidation. It is independent of the first reaction, probably possesses no significance for the retinal cycle, and is disregarded in the present paper.

The denial of the first of these stages by Köttgen and Abelsdorff and by Trendelenburg (1904) led to an extended theoretical excursion. If, as these authors believed, rhodopsin bleaches directly to colorless products, the final absorption of a bleached preparation may be assumed due to impurities present in the original solution. The difference between this residual absorption and the spectrum of the unbleached preparation should constitute the absorption spectrum of pure rhodopsin. Such "difference spectra" were found to agree in form with the sensitivity to monochromatic lights of rhodopsin solutions (Trendelenburg) and of rod vision (König, 1894; Trendelenburg, 1904; Hecht and Williams, 1922–23).

The assertion that rhodopsin bleaches to colorless products rested on wholly inadequate evidence. Garten (1906) and more recently Hosoya and Bayerl (1933),[3] have entirely confirmed Kühne's description. As Garten indicated, Köttgen and Abelsdorff's own results contain abundant evidence of the formation of yellow products in bleaching. Their conclusion to the contrary seems to have

---

not clear to me. Unfortunately the authors measured, not the direct absorption spectra, but differences in absorption between unbleached and bleached solutions. This may at least in part account for their results. It is noteworthy that one of their fishes showing apparently typical porphyropsin behavior is a gurnard, *Trigla hirundo*; our closely related American gurnard, *Prionotus*, possesses a typical rhodopsin system. I have suggested the possibility that the English preparations contained mixtures of rhodopsin and porphyropsin (Wald, 1937 *b*); but this is excluded in the present experiments by the carotenoid extraction data, which show the presence of vitamin A and retinene alone (Wald, 1936–37).

[2] Kühne, 1878, p. 49; 1879, p. 269.

[3] It is noteworthy that these authors "neigen—der schon von Köttgen und Abelsdorff geäuszerten Meinung zu, dass es sich bei dieser Ausbleichung nicht um die Entstehung eines neuen Farbstoffes handelt, sondern lediglich um das Verschwinden des roten Farbstoffes, ein Vorgang der nach unserer Meinung gut durch eine im Licht stattfindende physikalisch-chemische Veränderung, z.B. eine Dispersitäts-Änderung des Sehpurpurs erklärt werden kann."

been influenced by two attractive considerations: (1) the possibility of deriving, by simple spectrophotometric manipulation, "pure" absorption spectra from impure preparations; and (2) the close correspondence between such difference spectra and human rod visibility. For a long period this comparison constituted the only direct evidence that rhodopsin is a *visual* pigment.

It is now clear that under all known conditions the bleaching of rhodopsin yields colored products (Lythgoe, 1937; Wald, 1937*a*). Difference spectra, therefore, though on occasion useful for descriptive purposes, possess no physical meaning. The absorption spectrum of pure rhodopsin is still unknown.

Recently the belief that rhodopsin bleaching is exclusively photochemical has been questioned, due to the discovery that following irradiation, dried gelatin films of rhodopsin (Weigert and Nakashima, 1930) and aqueous solutions (Hosoya, 1933) continue to fade in darkness.

The present research is concerned with the course of bleaching of rhodopsin in aqueous solution; the contribution to it of thermal ("dark") processes; its products; and the form and significance of the rhodopsin absorption spectrum.

II

Methods

Rhodopsin solutions were prepared by leeching retinas of dark adapted animals for various periods at room temperatures with a 2 per cent aqueous solution of crystalline digitonin (Tansley, 1931). Special care was taken during the dissection of the tissues to remove all apparent traces of choroid and pigment epithelium. Two general methods of preparation were employed:

1. Retinas were dropped directly, or after preliminary soaking in 5 to 10 per cent salt solutions, into digitonin. The mixture was left in darkness for several hours with no other disturbance than occasional gentle rotation, and was then centrifuged. These preparations were slightly opalescent, and displayed reasonably constant properties, the most specific of which was an extinction at 400 mμ about 0.5 to 0.6 of that at the maximum.

2. Directly following dissection, retinas were dropped into 4 per cent aluminum potassium sulfate (alum) solution, and left to harden for 3 hours or longer. The mixture was centrifuged, the alum solution poured off, and the tissues washed twice in distilled water, then twice more in buffer mixtures to bring to the desired pH. Finally they were ground with a glass rod in digitonin solution, let stand for several hours, and centrifuged. These preparations appeared to be perfectly clear, and were distinguished by extinctions at 400 mμ only 0.32 to 0.35 of those at the maximum.

Spectra were measured at the Color Measurements Laboratory of Professor A. C. Hardy at the Massachusetts Institute of Technology, with a recording

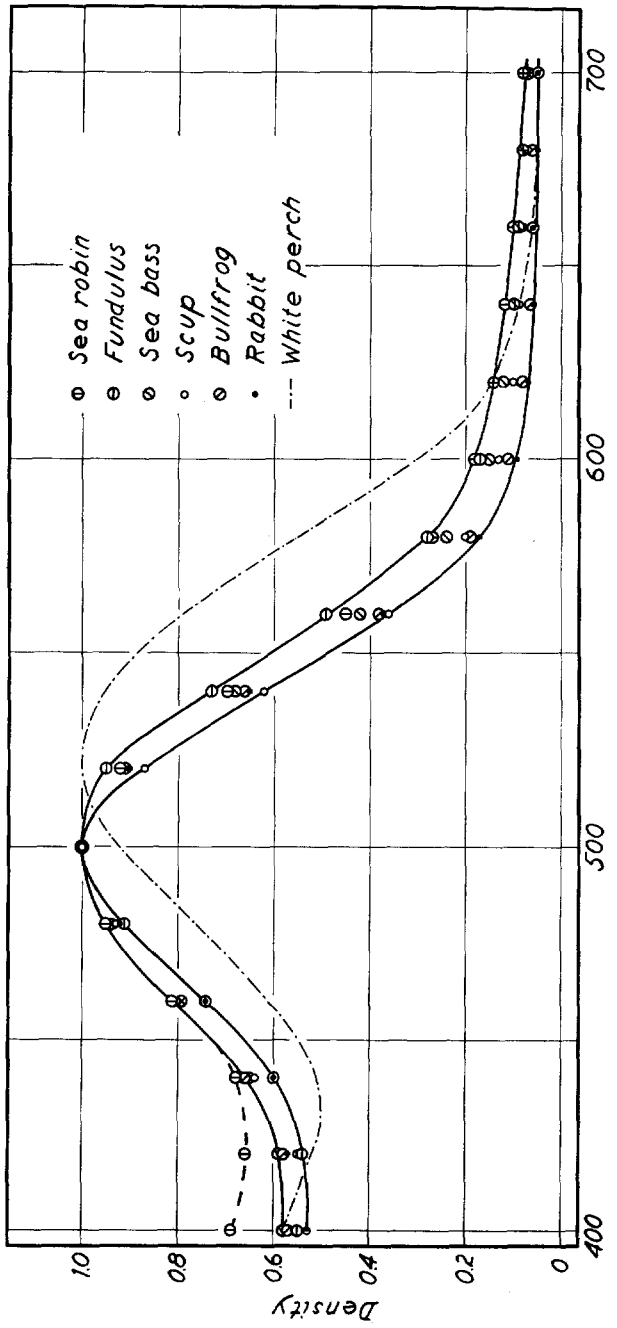


FIG. 1. Spectra of rhodopsin preparations from the rabbit, bullfrog, and four marine fishes; and of a typical porphyropsin preparation from the fresh-water white perch (*Morone americana*). All but the bullfrog preparation were obtained from retinas pretreated with alum. The absorption is plotted as density or extinction, $\log \frac{I_o}{I}$, in which I_o is the incident and I the transmitted intensity.

photoelectric spectrophotometer (Hardy, 1935). The spectra shown in Figs. 2, 3, and 5–8 were drawn by the instrument itself. This machine, in addition to complete objectivity in performance, possesses two special advantages for the present research: (1) it is extraordinarily rapid. The spectrum from 400 to 700 mμ is recorded within about 2 minutes. (2) It is economical of light. Since the spectrum is dispersed at the source, only the narrow wave length band the absorption of which is being measured passes through the test solution. This causes no distinguishable bleaching of rhodopsin during the measurement of an entire absorption spectrum; indeed, the spectrum may be retraced completely without perceptible change in absorption. With respect to the preparation, therefore, the period of measurement is equivalent to a period in darkness.

The general procedure in bleaching experiments was to measure the spectrum of the rhodopsin preparation, then irradiate it in position in the spectrophotometer, and re-measure its spectra thereafter periodically without otherwise disturbing it.

All of the spectra recorded in the following pages show the differences in absorption between test solutions and equal depths of pure solvent. The absorption is expressed either as a percentage of the incident radiation, or as extinction or optical density, $\log I_o/I$, in which I_o is the incident and I the transmitted intensity. The latter quantity is directly proportional to the concentration and to the depth of absorbent.

III

The Bleaching of Rhodopsin

Rhodopsin bleaches in a succession of light and "dark"—photic and thermal—reactions.[4] The velocity of the light component depends principally on the intensity of irradiation, that of the dark component on the temperature. In dim light the light reaction may be so slow as to limit the speed of the entire transformation. In this case, which characterizes almost all earlier studies of bleaching, the contribution of dark processes is obscured and usually overlooked. In the present experiments lights were used sufficiently bright to complete the photoprocess within a few seconds. Following such exposure the isolated dark reactions may be examined through a long succeeding interval.

