IODOPSIN

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Of the two groups of light receptors found in the vertebrate retina—the rods and cones—there is every reason to be more interested functionally in the cones. These are the organs of daylight vision. We depend upon them principally for the appreciation of form, and perhaps entirely for the appreciation of color.

Nevertheless, until very recently the rods provided almost all that is known of visual substances and processes. The reason is that rods contain much more visual pigment than cones. Rods when dark-adapted are brightly colored, either red, owing to rhodopsin; or purple, owing to porphyropsin. Cones, however, look colorless under all circumstances (*cf.* Kühne, 1879). Kühne concluded that the cones lack light-sensitive pigments; but that is impossible. Without photosensitive pigments there can be no vision. For light to act in any fashion, it must be absorbed; and for visible light, this demands a pigment. If one fails to see such a pigment in cones, this can mean only that it is too dilute to be apparent. Precisely because the cones contain so little light-sensitive pigment, intense light is required to stimulate them. It is this that makes them the organs of daylight vision.

Hence the first attempt to extract a photosensitive pigment from cones was made with the chicken retina, which contains a few rods among a large predominance of cones (Wald, 1937 *b*). From it an impure mixture of rhodopsin and the cone pigment was extracted. The cone pigment was identified by its special sensitivity to deep red light, which scarcely affects rhodopsin. An extract of dark-adapted chicken retinas exposed to red light of wave lengths longer than 650 mμ bleaches slightly, the extinction falling maximally at 560 to 575 mμ, depending upon the pH. This is the cone pigment. Judging from its spectral properties, it is violet in color. It was therefore called *iodopsin* (Greek *ion* = violet). After the bleaching in red light is completed, the residue, exposed to white light, bleaches further, the extinction now falling maximally at 505 to 510 mμ; this is rhodopsin. These observations were confirmed in detail

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by Bliss (1945–46 *a*), who showed also that iodopsin is most sensitive to light at about 560 mμ, close to its apparent absorption maximum.

Iodopsin is so like rhodopsin and porphyropsin in its general properties as to suggest that it too is a carotenoid-protein (Wald, 1937 *b*). In human vitamin A deficiency, cone vision deteriorates in parallel with rod vision; and both repair together on administration of vitamin A. It seemed probable therefore that iodopsin, like rhodopsin, is derived from vitamin A_1 (Wald, Jeghers, and Arminio, 1938; Hecht and Mandelbaum, 1938).

This suggestion received some support from Bliss's observation that a brei of chicken retinas exposed to red light liberates a lipid with maximal absorption at 390 mμ, resembling retinene (1945–46 *b*). The present experiments establish this point unequivocally. The bleaching of iodopsin, as of rhodopsin, yields retinene as one of its products.

Indeed all the carotenoid components of the rhodopsin and iodopsin systems are identical. What differentiates these systems chemically is the protein—the opsin—with which the carotenoid combines. The opsins of cone vision may be called *photopsins* to distinguish them from the *scotopsins* of the rods. It is the substitution of photopsin for scotopsin that converts the rhodopsin to the iodopsin system.

The Product of Bleaching Iodopsin

The bleaching of rhodopsin liberates retinene, in the sense that it can be extracted from the bleached retina with such an indifferent fat solvent as petroleum ether. Retinene is identified by its absorption maximum (λ_{max}) in hexane at about 365 mμ or in chloroform at about 387 mμ; and by the blue color which it yields when mixed with antimony chloride, associated with a specific absorption maximum at 664 mμ.

In the chicken retina, which contains both rhodopsin and iodopsin, exposure to red light might be expected to reveal the product of bleaching iodopsin alone; while subsequent exposure to white light, by bleaching rhodopsin, should yield retinene.

Bliss (1945–46 *b*) has described such an experiment. Chicken retinas were frozen-dried, and preextracted with petroleum ether to remove the carotenoid pigments of the cone oil globules. Part of the dry retinal powder which resulted was suspended in water and irradiated with red light, to bleach iodopsin. Another portion was similarly irradiated with yellow light, to bleach iodopsin and rhodopsin. Both preparations were extracted with petroleum ether containing 2 per cent ethyl alcohol; and the absorption spectra of the extracts measured in chloroform. In both cases a principal absorption maximum was found at about 390 mμ, close therefore to that of retinene. The two spectra, however, differed in shape; and Bliss was unable to obtain satisfactory antimony chloride tests from either of these preparations. He concluded that the

bleaching of iodopsin yields a product similar to retinene, though not specifically identified.