The course of bleaching in neutral solutions of bullfrog rhodopsin is shown in Figs. 2 and 3. It possesses the following components:

Photoprocess.—The first recorded product of irradiation is an

[4] Throughout this paper reactions are characterized as "light" or "dark" synonymously with the more accurate terms, photic and thermal. Dark reactions, of course, proceed equally well in light or darkness.

orange-colored pigment, the spectrum of which is shown in curves *b*. It possesses a broad absorption hump at about 480 mμ, and higher absorption than rhodopsin itself below 430 to 440 mμ. The change in extinction due to the photoprocess, revealed by subtracting curve *b* from *a*, may be plotted as a "difference spectrum." This

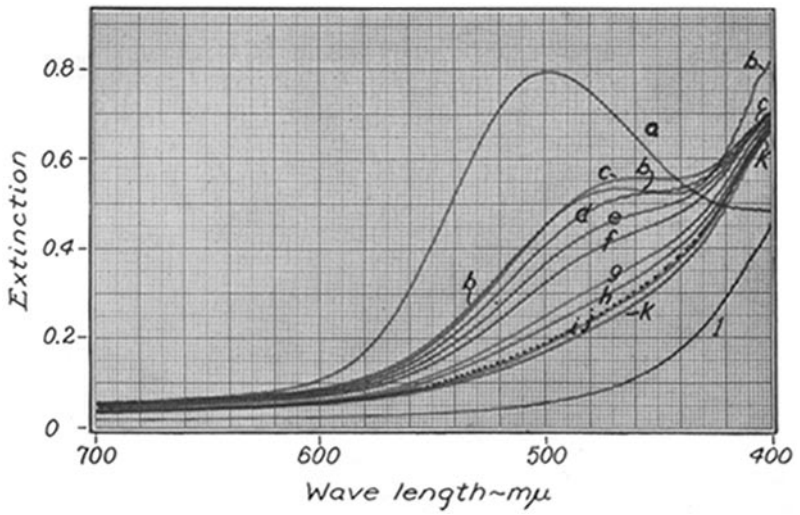


FIG. 2. Bleaching of neutral rhodopsin. Extract of 6 bullfrog retinas in 3.8 cc. of 1 per cent digitonin solution buffered at pH 6.9. Spectra of a 1 cm. layer recorded at 25.3°C., 6 hours after the beginning of extraction. The unbleached solution (*a*) was exposed for 25 seconds to 700 foot-candles and spectra recorded periodically thereafter in the dark: *b* at 1.5 minutes, *c* at 4.5, *d* at 7.5, *e* at 10.5, *f* at 15.0, *g* at 32.3, and *h* at 59.5 minutes from the beginning of irradiation. At 61 minutes the solution was re-irradiated for 40 seconds; its spectrum fell to *i*. After a further 22 minutes in darkness it had risen to *j* (broken line); and on a third irradiation for 40 seconds dropped to *k*. The spectrum of the benzine-soluble portion of the final bleached residue in 3.8 cc. of a mixture of 1 part methanol and 2.8 parts digitonin solution, buffered at pH 6.9, is shown in curve *l*.

has been done with the data of Fig. 3B; the result is shown in Fig. 4 (open circles). The fall in extinction due to light is maximal at about 510 mμ, and is accompanied by a rise at 400 mμ which may be almost equally great.

Hosoya (1933; Hosoya and Bayerl, 1933; Hosoya and Saito, 1935)

has reported that on irradiation of rhodopsin the absorption rises also in the red, above 600–620 mμ. This phenomenon has never appeared in the present experiments.

Dark Process I.—This is shown in Fig. 2, curves *b* to *c*. The ex-

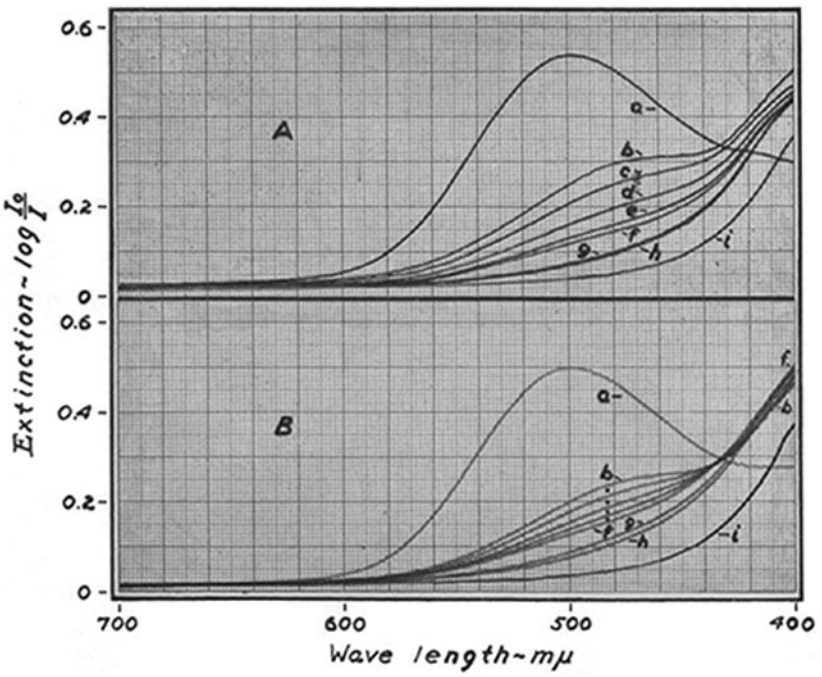


FIG. 3. Bleaching of neutral rhodopsin in fresh and aged solutions. Extract of 7 bullfrog retinas in 7.6 cc. of 1 per cent digitonin solution buffered at pH 6.9. Half of this preparation was measured 4 hours after extraction was begun (A), the remainder one week later (B). During the storage period of B a small amount of colorless material came out of solution and was centrifuged off. Spectra of a 1 cm. layer of the unbleached solutions are shown in curves *a*. After $\frac{1}{2}$ minute exposure to 700 foot-candles spectra were measured periodically in darkness, curves *b* at 1.2 minutes, *c* at 3.2 and 3.7, *d* at 7.2 and 8, *e* at 13.8 and 15.7, and *f* at 32.2 and 32.8 minutes (when two times are stated, the first refers to A). At 35 minutes the preparations were re-irradiated as before for 40 seconds and their spectra recorded (*g*, 37 minutes). At 38.5 minutes they were exposed for a third time, and yielded curves *h* (40 minutes). Temperatures: A, 29.3°C; B, 26.0°C. Curves *i* show the spectra of the benzine-soluble portions of the bleached residues, each in 3.8 cc. of a mixture of 1 part methanol and 2 parts digitonin solution, buffered at pH 6.9.

tinction rises rapidly in darkness between about 420 and 500 mμ, maximally at about 440 mμ, and falls to either side of this region. This component is most prominent in the bleaching of acidic rhodopsin. It has not been detected at all in alkaline solutions, and often is missing from neutral preparations, as in Fig. 3. When present it appears equally prominently in all samples of a preparation, and apparently is unaffected by aging of the solution. I believe this to be a normal component of the bleaching of neutral rhodopsin, and that it is missed in some preparations only because, for unknown reasons, it passes too swiftly to be recorded.

Dark Process II.—This is shown isolated in Fig. 3B, curves *b* to *f*. The extinction falls slowly between about 435 and 600 mμ, maximally at about 480 mμ, and rises simultaneously below about 435 mμ. The solution changes in color from orange to yellow, as the initial photoproduct maximum at 480 mμ is entirely obliterated. The difference spectrum of this change, obtained by subtracting curve *f* from *b* in Fig. 3B, is shown in Fig. 4 (closed circles). It resembles closely that due to the photoprocess except for the shift of maximum from about 510 to about 480 mμ. This dark process is an invariable component of the bleaching of neutral rhodopsin. In Fig. 3B it accounts for 19 per cent of the total fall in extinction from curves *a* to *f* at 500 mμ, and 26 per cent of that at 480 mμ.

Dark Process III.—Hosoya (1933) discovered a slow, general fall in the entire spectrum of bleached products of rhodopsin following prolonged low intensity irradiation. I find that this phenomenon appears clearly only in fresh neutral preparations. It is prominent in Figs. 2 and 3A, recorded within several hours following extraction, but has vanished completely after a week of storage (Fig. 3B). This component is not prominent even in fresh solutions from retinas pre-treated with alum, or at pH's below 5.

A difference spectrum showing the changes in extinction due to the combination of this process with dark component II is obtained by subtracting curve *f* from *b* in Fig. 3A. This is shown in Fig. 4 (half-filled circles). The fall in extinction is maximal at about 480 mμ, and no further rise occurs at 400 mμ.

Contribution of Dark Processes to Bleaching.—Of the total fall in extinction from curves *a* to *f* in Fig. 3A, 31 per cent at 500 mμ and

42 per cent at 480 mμ are due to dark processes. The corresponding values from Fig. 2 are 40 and 52 per cent. In a rhodopsin preparation which had been exposed to the 0.02–0.04 second flash of a magnesium foil lamp (Photoflash No. 20), the fall in extinction at 500 mμ during a subsequent 1.2 to 98 minutes in darkness was 48 per cent of

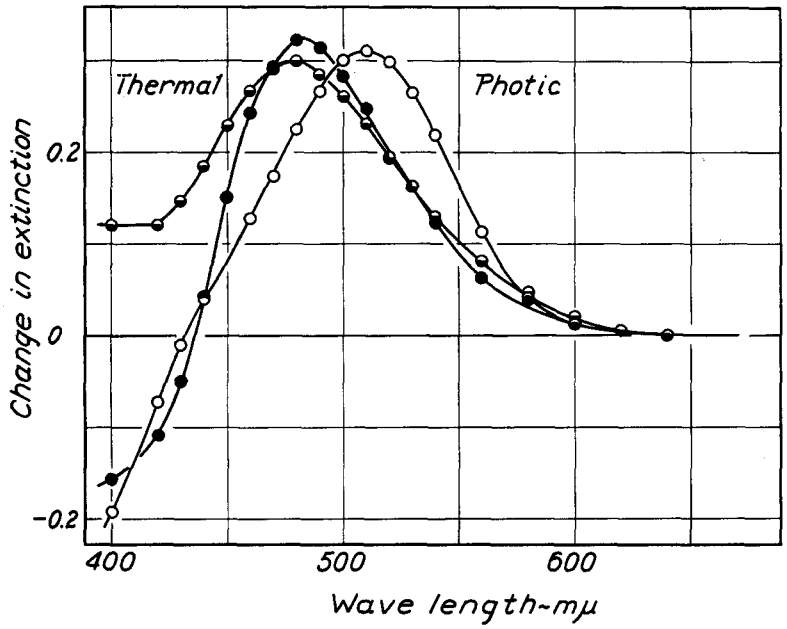


FIG. 4. "Difference spectra" of rhodopsin, from the data of Fig. 3. Open circles show changes in extinction due to the photoprocess (Fig. 3 B, *a–b*). Half-closed circles show those due to thermal processes in fresh solutions (Fig. 3 A, *b–f*); closed circles those due to the thermal process in aged solutions (Fig. 3 B, *b–f*). To make these curves conveniently comparable the ordinates of the second function have been multiplied throughout by 2, those of the third by 4. Bleaching due to light is maximal at about 510 mμ, that due to thermal reactions at about 480 mμ.

the total (22.4°C.). The contribution of dark processes to bleaching actually rises above any of these estimates, since it includes changes which occur before the first and following the final measurement, and some correction for whatever regeneration of rhodopsin may have occurred in the dark period. It may be concluded that in the bleaching

of neutral rhodopsin solutions about half the fall in extinction at 500 mμ and a considerably greater portion at 480 mμ are due to dark reactions.

Regeneration.—Ewald and Kühne (1878) found that solutions of rhodopsin which had been bleached in bright light and replaced in darkness regenerated appreciable quantities of the visual pigment. Hecht, Chase, Shlaer, and Haig (1936) have recently confirmed this observation and specified certain conditions which favor it.

In the present experiments, renewed exposure to light at the close of the dark processes always resulted in some further bleaching (Fig. 2, curves *h–k*; Fig. 3, curves *f–h*). Some of this might have been due to failure to complete the photoprocess in the original irradiation. It was principally due, however, to the regeneration of rhodopsin during the period in darkness.

In Fig. 2, upon re-irradiation following an hour in darkness, the absorption fell from *h* to *i*. During a subsequent 22 minutes in darkness, the absorption rose slightly to *j*; this could only have been due to regenerative processes which had by this time overtaken the residual dark components of bleaching. On re-irradiation, the spectrum fell to *k*. Any bleached residue of the original irradiation must have been bleached by the second exposure to light; therefore, bleaching due to the third exposure must have involved only regenerated rhodopsin.

The regeneration of rhodopsin therefore constitutes a fourth dark process in irradiated rhodopsin solutions, which reverses to a small but appreciable degree the processes of bleaching.

Acid and Alkaline Solutions.—Chase (1935–36) has studied the bleaching of rhodopsin solutions at various pH's and moderately low intensities of irradiation. In alkaline solution bleaching was found to be rapid and kinetically simple; in acid solution, slow and relatively complex. The final bleached residue was reported to be colorless in alkaline and yellow in acid solution, confirming earlier observations of Nakashima (1929).

The present experiments extend and in part clarify these results through separation of the light and dark components of bleaching. Changes in pH do not affect rhodopsin itself appreciably but modify greatly the velocities of the thermal components of bleaching and the spectra of the reactants.

The response of rhodopsin itself to changes in pH is shown in curves *a* of Figs. 5 and 7. A bullfrog extract had been divided into two equal portions. One was brought to a pH about 5 with monopotassium phosphate; its spectrum is shown as the solid line, curve *a* of Fig. 5. This is almost indistinguishable from the spectrum of the second portion in neutral solution; departures of the latter are shown as broken lines in curve *a* of Fig. 5. This second portion was brought to a pH about 11 with sodium carbonate. The solution, which had

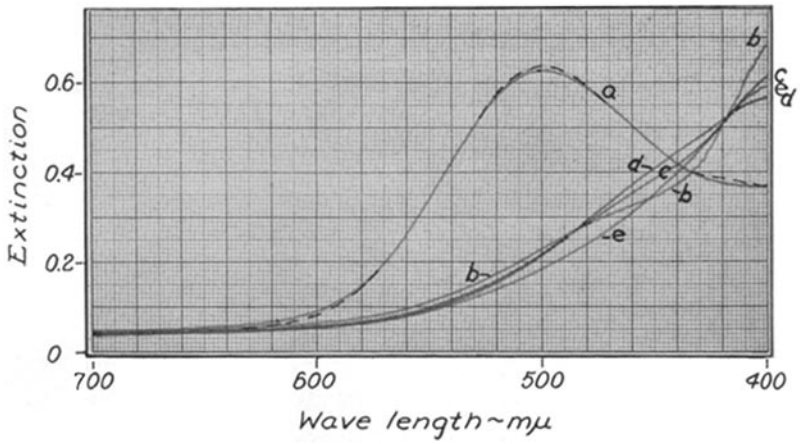


FIG. 5. Neutral and acidic rhodopsin. Extract of 5 bullfrog retinas in 4 cc. of digitonin solution, divided in halves, and measured at about 25°C. in 5 mm. layers. One half brought to pH 5 with monopotassium phosphate (*a*, solid line). Other half measured in neutral solution (departures are shown by broken lines in *a*). The acidic portion was exposed to 700 foot-candles for 25 seconds, and spectra thereafter recorded in darkness, *b* at 1.5 minutes, *c* at 5.3, *d* at 11.3, and *e* at 51 minutes.

been slightly opalescent, cleared. Its spectrum is shown in curve *a* of Fig. 7. The apparent absorption has fallen almost uniformly throughout the spectrum, probably due to the decrease in opalescence, but has not otherwise changed significantly. It is clear that the color of rhodopsin itself is not pH-labile (*cf.* Lythgoe, 1937).

The bleaching of the acidic portion of this preparation is shown in Fig. 5. For the first few minutes in darkness following irradiation, the absorption of the initial photoproduct (*b*) rises in the region of 440 mμ, simultaneously falling to either side of nodes at 417 and 485 mμ.

This is clearly analogous to thermal component I of bleaching in neutral solution. In acid solution it appears invariably and very prominently, and its velocity is greatly retarded.

This process is followed by a slow, regular fall in absorption, maximal at 460 mμ ,with simultaneous rise below about 416 mμ. This is the acidic analogue of thermal component II in neutral solution; at

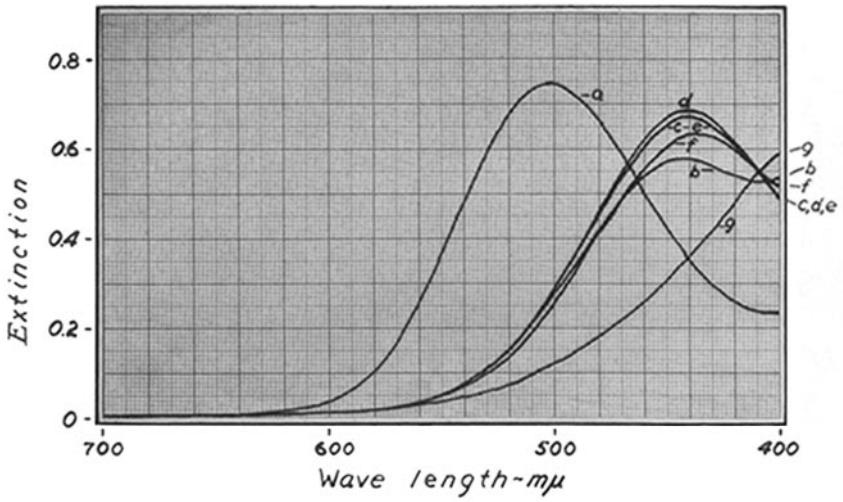


FIG. 6. Acidic rhodopsin, alum preparation. 6 bullfrog retinas soaked in 5 per cent alum for 3 days at 5°C. Washed with water, soaked in 10 per cent saline for 3 hours, then ground in 3.8 cc. of digitonin solution. The pH of the extract, measured before and after bleaching, remained constant at 3.9. The preparation had been at this pH a total of 6 days. Spectra of a 10 mm. layer are shown. The unbleached solution (*a*) was exposed to 700 foot-candles for 30 seconds, and spectra recorded in darkness, *b* at 1.3 minutes, *c* at 3.8, *d* at 8.2, *e* at 15 (retraces *c*) *f* at 34.3 minutes from the beginning of irradiation (25.9°C.). Final solution left in darkness for 28 hours longer (*g*).

pH 5 it is shifted about 20 mμ toward shorter wave lengths, and has ceased to be the most prominent dark factor in bleaching.

At higher acidities the peculiarities of Fig. 5 are accentuated. The bleaching of rhodopsin at pH 3.9 is shown in Fig. 6. The initial photoproduct (*b*) possesses a distinct maximum at about 443 mμ. During the first 8 to 10 minutes in darkness this rises rapidly, finally assuming a quite symmetrical form (*c–c*). Simultaneously the photo-

product maximum shifts gradually toward shorter wave lengths. This initial rise in the region of 440 mμ obviously is homologous with the first thermal component in less acidic solutions. Decrease in pH has further retarded its speed and changed the spectra of its components.

The remaining dark changes at pH 3.9 consist in a slow decrease in absorption in the region of 440 mμ, with simultaneous rise at 400 mμ. The maximum of the initial photoproduct slowly travels into the

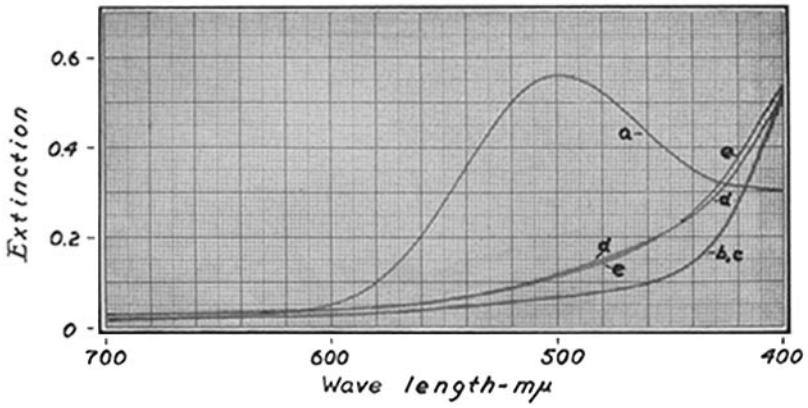


FIG. 7. Alkaline rhodopsin. The neutral half of the preparation which yielded Fig. 5 was brought to pH 11 with sodium carbonate, and its spectrum measured at about 25°C. in a 5 mm. layer (*a*). It was exposed to 700 foot-candles for 15 seconds, and spectra recorded thereafter in darkness, *b* at 1 minute, and *c* at 6.5 minutes from the beginning of irradiation. The solution was quickly neutralized with a prepared quantity of monopotassium phosphate, and its spectrum remeasured without further exposure to light, *d* at 4 minutes, and *e* at 13 minutes following neutralization.

ultraviolet. Finally only end absorption remains (*g*), resembling that of the final product in less acidic solutions.

The bleaching of alkaline rhodopsin is shown in Fig. 7. Within 1 minute from the beginning of a 15 second irradiation the spectrum has already attained the stable form, *b*. After 6.5 minutes in darkness this has scarcely changed (*c*). The final product is not colorless but light yellow, and absorbs light more strongly than rhodopsin itself below 417 mμ.

To make certain that bleaching under these conditions is really

complete and not simply retarded in some intermediate condition, this solution was rapidly neutralized with a prepared quantity of monopotassium phosphate, and its spectrum remeasured within 4 minutes following neutralization (*d*), and again 9 minutes later (*e*), all in darkness. A very small fall in absorption at about 480 mμ and rise below about 450 mμ occurred from curves *d* to *e*, but these changes are almost negligible compared with those occurring in a comparable period in neutral solution. One may conclude that at pH 11 thermal components of bleaching are almost complete within 1 minute following short exposure to light.