We had performed comparable experiments, but questioned them on another score (Wald, 1949). The chicken retina contains more rhodopsin than iodopsin, and it is difficult to be sure that the latter rather than the former is the source of the small quantity of retinene which emerges. It should be noted in this regard that the Wratten 70 filter which Bliss used for irradiation with red light is not suitable for this experiment. It has a serious radiation leak in the violet and blue (350 to 490 mμ) (Wald, 1945 *a*); and the light which it transmits bleaches rhodopsin at an appreciable rate.

Figs. 1 and 2 show the results of an experiment which attempts to meet these difficulties. A chicken retinal preparation was exposed to red light known not to bleach rhodopsin in solution; and was subsequently exposed to white light to bleach rhodopsin. The direct spectra and the spectra of the antimony chloride tests show the product of both irradiations to be retinene.

Experiment.—Twenty-two adult chickens were dark-adapted overnight. Their retinas were dissected out under dim red light, and were frozen at once in dry ice. They were lyophilized overnight in the dark. The dry tissue was broken up, and extracted 3½ hours by soxhlet apparatus in the dark with petroleum ether (b.p. 20–40°C.), to remove colored carotenoids. The residue was ground to a fine powder, 4 ml. of hexane added,[1] and this mixture stirred slowly by motor in the dark for 30 minutes. Then it was centrifuged and the hexane extract decanted, and replaced with 4 ml. of fresh hexane. The extraction was repeated exactly as before, in the red light from a 100 watt lamp, passing through a red Corning 2403 filter and a Jena BG19 heat filter. After centrifuging and decanting this second extract, the residue was extracted a third time exactly as before, but in bright white light. That is, the same retinal tissue was extracted three times with hexane, first in the dark, then in red, and finally in white light.

The absorption spectra of these extracts are shown in the upper part of Fig. 1. The "dark"extract displays the absorption spectrum of a retinal carotenoid which, despite the long preextraction with petroleum ether, is still emerging. In the successive hexane extracts this pigment appears in smaller and smaller amount; but the irradiation with red light produces a distinct *rise* in absorption throughout the near ultraviolet, and the final irradiation with white light brings out a high band in this region, maximal at about 368 mμ.

The colored carotenoid present in all these extracts is easily eliminated from the result. Above about 450 mμ, the absorption spectra of all three extracts involve this contaminant alone, and are simple multiples of one another in the ratio, dark/red/white = 1.0/0.75/0.54. Hence one has only to subtract 0.75

[1] We use the term *hexane* to denote a fraction of petroleum ether which boils in the range of 60–80°C., and which consists primarily of hexane.