In several bleaching experiments with rhodopsin at pH about 9, the thermal fall in extinction in the region of 480 mμ with simultaneous rise at 400 mμ characteristic of neutral solutions (dark process II) was found to be still prominent, though greatly increased in speed. We may assume that at pH 11 the normal sequence of changes during bleaching is unaltered, but that due to their very high velocity, intermediate stages are not recorded.

Recapitulation

Four components have been distinguished in the bleaching of rhodopsin solutions:

1. Photic reaction. In neutral solution the absorption falls maximally at about 510 mμ, simultaneously rising below a node at about 430–440 mμ. In alkaline solutions the node is displaced toward 410 mμ, in acidic solutions toward 470 mμ.

2. Thermal component I. In neutral solution the absorption rises maximally at about 440 mμ, simultaneously decreasing to either side of nodes at about 420 and 495 mμ. Above pH 7 this component has not yet been recorded. With increase in acidity it grows increasingly prominent, its velocity falls sharply, and the nodes are displaced toward shorter wave lengths.

3. Thermal component II. In neutral solutions the absorption falls maximally at about 480 mμ, and rises below a node at about 435 mμ. This process is recorded at pH's 9 and below. Its velocity decreases markedly with increase in acidity; simultaneously the region of maximal bleaching and the node are displaced toward shorter wave lengths.

4. Thermal component III. The absorption of the bleached products decreases throughout the spectrum. This change appears prominently only in fresh neutral preparations.

Within this succession of photic and thermal reactions, the latter account for about half the total fall in extinction at 500 mμ. Bleaching as a whole may be regarded as a slow explosion, touched off by an appropriate quantity of light in as short a period as desired, and proceeding to completion on internal energy reserves during a long subsequent interval.

Though the spectrum of rhodopsin itself is unaffected by pH changes, the spectra of all stages of bleaching are highly pH-labile. Rhodopsin apparently does not possess an acidic or basic grouping in close association with its chromophore, but such a grouping is exposed as a first result of irradiation (*cf.* Lythgoe, 1937). The effects of pH change on the absorption spectra of bleached products are in some cases large and peculiar, probably because they involve complicated mixtures of pigments.

IV

Final Products of Bleaching

The final yellow product of bleaching of rhodopsin in solution owes its color to a benzine-soluble pigment, most of which is attached loosely to protein. It is extracted by the following procedure.

To the yellow bleached residue an equal volume of acetone or alcohol is added to precipitate proteins. On centrifuging, most of the yellow color descends with the precipitate. The precipitate is shaken with benzine[5] containing about 1 per cent ethanol, the solution separately with benzine. All color enters the benzine extracts.

The benzine extract is faint greenish yellow in color. Partitioned between benzine and 90 per cent methanol, either before or after saponification, about three-fourths the pigment seeks the benzine layer (epiphasic). In chloroform its absorption rises regularly throughout the visible spectrum, and possesses a small inflection at the edge of the ultraviolet, which develops into a broad maximum at about 385 mμ. Tested in chloroform solution with saturated antimony

[5] Petrol-ether, boiling range 30 to 60°C.

trichloride, it yields a deep blue color due to a sharp band at 664 mμ. These properties identify it as retinene (Wald. 1935–36).

Retinene has so far been examined only in impure condition. It is impossible, therefore, to be certain that any two properties of its solutions are due to a single substance. I have defined retinene specifically by the antimony trichloride band at 664 mμ; one may assume tentatively that other properties which invariably and proportionately accompany this one are due to retinene. The yellow color is such a property. In a series of retinene preparations obtained at various times and by various procedures, the densities of the yellow color and of the antimony trichloride band have been measured with a Pulfrich photometer (Zeiss). These quantities are directly proportional to each other. If a solution in chloroform possesses a density of 1.0 measured with the S43 filter in a layer 1 cm. in depth, then 0.3 cc. of it mixed with 3 cc. of antimony chloride reagent possesses an immediate extinction with the S66.6 filter of about 0.4 in a 1 cm. layer. This is true of extracts of retinas and of bleached rhodopsin solutions before and after saponification; and of all fractions of the extracts in partition between benzine and 90 per cent methanol.

It is shown below that a third property, change in color with pH, accompanies these solutions. I see no better alternative at present than to ascribe this also to retinene. It is an unusual property in a carotenoid, and therefore particularly interesting that it is exhibited also, though less markedly, by the tetra-keto-β-carotene, astacene. I find astacene in acidic methanol solution to be deep orange-red in color, and to shift in hue markedly toward yellow in alkaline solution. Spectrophotometric measurements show this change to be due to a displacement of the entire spectrum 4 to 8 mμ toward shorter wave lengths. The long wave length limb of the astacene absorption band—comparable with the portion of the retinene spectrum measured in the present experiments—changes with pH as does retinene itself.

In the intact retina, retinene is converted quantitatively to vitamin A, characterized by an antimony trichloride band at about 615 mμ (Wald, 1935–36). This process occurs only slightly if at all in rhodopsin solutions. The antimony trichloride test with extracts of bleached solutions does possess a small hump at about 615 mμ superimposed on the high 664 mμ retinene band. This, however, has appeared heretofore in all retinene tests, and even if due wholly or in part to vitamin A is still no higher than occurs in extracts of dark adapted retinas (Wald, 1935–36). There is no present evidence that vitamin A is formed in the bleaching of rhodopsin solutions.

The benzine-soluble pigment from bleached rhodopsin exhibits the

color properties and pH changes of the entire residue. Spectra of a methanol solution of this pigment are shown in Fig. 8, (*a*) neutral, (*b*) saturated with carbon dioxide at 25°C., (*c*) in 0.25 N acetic acid, (*d*) in 0.56 N acetic acid, (*e*) in 0.6 N ammonia, and (*f*) in 1.3 N ammonia. Clearly the pH-lability of the final product of rhodopsin bleaching is due principally to this lipoidal pigment.

To compare accurately the spectrum of the benzine-soluble pigment with that of the whole bleached residue, both solvents and pH should be identical. This condition is approximated by diluting

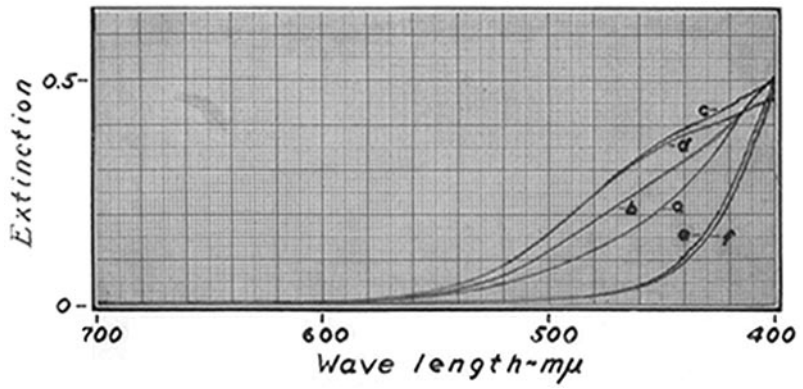


FIG. 8. Benzine-soluble pigment from the final residue of bleaching. Spectra of methanol solutions, (*a*) neutral; (*b*) saturated with carbon dioxide; (*c*) in 0.25 N acetic acid; (*d*) in 0.56 N acetic acid; (*e*) in 0.6 N ammonia; and (*f*) in 1.3 N ammonia. To bring to equivalent concentrations of pigment, multiply all ordinates of (*c*) by 1.02, (*d*) by 1.04, (*e*) by 1.04, and (*f*) by 1.1.

methanol solutions of the lipoidal pigment with buffered digitonin solution. The latter alone can dissolve small concentrations of retinene, forming solutions which differ very little spectroscopically from methanol solutions.

Curves *l* in Fig. 2 and *i* in Fig. 3 show spectra of the benzine-soluble fractions of final bleached residues, brought to the original volumes in mixtures of methanol and aqueous digitonin at pH 6.9. Such solutions resemble very closely the whole residues, but display considerably lower absorptions. The whole residues contain, in addition to benzine-soluble pigment and salts, various proteins, including

the protein component of rhodopsin itself, which absorb and scatter light principally at low wave lengths. The difference in extinction between the whole residue and its benzine-soluble fraction is a measure of the apparent absorption due to these non-lipoidal constituents. This matter is discussed in detail below.

State of Retinene in the Bleached Residue.—I have assembled elsewhere the evidence that rhodopsin is a conjugated protein, containing retinene or its carotenoid precursor as prosthetic group (Wald, 1935–36). At the close of bleaching the color and behavior of the residue closely resemble those of free retinene. The following experiments were undertaken to examine the association of retinene with protein in rhodopsin and in bleached solutions.

Free retinene dissolves very readily in benzine. Irradiated retinas yield their retinene quantitatively on simply shaking with this solvent. One cannot proceed so simply with rhodopsin solutions; to avoid the formation of viscous emulsions a special procedure is needed. Acetone quickly destroys rhodopsin at room temperatures liberating its retinene content in the process. I have found, however, that rhodopsin may be precipitated from solution quantitatively in the cold by adding an equal volume of acetone, with little immediate injury to the molecule. Precipitate and supernatant solution may be extracted separately in the cold with benzine or with benzine-acetone mixtures, still without decolorizing the rhodopsin. By this means the state of retinene may be examined in solutions of rhodopsin and of its bleached products.

When rhodopsin is precipitated in the cold with acetone practically all color descends with the precipitate. If precipitate and solution are extracted in the cold, the combined extract contains a very small amount of yellow pigment. This has never been obtained in quantities sufficient for identification. Its spectrum displays simple end-absorption. It may be retinene or some lipoidal impurity of the original solution. Its density at 400 mμ is only about 5 per cent of that of the total benzine-soluble fraction of the bleached residue.

The addition of an equal volume of acetone to a bleached solution yields a precipitate containing protein and most of the yellow color. The retinene which descends with this precipitate must be attached

to protein in the final bleached product. Free retinene would not be precipitated by addition of acetone; and the possibility that the retinene is adsorbed and carried down during the formation of the protein precipitate must be rejected, since carotenoids are not ordinarily adsorbed appreciably in the presence of high concentrations of acetone.

The extraction of retinene from this precipitate with benzine is subject to two conditions: (*a*) the benzine must contain a small quantity (1 to 2 per cent) of acetone or alcohol, preferably the latter; and (*b*) the extraction is greatly retarded at low temperatures. This behavior suggests two possibilities: (1) Retinene may remain in part adsorbed on the protein residue of the rhodopsin molecule after bleaching has broken its chemical bond. Small quantities of the typical carotenoid eluents, alcohol and acetone, liberate retinene by simple displacement from the adsorbent. (2) Retinene may remain in part chemically bound to protein in the final bleached product. Alcohol and acetone disrupt this complex just as they destroy rhodopsin itself in the warm, by attacking the protein.

By adding equal volumes of acetone to rhodopsin solutions in various stages of bleaching, and determining separately the retinene contents of the precipitate and of the supernatant solution, one may estimate the extent to which retinene is liberated during bleaching. Immediately following irradiation about five-sixths of the retinene is found to descend with the protein precipitate, and after all thermal components of bleaching are complete this fraction has fallen only to about two-thirds.

The final product of bleaching of rhodopsin in solution is therefore a mixture of retinene and protein, about two-thirds still loosely attached to each other in some manner that scarcely alters the retinene spectrum. It is this mixture to which Kühne at times applied the term "visual yellow," and which Lythgoe (1937) has suggested be called "indicator yellow." Both terms are ambiguous. The former has been applied at various times to probably all orange or yellow products of bleaching in both retinas and solutions; the latter fails to distinguish the final mixture from intermediate yellow or orange products of bleaching, all of which appear to be pH indicators.

V

The Spectrum of Rhodopsin

Rhodopsin solutions are principally contaminated with yellow and colloidal impurities, both of which, the former by absorption, the latter by differential scattering, raise the apparent extinction principally in the blue and violet. The ratio of the extinction at 400 mμ to that at the maximum, 500 mμ ("400/500 ratio"), therefore offers a convenient criterion of the purity of a preparation. The lower this ratio, the purer is the solution.

My preparations form two groups in this respect. Solutions of rhodopsin from bull frog retinas extracted directly or after preliminary soaking in brine exhibited 400/500 ratios of 0.52–0.59. Preparations from other animals, derived from retinas pretreated with alum, also fall into this group. A representative series of such spectra has been shown in Fig. 1. The relative constancy of these spectra in spite of considerable variation in the details of preparation suggested for a time the possibility that the solutions were approximately pure.

However, almost simultaneously a number of investigators including myself prepared rhodopsin solutions possessing 400/500 ratios of 0.30 to 0.35. These were derived from retinas pretreated with acid (Lythgoe, 1937), or with acidic alum solutions (Chase and Haig, 1937–38; present experiments), or by purifying initial extracts by fractional precipitation (Krause and Sidwell, 1938). The efficacy of alum treatment appears to be due to the fact that proteins other than rhodopsin are rendered insoluble. It is possible that rhodopsin itself is significantly changed by this treatment; but no specific difference in behavior has yet been detected between these and non-alum-treated preparations. For the present it may be assumed, therefore, that alum treatment does not significantly affect those properties of rhodopsin with which we are concerned. The spectrum of my best preparation from alum-treated retinas is shown in curve *a* of Fig. 6. Its 400/500 ratio is 0.32.

These rhodopsin solutions still contain unknown quantities of impurities, some of which may contribute to their absorption in the visible spectrum. It is possible, therefore, that the spectrum of pure rhodopsin lies considerably below that of any preparation yet meas-

ured. There is a strong disposition to believe that the pure rhodopsin spectrum is identical in form with the visibility function of the rods; in this case its absorption at 400 mμ should be only about 5 per cent of that at the maximum (*cf.* Lythgoe, 1937).

Hosoya and Bayerl (1933) have attempted to estimate empirically the absorption due to impurities in rhodopsin solutions. From the spectrum of a rhodopsin preparation they subtracted the spectrum of an extract of light-adapted retinas prepared in the same way. The resulting curve was unsymmetrical, maximal at about 500 mμ, and possessed a 440/500 ratio of 0.47. If this computation is reliable, the spectrum shown in curve *a* of Fig. 6 approximates that of pure rhodopsin, for its 440/500 ratio is almost precisely 0.47.

Hosoya and Bayerl's procedure, however, is open to serious question. The spectra of all their rhodopsin preparations indicate the presence of large and variable quantities of impurities. Presumably, control extracts of light-adapted retinas varied similarly. Large inaccuracies might consequently be expected to accompany estimation of the impurities in one preparation by those found in another. Only a single experiment of this sort is mentioned, and its outcome might possibly have been largely fortuitous.

With the aid of data presented in the foregoing pages the spectrum of pure rhodopsin may be established within narrow limits. Its upper limit obviously is the directly measured total absorption of a rhodopsin preparation. Its lower limit is fixed by the following procedure.

It is assumed that during the bleaching of rhodopsin in solution no other colored component changes its spectral characteristics. The final bleached residue contains newly formed retinene and protein, in addition to impurities present in the original solution. To a first approximation, retinene is the only benzine-soluble pigment in this mixture. This is extracted and its spectrum measured in the original volume of solvent. The difference between its absorption and that of the entire bleached residue is due to the protein product of bleaching and to impurities present in the original solution. This difference, therefore, sets an upper limit to the absorption in the original solution which may be ascribed to impurities or to the absorption or scattering of light by the protein fraction of rhodopsin itself. If this difference is subtracted from the spectrum of the rhodopsin preparation, the

resulting curve is the lower limit for the absorption of pure rhodopsin; or, expressed more precisely, the lower limit of absorption of the rhodopsin chromophore.

An example may clarify this procedure. Curve *a* of Fig. 3B possesses a 400/500 ratio of 0.56. The difference in absorption between its final bleached product (curve *h*) and its benzine-soluble fraction in clear solution (curve *i*) constitutes the upper limit of absorption in the bleached residue due to impurities and scattering of light by colloidal particles. If this difference is subtracted from spectrum *a*, the approximate lower limit of pure rhodopsin absorption is obtained. Its 400/500 ratio is 0.335. In five other preparations a similar procedure has yielded lower limit curves with 400/500 ratios of 0.30 to 0.34, with an average value of 0.32. The 440/500 ratios of these functions are close to 0.47, agreeing very well therefore with Hosoya and Bayerl's corrected spectrum and with the uncorrected spectra of preparations from alum-treated retinas.

This approximate calculation, however, requires some further adjustment. Ideally the benzine-soluble fraction should contain only the retinene produced by bleaching in free solution in buffered, colorless, 1 per cent digitonin. Actually it includes all benzine-soluble substances in the final bleached residue, in a buffered solution of digitonin in methanol-water mixtures. The discrepancies between these situations are counterbalanced as follows:

1. The digitonin used to prepare the solutions of Fig. 3 B itself absorbed light very slightly at low wave lengths. Its density in a 1.3 per cent solution—the concentration used in curve *i*—was measured and subtracted from curve *i*.

2. The benzine-soluble fraction in true solution in alcohol or chloroform does not absorb light appreciably at 700 mμ. In aqueous mixtures it does register a small absorption at this wave length, either real or due to scattering of light by the solution. I have compensated for the possibility that this absorption is an artifact by arbitrarily bringing the extinction at 700 mμ to zero; in the case of Fig. 3 B subtracting 0.003 from all values of curve *i*.

When these two corrections have been made the 400/500 ratio of the lower limit of rhodopsin absorption in Fig. 3B has fallen to 0.27.

3. There is some doubt that the benzine-soluble residue is in true

solution in aqueous methanol-digitonin mixtures. If this is really a colloidal emulsion or suspension, some of its apparent absorption might be due to scattering of light. Solution *i* in Fig. 3B appeared clear to the eye; but on more rigid examination it might have revealed some optical heterogeneity.

These first three difficulties do not arise at all in two experiments in which the absorption of the benzine-soluble pigment was measured in absolute methanol. Undoubtedly the solutions were "true," the methanol possessed no intrinsic absorption, and the extinctions of the solutions at 700 mμ were zero. The lower limits of rhodopsin absorption computed from these experiments possessed 400/500 ratios of 0.30. This is in good agreement with the above, probably somewhat over-corrected result.

4. The final correction is most important. Benzine-soluble colored substances may have been present in the original unbleached solution, and these must be estimated and reckoned among the impurities. For this purpose several rhodopsin solutions were precipitated with cold acetone and the precipitate and supernatant solution were extracted with benzine-acetone mixtures in the cold, as described in the preceding section. The absorptions of such extracts in two experiments were found to be negligible at 500 mμ; and at 400 mμ were 4.4 and 5.6 per cent of the extinctions at 500 mμ of the original rhodopsin. One may therefore correct for this factor approximately by subtracting from the extinction at 400 mμ of, for example, curve *i* of Fig. 3B, 5 per cent of the extinction at 500 mμ of curve *a*.

When this final correction has been applied to curve *i*, the difference between it and curve *h* represents the thoroughly corrected upper limit which may be assigned to absorption and scattering by impurities and by the protein component of rhodopsin. This absorption, subtracted from curve *a*, yields a lower limit spectrum for the pure rhodopsin chromophore possessing a 400/500 ratio of 0.20. Similar corrections applied to the two experiments in which the benzine-soluble pigment from bleached residues was measured in methanol solution reduce their lower limit 400/500 ratios to 0.23 and 0.24.

Fig. 9 shows the spectrum of the whole rhodopsin preparation, taken from Fig. 3B, and in broken lines the lower limit absorption spectrum derived from it. Both curves have been given the same

ordinate height by plotting extinctions on a percentage basis. The spectrum of pure rhodopsin lies within the limits set by these spectra.