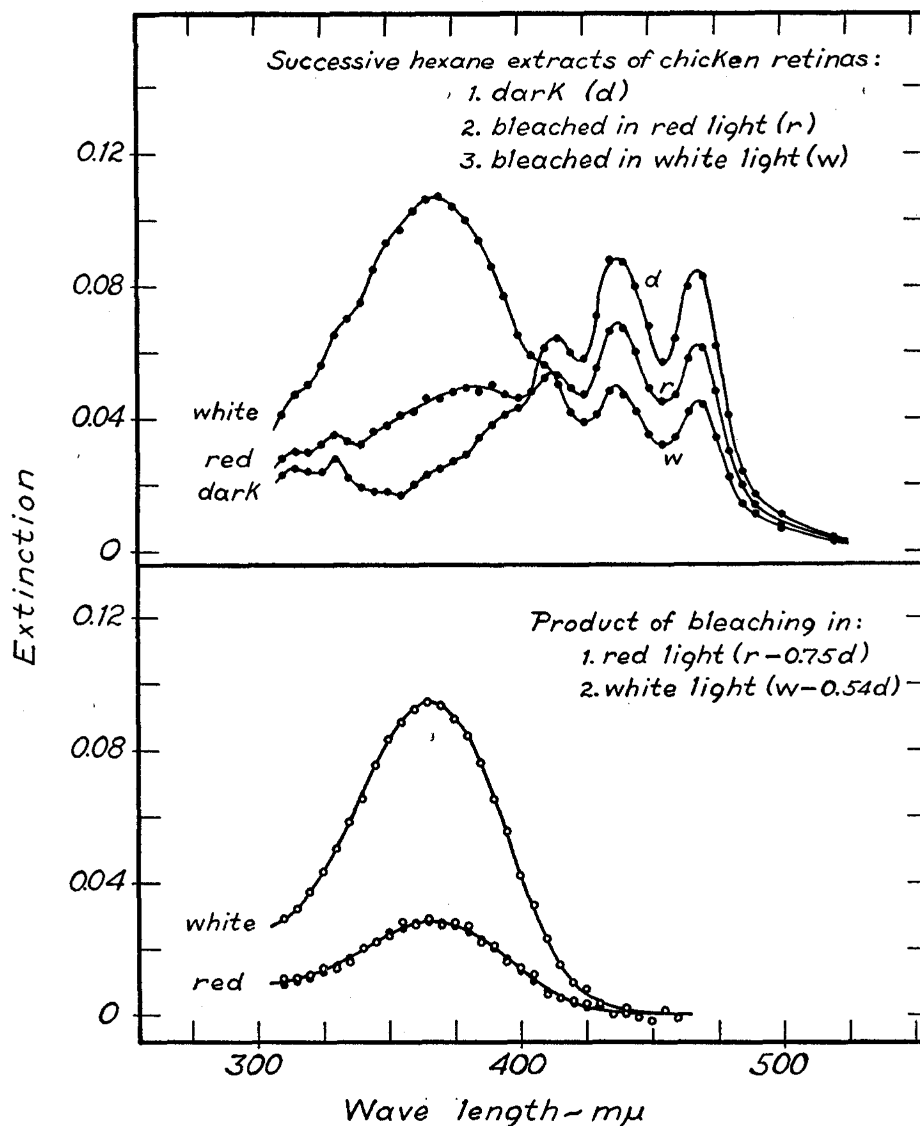


FIG. 1. *Top*, absorption spectra of successive hexane extractions of a chicken retina powder: (*d*) in the dark; (*r*) in deep red light, which bleaches iodopsin; and (*w*) in white light, which bleaches rhodopsin. The "dark" extract exhibits the three-banded spectrum of a retinal carotenoid. In the successive extractions, this declines regularly, while a new absorption rises in the near ultraviolet in response to the bleaching of the visual pigments. *Bottom*, to evaluate the products of bleaching, proper amounts of the spectrum of the indifferent carotenoid (*d*) are subtracted from (*r*) and (*w*). The curves drawn through the open circles result, representing the products of bleaching iodopsin (red) and rhodopsin (white). Their identity of shape is demonstrated by plotting together with the "red" curve, 0.30 times the "white" curve (solid circles). Iodopsin and rhodopsin yield the same product, retinene$_1$.

times the "dark" spectrum from that obtained in red light, and 0.54 times the "dark" spectrum from that obtained in white light, to have the spectra of the products of irradiation alone.

These are shown in the lower part of Fig. 1. Both spectra have absorption maxima at 365 mμ, the λ_{max} of retinene$_1$ in hexane.[2] To compare their shapes, the "white" curve has been reduced to the same height as the "red" by multiplying all its ordinates by 0.30; these points are plotted as solid circles. It is clear that both spectra are identical.

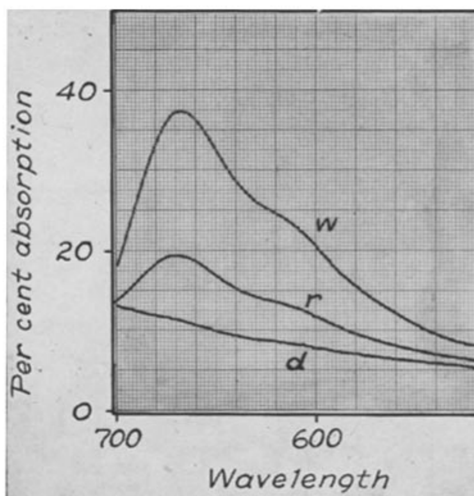


FIG. 2. Absorption spectra of antimony chloride tests with the three successive extracts of chicken retinal tissue shown in Fig. 1 (top). The "dark" extract (*d*) yields only unspecific absorption. The "red" and "white" extracts (*r* and *w*) both display the specific band of retinene$_1$ in this test. The curves were drawn by a recording photoelectric spectrophotometer.