The spectrum of a whole preparation from alum-treated retinas (from curve *a* of Fig. 6) is also shown in Fig. 9. If this be accepted as the upper limit of rhodopsin absorption—and there is no known reason to deny it this significance—then the spectrum of pure rhodopsin lies within the very narrow hatched area enclosed between this and the lower limit spectrum. One may with considerable confidence conclude from these observations that the extinction of pure rhodopsin

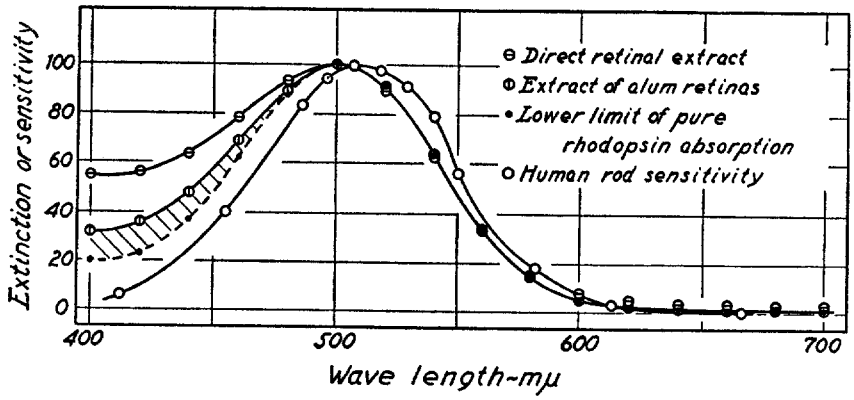


FIG. 9. Limits of absorption of pure rhodopsin, and human rod visibility. The latter function is from Hecht and Williams (1922–23). The direct retinal extract spectrum is from curve *a*, Fig. 3 B. The broken line shows the lower limit of rhodopsin absorption derived from it as described in the text. Between these curves is drawn the direct spectrum of an extract of alum-treated retinas, from curve *a* of Fig. 6. The spectrum of pure rhodopsin lies within the limits of the hatched area.

at 400 mμ lies between 0.20 and 0.32 of that at 500 mμ, and this exclusive of any absorption or scattering of light due to the protein residue of the rhodopsin molecule.

VI

Concentrations

Extracts of rhodopsin in the present experiments, though not complete, contained reasonably reproducible quantities of pigment. Extinctions of extracts of alum-treated retinas from bullfrogs, rabbits,

and rats are shown in Table I. These data were selected for maximal purity of the preparations, as evidenced by the absorption spectra. Extracts from non-alum-treated tissues were more variable, but contained about the same average quantities of pigment.

The third column of Table I presents the average extinction at 500 mμ of the total extract from one retina, concentrated in 1 cc., and

TABLE I

Extinctions at 500 mμ of Extracted Rhodopsin

Column III shows the extinction per retina, for the total extract in 1 cc., measured in a 1 cm. layer. By dividing the averages in this column by the retinal areas (column IV), the extinction of the total extract spread evenly over the retinal surface is obtained (column V). In column VI these values are reduced to percentages absorbed.

I	II	III	IV	V	VI
Animal	No. of retinas	Extinction per retina-1 cc., 1 cm.	Retinal area	Extinction per retinal surface	Absorption per retinal surface
			sq. cm.		*per cent*
Bullfrog	6	0.440			
	6	0.604			
	6	0.468			
	6	0.380			
	8	0.456			
	6	0.496			
Averages		0.472	2.65	0.178	33.6
Rabbit	8	0.116			
	8	0.104			
Averages		0.110	5.65	0.019	4.2
Rat	11	0.040	0.66	0.061	13.0

measured in a layer 1 cm. in depth. Expressed differently, this is the extinction of extracted rhodopsin per retina, spread uniformly over a surface 1 sq. cm. in area. These figures provide no relative measure of retinal concentrations until corrected with the appropriate retinal areas.

In the fourth column of the table the average areas of the retinas

are shown. They were obtained by measuring the diameter of each type of fundus and computing area as though the retina were spread over the surface of a hemisphere of this diameter.

By dividing the extinction per retina at area 1 sq. cm. by the retinal area, one obtains the values shown in the fifth column. These are the extinctions of extracted pigment per retina, as though spread in a uniform layer over the retinal surface. They are re-stated as percentage absorption in the sixth column. They show the bullfrog retina to possess about three times as great a density of rhodopsin as the rat retina, and this in turn about three times that of the rabbit retina.

These values constitute the lower limit which may be assigned to the extinction of rhodopsin *in situ*. The true extinction *in situ* is certainly higher, since the extractions were not quantitative; and since rhodopsin is not uniformly distributed in the retina, but is concentrated at discrete points, the outer limbs of the rods. This latter factor introduces a much greater discrepancy in the rabbit retina, which is considerably diluted with cones, than in the rat, which possesses very nearly a pure rod retina (Lashley, 1932; Walls, 1934).

VII

DISCUSSION

Rhodopsin Absorption and Rod Visibility.—The absorption spectrum of rhodopsin has been believed to resemble almost exactly the effectiveness of monochromatic lights in bleaching this pigment (Trendelenburg, 1904) and in stimulating the rods (König, 1894; Hecht and Williams, 1922–23).

This comparison has been based, not upon the absorption spectrum, but on the "difference spectrum" of rhodopsin. As Fig. 9 shows, the absorption spectrum of pure rhodopsin differs in form and position from the rod visibility curve.

A second consideration, however, negates these differences, and restores the original conclusion. The original comparison involved not only a mistaken absorption spectrum, but apparently also an inappropriate visibility function.

Hecht and Williams have accurately determined the rod visibility in a group of forty-eight observers of average age 25 years. Their

data are reproduced in the first two columns of Table II, and in Fig. 9. They show the visibility as reciprocal of relative energy incident on the corneal surface of the eye, required to stimulate a constant sensation of brightness.

TABLE II

Rod Visibility and the Limits of Absorption of Pure Rhodopsin

Columns I and II are from Hecht and Williams (1922–23). Column III is the dividend of column II by I. Column IV is from Roggenbau and Wetthauer (1927). Column V is the dividend of III by IV. Column VII is computed from Fig. 6 *a*, column VIII from Fig. 9, as explained in the text. All visibilities and absorptions have been reduced to a common percentage basis for comparison.

I	II	III	IV	V	VI	VII	VIII
Wave length	Energy visibility at cornea	Quantum visibility at cornea	Transmission of ocular media	Quantum visibility at retina	Wave length	Rhodopsin absorption (upper limit)	Rhodopsin absorption (lower limit)
mu			*per cent*		*mu*	*per cent*	*per cent*
412	6.32	7.82	19.3	18.3	400	35.2	22.0
455	39.95	44.7	33.0	61.4	420	39.4	23.5
486	83.40	87.5	40.5	94.2	440	51.9	39.4
496	93.90	96.5	43.8	100	460	72.5	64.3
507	99.35	100	45.7	99.0	480	90.8	88.3
518	97.30	95.8	47.8	90.9	500	100	100
529	91.10	87.8	49.9	79.9	520	91.7	91.7
540	78.78	74.4	51.0	65.7	540	66.3	67.2
550	55.60	51.5	52.8	43.4	560	37.3	36.5
582	17.78	15.6	57.5	12.3	580	15.8	14.9
613	2.72	2.26	58.7	1.75	600	5.8	5.8
666	0.181	0.138	66.5	0.093	620	3.3	3.3
					640	2.1	2.1
					660	1.7	1.7
					680	1.2	1.2
					700	0.8	0.8

The incident energy is absorbed by rhodopsin in discrete quanta, which vary in size inversely with the wave length, but are very likely equivalent in their effects. To appreciate its action on rhodopsin, incident energy should be converted into relative numbers of incident quanta. This is done by multiplying the energies by the respective wave lengths, or alternatively by dividing the respective visibilities by the wave lengths. The results are shown in column III of Table II.

The change from energy visibility to quantum visibility is very small, but in the direction of better fit with the absorption data (Dartnall and Goodeve, 1937).

To this point the visibility data deal only with the numbers of quanta incident on the corneal surface. For comparison with their effects on the retinal rhodopsin it is necessary to know the proportions of incident quanta at each wave length which penetrate to the outer limbs of the rods.

It is commonly stated in the ophthalmological literature that the transmission of human ocular media is practically complete throughout the visible spectrum. This statement is based upon very rough qualitative observations. Recent exact measurements show that actually the ocular tissues absorb a large fraction of the incident light, particularly at low wave lengths. Roggenbau and Wetthauer (1927), in a careful examination of the transmission of cornea, vitreous, and lens in cattle, have shown that at 400 mμ only about 16 per cent of the incident energy is transmitted to the retinal surface, while at 500 mμ the transmission has risen only to about 44 per cent. Ludvigh and McCarthy at the Howe Laboratories of Ophthalmology in Boston have recently determined the transmission of human ocular tissues in the visible. These data are not yet published, and have not been available to me. However, Dr. Ludvigh has kindly informed me that the human data for an age group averaging 22 years resemble closely the data of Roggenbau and Wetthauer without correction. In younger human eyes the average transmissions are higher, in older eyes lower.

One may therefore, as a first approximation, use the Roggenbau and Wetthauer transmission data to correct the Hecht and Williams visibility function. This is almost certainly in part an under-correction, since the transmission data go only to the retinal inner surface, and so do not include absorption of light by the retinal tissues which screen the outer limbs of the rods. Particularly in the mammalian retina, with its rich network of capillaries, this factor might involve a large additional absorption of light.

Transmission of light to the retinal surface, taken from the data of Roggenbau and Wetthauer, is presented in column IV of Table II. The numbers in column III, divided by these factors, yield the "ret-

inal visibility," the reciprocals of relative numbers of quanta incident on the surface of the retina, required to stimulate a constant brightness sensation. These are shown in column V, and in Fig. 10.

It may be noted that it is only the retinal visibility, ideally the reciprocals of numbers of quanta incident on the outer limbs of the rods, that should possess a unique form. Any more peripheral type of visibility curve should vary with the individual transmission char-

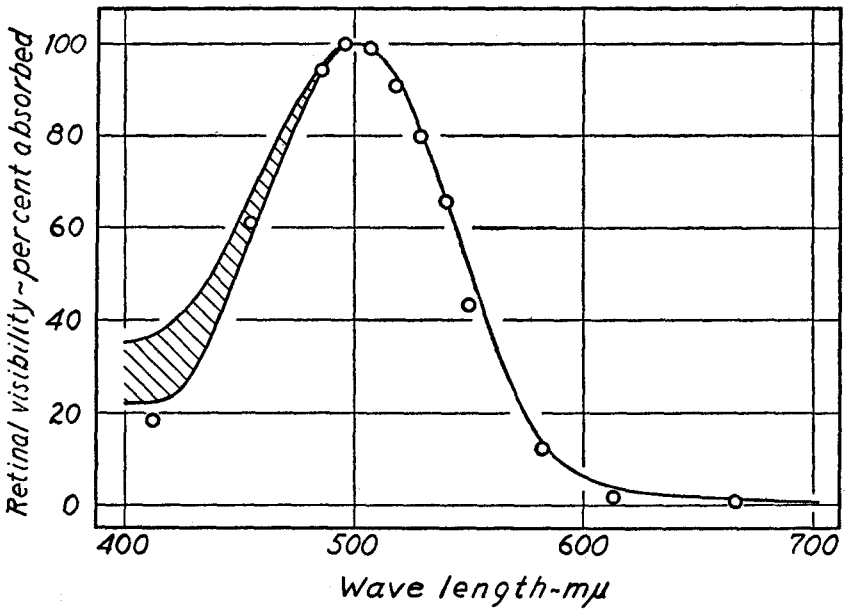


FIG. 10. Human rod visibility at the inner retinal surface (circles), and the absorption limits of pure rhodopsin *in situ* (hatched area), computed as described in the text. To facilitate comparison, these functions are plotted as percentages of their respective maxima.

acteristics of the observer's eye. Such variation has been clearly demonstrated in Abney and Watson's measurements of "external" rod visibility in various retinal areas of eight observers (1915). In fields 10° above the fovea, for example, the wave length of maximum visibility varied from about 495 mμ in the case of a student, *Ar*, to about 515 mμ in Abney himself; and the visibilities at 425 mμ in these two instances varied from 13 to 5.6 per cent of those at the re-

spective maxima. A thoroughly corrected retinal visibility function should be absolute and invariant; but computation of such a relation must await the collection of visibility and ocular transmission data in statistically significant quantities.