To complete the identification, all three hexane extracts were transferred to chloroform and tested by mixing with antimony trichloride reagent. The spectra of the resulting solutions, measured at once in a recording photoelectric spectrophotometer, are shown in Fig. 2. The dark extraction yields only unspecific absorption. The "red" and "white" extracts yield the specific antimony chloride band of retinene$_1$. The band maxima appear here at 667 to 669 mμ instead of at the usual retinene position, 664 mμ, because slightly skewed by being superimposed on such rising base lines as represented by the "dark" curve.

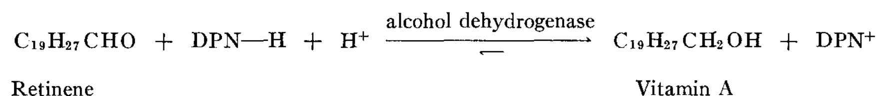
[2] This is the λ_{max} of the equilibrium mixture of cis-trans isomers which results when solutions of retinene are exposed to light (Hubbard, Gregerman, and Wald, 1952–53).

Quantitative relations also are consistent throughout. As already noted, the direct spectra of Fig. 1 show the ratio of retinene liberated in red and white light to be 0.30. If the curves of Fig. 2 are corrected for extraneous absorption as was done in Fig. 1, by subtracting 0.75 times the "dark" spectrum from the "red" curve, and 0.54 times the "dark" spectrum from the "white" curve, the ratio of extinctions at 664 mμ is red/white = 0.054/0.175 = 0.31.

It is clear that the irradiation of chicken retinal tissue with red light as with white light yields retinene$_1$. That iodopsin specifically is the source of the retinene liberated in red light is confirmed unequivocally by the experiments on solutions of the visual pigments, described below. It can be concluded that the bleaching of iodopsin as of rhodopsin yields retinene$_1$ as product.

Reduction of Retinene to Vitamin A in Cones

Retinene is vitamin A aldehyde. Its normal fate in the rods is to be reduced to vitamin A by alcohol dehydrogenase and reduced cozymase (coenzyme I; DPN-H):[3]



$$C_{19}H_{27}CHO + DPN{-}H + H^+ \underset{}{\overset{\text{alcohol dehydrogenase}}{\rightleftharpoons}} C_{19}H_{27}CH_2OH + DPN^+$$

Retinene — Vitamin A

It is easy to show that the chicken retina contains alcohol dehydrogenase. A group of 150 retinas was homogenized in 0.9 per cent sodium chloride solution, and the solution, which contains the enzyme, clarified by centrifuging. When retinene in digitonin solution and reduced DPN are added, the retinene is reduced to vitamin A. Omitting any of these ingredients, or boiling the enzyme solution for 5 minutes, abolishes the reaction.

It is much more difficult to demonstrate that the retinene liberated specifically in the cones by the bleaching of iodopsin is reduced to vitamin A by this enzyme system. This was finally accomplished in an experiment, which compared the product of bleaching iodopsin in dried retinal tissue, in which alcohol dehydrogenase cannot function, with the product of its bleaching in the intact retina. The results are shown in Fig. 3.

Experiment.—Sixty dark-adapted chicken retinas were dissected into normal saline, and divided into two equal groups. One group was centrifuged, the medium decanted, and the tissue dried by grinding with anhydrous sodium sulfate. The powder which resulted was extracted thoroughly with petroleum ether by stirring by motor for 1 hour at room temperature in deep red light (Jena RG5 filter). During this hour the petroleum ether was decanted and replaced with fresh solvent about 10 times. At the end all these fractions were pooled.

The second group of retinas, suspended intact in normal saline, was exposed to the

[3] References will be found in the review by Wald (1953 *a*).

same red light for the same time. Then the tissues were centrifuged, dried, and extracted exactly as before.

Each of the pooled petroleum ether extracts was run through a column of calcium carbonate, which adsorbed the colored carotenoids, and eventually allowed vitamin A and retinene to run through. The filtrates were collected from each column in four parallel fractions. Each fraction was transferred to chloroform, and half of it used

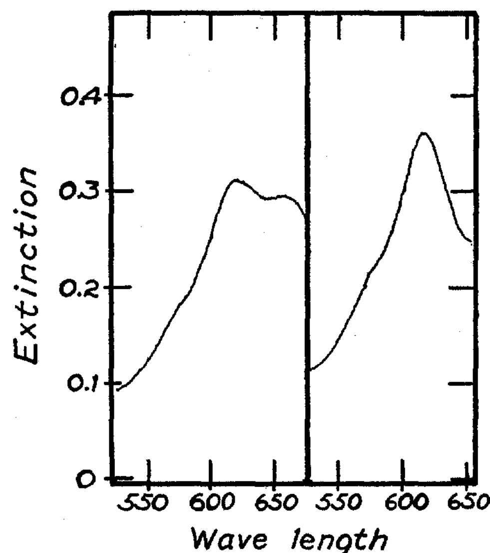


FIG. 3. Retinene liberated from iodopsin in the cones is reduced to vitamin A. Absorption spectra, recorded photoelectrically, of antimony chloride tests with extracts of chicken retinal tissue, in which the iodopsin had been bleached in deep red light. At the left, the tissue was bleached in the dry state, in which no enzymes could act. The extract shows the presence of vitamin A (λ_{max} 620 mμ), some of which is always found in dark-adapted retinas; and retinene (λ_{max} 664 mμ), liberated by the bleaching of iodopsin. At the right, the retinal tissue had been bleached in the fresh condition. Here vitamin A alone appears, showing that the retinene liberated from iodopsin had been reduced to vitamin A.

in an antimony chloride test, the spectrum of which was recorded with the Cary spectrophotometer. The first fraction to run through in both columns yielded no antimony chloride test; the second contained a little vitamin A (λ_{max} 620 mμ); the third contained the bulk of the vitamin A and retinene; and the fourth again yielded no test.

The spectra of the third fraction are shown in Fig. 3, that from dried tissue on the left, that from intact tissue on the right. It should be understood that the dark-adapted chicken retina, like all others, contains a small amount of free vitamin A, which is expected to appear equally in both extracts.

Since only deep red light was used to irradiate these tissues, only iodopsin was bleached. The dried tissue, in which alcohol dehydrogenase cannot act, yielded a mixture of vitamin A and retinene, the former a normal component of the dark-adapted retina, the latter the product of bleaching iodopsin (Fig. 3, left). The retinas suspended in saline yielded vitamin A alone, in somewhat larger quantity. In these intact tissues, the retinene liberated in the cones by the bleaching of iodopsin had been reduced to vitamin A.

Preparation of the Visual Pigments in Solution

Three procedures have been used in our laboratory to prepare the visual pigments of the chicken retina. The earliest (Wald, 1937 *b*) was designed primarily to coax into solution whatever would come. It extracted iodopsin, but with it much else that the retina contained.

Chickens were dark-adapted overnight in the laboratory, beheaded, and their retinas dissected out in dim red light into Ringer solution. They were transferred directly to 2 per cent digitonin solution containing about 2 per cent sodium chloride. The tissue was ground in this mixture with a glass rod, and left at room temperature in the dark to extract overnight. It was centrifuged, and the slightly opalescent supernatant solution decanted for measurement.

This procedure yielded very impure preparations. The extraneous absorption was so great as to make accurate measurements in the violet and near ultraviolet impossible. The yields of iodopsin and rhodopsin obtained by this method are summarized in Table I (preparations 1 to 4).