The retinal visibility is to be compared with the absorption spectrum of pure rhodopsin in solution. Hecht has stated the theoretical basis for the belief that visibility varies proportionately with the *percentage absorption* (Hecht and Williams, 1922–23).[6] Though the form of an extinction spectrum is independent of concentration and depth of the absorbing layer—since the extinction is directly proportional to both these parameters—the shape of a percentage absorption spectrum is specific for one concentration and depth of layer. For accurate comparison it is necessary, therefore, to estimate the true percentage absorption of rhodopsin *in situ* in the retina under consideration.

It was shown above (Table I) that the extinction at 500 mμ of rhodopsin extracted from rat retinas, computed as though spread in a homogeneous layer over the retinal surface, is 0.061; and it was indicated that the extinction of rhodopsin in the rod outer limbs is surely considerably greater. We may estimate roughly the extinction *in situ* as double this value, or about 0.12.[7]

[6] It is assumed that to produce a constant visual effect, a constant quantity of light—or more accurately a constant number of quanta—must be absorbed. If I is the incident intensity at any wave length, and α the percentage absorption, then for constant visual effect, $\alpha I = constant$. But $1/I$ for constant visual effect is the visibility. Hence the latter is directly proportional to α. Recently Hecht (1937) has pointed out that α is related to ϵ, the absorption coefficient (= 2.303 × extinction) by an expansion of the form, $\alpha = \epsilon - \frac{\epsilon^2}{2!} + \frac{\epsilon^3}{3!} \ldots$. For small values of ϵ all but the first member of this series may be neglected and ϵ—or extinction—substituted directly for α in comparisons with the visibility. In the present computations, however, this substitution entails considerable error. I have preferred, therefore, to use the older, precise relation.

[7] I have chosen the rat retina for this estimate, as a structure in which the rods approximate those of the human retina and are almost undiluted with cones. Dartnall and Goodeve (1937), starting with data of frog extractions, have arrived at 0.1 as a probable extinction of rhodopsin *in situ* in the human eye. The close correspondence between their estimate and mine is fortuitous. As these authors properly indicate, doubling this estimate would not appreciably affect the argument.

The limits of rhodopsin absorption, shown as the hatched area in Fig. 9, have therefore been re-computed with extinctions at 500 mμ set at 0.12. They have then been converted into percentages absorbed, and the maxima arbitrarily set at 100 for purposes of comparison. They are presented in this form in the last three columns of Table II and in Fig. 10.

It is clear that the limits here assigned for rhodopsin absorption *in situ* agree very closely with the provisional retinal visibility. The small departure of the point at 412 mμ is scarcely to be regarded seriously. As already indicated, the visibility function is almost certainly under-corrected, and all of the low wave length points should probably be raised. Within the limitations of available information, therefore, it may be concluded that the retinal visibility agrees in both form and position with the absorption spectrum of pure rhodopsin.

Kinetics.—The present results show the complete process of bleaching of rhodopsin in solution to be complicated beyond any present hope of useful kinetic analysis. It should be possible, however, to regulate bleaching so that its over-all velocity is limited by the speed of the photoprocess alone. In this way the kinetics of the isolated light reaction might be explored.

Chase (1935–36), using irradiation intensities much lower than those of the present research, found the kinetics of bleaching in acid and cold solutions to present "anomalies," while in warmer and more alkaline solutions the results permitted simpler interpretation. Chase ascribed these differences to the appearance of yellow reaction products under the former conditions, their absence under the latter. As shown above, new yellow products actually appear in all known circumstances.

The conditions for precise measurement of the kinetics of the photoprocess may be stated as follows:

1. The velocity of the light reaction must limit that of the entire transformation. This is accomplished by decreasing its speed by lowering the irradiation intensity, while simultaneously hastening the thermal components by raising the temperature and alkalinity. These conditions ensure that at all stages of bleaching only rhodopsin and the final yellow product (retinene-protein) are present in the reaction mixture.

2. The concentration of rhodopsin in this mixture must be meas-

ured. Obviously this is not given directly by the extinction at any wave length at which the yellow product itself absorbs light. It is clear from Fig. 8 that this prohibition involves appreciably all wave lengths below about 580 mμ in neutral or acid solutions, and below about 500 mμ in alkaline solutions. The error introduced in this way in measurements of decrease in extinctions of neutral solutions at 500 mμ is of the order of 10 to 15 per cent. Trendelenburg's measurements of bleaching in presumably neutral solution at 589 mμ, and Chase's measurements at 500 mμ and pH 9.3, are probably the only existent accurate spectrophotometric descriptions of the photoprocess.

Hecht's detailed measurements of rhodopsin bleaching kinetics still remain the most acceptable (1920–21, 1923–24). Hecht principally employed low intensities of irradiation at room temperatures, so that bleaching occupied from 1 to 2 hours. Under these conditions the speed of the light reaction almost certainly limits the velocity of bleaching. Concentrations were measured by the rough but otherwise unexceptionable method of visual comparison with standard mixtures of rhodopsin and the final yellow product. In a number of Hecht's experiments in which relatively high intensities and low temperatures were employed, the light reaction should not have limited the velocity of bleaching. Yet such experiments did not yield anomalous results. Apparently Hecht's colorimetric method succeeds in estimating the rhodopsin content of bleached solutions comparatively undisturbed by the state of the bleached products. It is curious that this superficially rough procedure seems much better suited to the purpose than the more elegant spectrophotometric methods since employed. There is no present reason for doubting Hecht's results. They show that the photoprocess is first order, and that its velocity is directly proportional to the light intensity, and is practically independent of the temperature between 6.1 and 36.1°C.

Bleaching and Denaturation.—Most of the available evidence that rhodopsin is a protein involves its sensitivity to common protein denaturants (Wald, 1935–36). Recently Mirsky (1936) has suggested that the bleaching of rhodopsin by light is a denaturation process.

The term denaturation includes a number of diverse changes: primarily the loss of solubility at the isoelectric point; and associated

with this in varying degree a loss of specificity, or of ability to crystallize or to spread on a surface, increase in the number of exposed reducing groups, and changes in acid-base properties (Neurath, 1936; Mirsky and Pauling, 1936; Bull and Neurath, 1937). None of these changes has yet been shown to accompany the bleaching of rhodopsin by light. Unless the definition of denaturation is expanded to include all changes in the prosthetic groups of conjugated proteins, I see no present reason for calling the photodecomposition of rhodopsin a denaturation.

It seems to me that denaturation of rhodopsin may occur with bleaching, or without bleaching, or that bleaching may occur without denaturation, depending upon what is done to the molecule. Strong acids and alkalies, alcohol, chloroform, acetone in the warm, and temperatures above 60°C. all convert rhodopsin irreversibly to yellow with denaturation of the protein residue. It may be that careful experimentation will eventually show some of these effects to be reversible. The behavior of the related carotenoid-protein, ovoverdin, foreshadows what may be expected of rhodopsin in this regard (Stern and Salomon, 1938).

On the other hand the protein residue of rhodopsin may be profoundly altered without a trace of bleaching; as when retinas are treated for long periods with 8 per cent formol, and probably also when they are soaked for days in 4 per cent alum, or are allowed to rot intensively at 40°C. (Ayres, 1882).

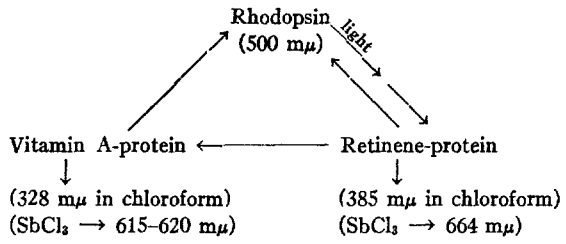
Finally, the prosthetic group of rhodopsin may be attacked directly by absorption of light. That in this case the protein residue is appreciably affected, apart from changes in the carotenoid-protein bond, is still to be demonstrated.

There is a particularly interesting situation in which denaturation processes may cooperate with photoprocesses to produce bleaching. This appears among the observations of Ewald and Kühne (1878).[8] Below about 37°C. bleaching is typically photochemical; it occurs only in light, and its velocity is practically independent of the temperature (see also Hecht, 1920–21). Above about 50°C. bleaching occurs by a typical denaturation process, in darkness, and possesses a very high temperature coefficient. In the intermediate range from

[8] Ewald and Kühne, 1878, IV, 440.

40 to 50°C., bleaching requires light, but possesses also a high temperature coefficient. It is possible that within this range the increase in internal energy of rhodopsin molecules effectively raises the quantum efficiency.

The Visual Cycle.—The final product of bleaching of rhodopsin in solution is a mixture of retinene and protein, in part loosely bound to each other. In the isolated retina, two reactions are added to the system *in vitro*: (1) more pronounced and regular reversion of retinene to rhodopsin; and (2) conversion of retinene to vitamin A. Finally, in the intact eye, the visual cycle is completed with reversion of vitamin A to rhodopsin (Wald, 1935–36; 1936–37). The entire cycle is expressed conveniently in the following skeletal form:



The bleaching of rhodopsin to retinene-protein has now been shown to proceed by a complex succession of photic and thermal processes. Other reactions in the retinal cycle may be similarly complicated. The arrangement of apparent precursors as written here is also not to be interpreted rigidly. Now that retinene is recognized to be only the last of a series of products of bleaching, it is clear that any of the intermediates might be the true precursor for formation of either rhodopsin or vitamin A.

It is noteworthy in this connection that the color of retinas in all stages of bleaching, following exposure to bright light, is orange, not yellow; and resembles therefore the color of the initial photoproducts rather than that of retinene. This distinction is apparently not simply a physical effect, for on strongly acidifying an orange retina the strikingly different yellow color of acidic retinene at once appears (Wald, 1936–37). It is quite possible, therefore, that the color of irradiated retinas is due principally to the initial photoproducts. Certainly retinene is present in the retina, since it may be extracted di-

rectly with benzine; but perhaps normally it occurs in low concentration. We may imagine retinene to be in mobile equilibrium in the irradiated retina with orange photoproducts; extraction displaces the equilibrium and removes all the pigment in the form of retinene. On the other hand, in solutions of irradiated rhodopsin, retinene accumulates because it is formed irreversibly. From this point of view retinene may occupy much the same position in the retinal cycle that hexose diphosphate does in muscle—that of an intermediate normally present in very small amounts, but capable of accumulating in large quantity when the normal system is reduced by poisoning of the tissue or by extraction (Meyerhof, 1937).

These are matters for further experimentation to decide. For the present the cyclic scheme presented above expresses correctly certain relations among the three most stable loci in the complex retinal cycle. It summarizes simply a large number of the empirical observations. It has already proved useful in describing the behavior of the functioning visual system (Winsor and Clark, 1936; Riggs, 1937; Hecht, Haig, and Chase, 1936–37; Wald and Clark, 1937–38).

SUMMARY

1. The properties of rhodopsin in solution have been examined in preparations from marine fishes, frogs, and mammals.

2. The bleaching of neutral rhodopsin in solution includes a photic and at least three thermal ("dark") processes. Thermal reactions account for approximately half the total fall in extinction at 500 mμ.

3. Bleaching has been investigated at various pH's from 3.9 to about 11. With increase in pH the velocity of the thermal components increases rapidly. Though the spectrum of rhodopsin itself is scarcely affected by change in pH, the spectra of all product-mixtures following irradiation are highly pH-labile.

4. The spectrum of pure rhodopsin—or of the rhodopsin chromophore—is fixed within narrow limits. The extinction at 400 mμ lies between 0.20 to 0.32 of that at the maximum.

5. Within the limitations of available data, the spectrum of pure rhodopsin corresponds in form and position with the spectral sensitivity of human rod vision, computed *at the retinal surface.*

6. The nature of bleaching of rhodopsin in solution, its kinetics, and its significance in the retinal cycle are discussed.

REFERENCES

Abney, W. deW., and Watson, W., The threshold of vision for different coloured lights, *Phil. Tr. Roy. Soc. London, Series A*, 1915, **216,** 91.

Ayres, W. C., Zum chemischen Verhalten des Sehpurpurs, *Untersuch. physiol. Inst. Heidelberg*, 1882, **2,** 444.

Bayliss, L. E., Lythgoe, R. J., and Tansley, K., Some new forms of visual purple found in sea fishes, *Proc. Roy. Soc. London, Series B*, 1936, **120,** 95.

Bull, H. B., and Neurath, H., Denaturation and hydration of proteins. II. Surface denaturation of egg albumin, *J. Biol. Chem.*, 1937, **118,** 163.

Chase, A. M., Anomalies in the absorption spectrum and bleaching kinetics of visual purple, *J. Gen Physiol.*, 1935–36, **19,** 577.

Chase, A. M., and Haig, C., The absorption spectrum of visual purple, *J. Gen. Physiol.*, 1937–38, **21,** 411.

Dartnall, H. J. A., and Goodeve, C. F., Scotopic luminosity curve and the absorption spectrum of visual purple, *Nature*, 1937, **139,** 409.

Ewald, A., and Kühne, W., Untersuchungen über den Sehpurpur. I–IV, *Untersuch. physiol. Inst. Heidelberg*, 1878, **1.**

Garten, S., Über die Veränderungen des Sehpurpurs durch Light, *Arch. Ophth., Leipsic*, 1906, **63,** 112.

Hardy, A. C., A new recording spectrophotometer, *J. Opt. Soc. America*, 1935, **25,** 305.

Hecht, S., Photochemistry of visual purple. I. The kinetics of the decomposition of visual purple by light, *J. Gen. Physiol.*, 1920–21, **3,** 1.

Hecht, S., Photochemistry of visual purple. II. The effect of temperature on the bleaching of visual purple by light, *J. Gen. Physiol.*, 1920–21, **3,** 285.

Hecht, S., Photochemistry of visual purple. III. The relation between the intensity of light and the rate of bleaching of visual purple, *J. Gen. Physiol.*, 1923–24, **6,** 731.

Hecht, S., Rods, cones, and the chemical basis of vision, *Physiol. Rev.*, 1937, **17,** 239.

Hecht, S., Chase, A. M., Shlaer, S., and Haig, C., The regeneration of visual purple in solution, *Science*, 1936, **84,** 331.

Hecht, S., Haig, C., and Chase, A. M., The influence of light adaptation on subsequent dark adaptation of the eye, *J. Gen. Physiol.*, 1936–37, **20,** 831.

Hecht, S., and Williams, R. E., The visibility of monochromatic radiation and the absorption spectrum of visual purple, *J. Gen. Physiol.*, 1922–23, **5,** 1.

Hosoya, Y., Absorptionsspektrum des Sehpurpurs und des Sehgelb. "Nachbleichung des Sehgelb im Dunkeln," *Arch. ges. Physiol.*, 1933, **233,** 57.

Hosoya, Y., and Bayerl, V., Spektrale Absorption des Sehpurpurs vor und nach der Belichtung, *Arch. ges. Physiol.*, 1933, **231,** 563.

Hosoya, Y., and Saito, Z., Untersuchungen über die Bildung des Sehgelbs und des sogennanten Sehweisses bei der Sehpurpurbleichung. I. Beeinflussung der spektralen Absorption des Sehpurpurs und des Sehgelbs durch verschiedene Bedingungen bei der Extraktion, *Tohoku J. Exp. Med.*, 1935, **27,** 172.

König, A., Ueber den menschlichen Sehpurpur und seine Bedeutung für das Sehen, *Sitzungsber. Akad. Wissensch. Berlin*, 1894, 577.

Köttgen, E., and Abelsdorff, G., Absorption und Zersetzung des Sehpurpurs bei den Wirbeltieren, *Z. Psychol. u. Physiol. Sinnesorgane*, 1896, **12**, 161.

Krause, A. C., and Sidwell, A. E., The absorption spectra of visual purple and its photodecomposition products, *Am. J. Physiol.*, 1938, **121**, 215.

Kühne, W., Über den Sehpurpur, *Untersuch. physiol. Inst. Heidelberg*, 1878, **1**, 15.

Kühne, W., Chemische Vorgänge in der Netzhaut, in Hermann, L., Handbuch der Physiologie, Leipsic, F. C. W. Vogel, 1879, **3**, 235.

Kühne, W., Zur Darstellung des Sehpurpurs, *Z. Biol.*, 1895, **32**, 21.

Lashley, K. S., The mechanism of vision. V. The structure and image-forming power of the rat's eye, *J. Comp. Psychol.*, 1932, **13**, 173.

Lythgoe, R. J., The absorption spectra of visual purple and of indicator yellow, *J. Physiol.*, 1937, **89**, 331.

Meyerhof, O., Über die Intermediärvorgänge der enzymatischen Kohlehydratspaltung, *Ergebn. Physiol.*, 1937, **39**, 10.

Mirsky, A. E., The visual cycle and protein denaturation, *Proc. Nat. Acad. Sc.*, 1936, **22**, 147.

Mirsky, A. E., and Pauling, L., On the structure of native, denatured, and coagulated proteins, *Proc. Nat. Acad. Sc.*, 1936, **22**, 439.

Nakashima, M., Beiträge zur Kenntnis des Sehpurpurs, *XIII Conc. Ophth.*, Amsterdam, 1929, **1**, 317.

Neurath, H., Influence of denaturation on the spreading of proteins on a water surface, *J. Physic. Chem.*, 1936, **40**, 361.

Riggs, L. A., Dark adaptation in the frog eye as determined by the electrical response of the retina, *J. Cell. and Comp. Physiol.*, 1937, **9**, 491.

Roggenbau, C., and Wetthauer, A., Zur Frage der Durchlässigkeit der Hornhaut, Linse und des Glaskörpers für Kurzwelliges Licht nach Untersuchungen an Rinds-und Kalbsaugen, *Klin. Monatsbl. Augenheilk*, 1927, **78**, 762.

Roggenbau, C., and Wetthauer, A., Über die Durchlässigkeit der brechenden Augenmedien für langwelliges Licht nach Untersuchungen am Rindsauge, *Klin. Monatsbl. Augenheilk.*, 1927, **79**, 456.

Stern, K. G., and Salomon, K., On ovoverdin, the carotenoid-protein of the egg of the lobster, *J. Biol. Chem.*, 1938, **122**, 461.

Tansley, K., The regeneration of visual purple: its relation to dark adaptation and night blindness, *J. Physiol.*, 1931, **71**, 442.

Trendelenburg, W., Quantitative Untersuchungen über die Bleichung des Sehpurpurs in monochromatischem Licht, *Z. Psych.*, 1904, **37**, 1.

Wald, G., Carotenoids and the visual cycle, *J. Gen. Physiol.*, 1935–36, **19**, 351.

Wald, G., Pigments of the retina. I. The bullfrog, *J. Gen. Physiol.*, 1935–36, **19**, 781.

Wald, G., Pigments of the retina. II. Sea robin, sea bass, and scup, *J. Gen. Physiol.*, 1936–37, **20**, 45.

Wald, G., Bleaching of visual purple in solution, *Nature*, 1937 *a*, **139**, 587.
Wald, G., Visual purple system in fresh-water fishes, *Nature*, 1937 *b*, **139**, 1017.
Wald, G., Photolabile pigments of the chicken retina, *Nature*, 1937 *c*, **140**, 545.
Wald, G., and Clark, A.-B., Visual adaptation and chemistry of the rods, *J. Gen. Physiol.*, 1937–38, **21**, 93.
Walls, G. L., The visual cells of the white rat, *J. Comp. Psychol.*, 1934, **18**, 363.
Weigert, F., and Nakashima, M., Photodichroismus und Photoanisotropie. VI. Die Farbenanpassung des Sehpurpurs, *Z. phys. Chem., Abt. B*, 1930, **7**, 25.
Winsor, C. P. and Clark, A.-B., Dark adaptation after varying degrees of light adaptation, *Proc. Nat. Acad. Sc.*, 1936, **22**, 400.