In 1942 we returned to the preparation of iodopsin, employing methods which had proved useful with mammalian tissues. The retinas were dissected as before, and transferred immediately to a test tube embedded in dry ice. They were lyophilized overnight, and the dry tissue was ground to a powder. This was extracted in a soxhlet apparatus for 2 to 3 hours in the dark with low boiling petroleum ether (b.p. 20–40°C.), to remove colored carotenoids and other lipids. The residue was freed under suction from all traces of petroleum ether. It was leached with 2 to 3 portions of neutral phosphate buffer to remove water-soluble material (the latter included a bright yellow carotene-protein). Finally the solid residue was stirred up with 2 per cent digitonin solution containing neutral phosphate buffer, and allowed to extract overnight in the dark at room temperature. The extract was clarified by centrifuging.

This procedure yielded considerably more iodopsin and rhodopsin than the previous method, and in much cleaner condition. The extraneous absorption at low wave lengths was still too high to permit accurate measurements in the near ultraviolet. The yields of visual pigment obtained with this method are summarized in Table I (preparations 5 and 6).

The third method of preparation has been used in our laboratory since 1951. It developed from procedures brought into use in the preparation of frog and

TABLE I

Yields of Iodopsin and Rhodopsin from the Chicken Retina

Iodopsin is measured by the change in extinction at 560 mμ (K_{560}) on exposure of the extract to red light; rhodopsin by the change at 500 mμ (K_{500}) on subsequent exposure to white light. The yields are expressed as K/retina/ml., as though the visual pigments from one retina were extracted into 1 ml. of solution and measured in a layer 1 cm. in depth. To obtain these values the observed extinctions of the extracts are divided by the number of retinas employed, and multiplied by the volume.

Preparation	Date	No. of retinas	Volume	Iodopsin K_{560}	Rhodopsin K_{500}	K/retina/ml.		Ratio
						Iodopsin	Rhodopsin	Rhodopsin/iodopsin
			ml.					
1	July 5/37	18	4.0	0.027	0.045	0.006	0.010	1.67
2	Aug. 6/37	20	4.0	0.060	0.069	0.012	0.014	1.15
3	Aug. 11/37	20	4.0	0.056	0.084	0.011	0.017	1.50
4	July 1/38	24	3.6	0.041	0.078	0.006	0.012	1.90
					Averages....	0.0088	0.013	1.56
5	May 15/42	32	5.1	0.12	0.19	0.019	0.030	1.58
6	June 3/42	40	4.0	0.11	0.16	0.011	0.016	1.45
					Averages....	0.015	0.023	1.52
7	July 4/51	24	3.0	0.039	0.042	0.0049	0.0053	1.08
8	Aug. 2/51	30	2.5	0.054	0.108	0.0045	0.0090	2.00
9	Aug. 10/51	80	3.4	0.063	0.140	0.0027	0.0060	2.22
10	Aug. 16/51	40	3.0	0.060	0.087	0.0045	0.0065	1.45
11	Oct. 1/51	150	3.0	0.300	0.304	0.0060	0.0061	1.01
12	Oct. 24/51	100	3.0	0.110	0.174	0.0033	0.0052	1.58
13	Nov. 16/51	170	3.5	0.332	0.469	0.0068	0.0097	1.41
14	Dec. 4/51	170	4.0	0.368	0.489	0.0086	0.0115	1.33
15	Jan. 4/52	110	4.0	0.312	0.760	0.0114	0.0277	2.44
16	Jan. 18/52	180	3.0	0.260	0.322	0.0043	0.0053	1.24
17	Jan. 28/52	180	2.0	0 480	0.806	0.0053	0.0090	1.68
18	Mar. 10/52	180	3.0	0.380	0.438	0.0063	0.0073	1.15
19	Apr. 10/52	96	5.3	0.202	0.261	0.0112	0.0144	1.29
20	Aug. 7/53	208	3.0	0.328	0.478	0.0047	0.0069	1.46
					Averages....	0.0060	0.0093	1.52

cattle rhodopsin (St. George, 1951–52; Wald and Brown, 1951–52). Only this method yielded preparations sufficiently pure to measure in the ultraviolet, and sufficiently concentrated to permit a wide variety of new experiments.

In this procedure, dark-adapted retinas are dissected as before, and are disintegrated by freezing and thawing and by grinding. From the resulting brei,

fragments of outer segments of the rods and cones are separated from the remaining retinal tissue by differential centrifugation. These particles, having already lost their water-soluble components in early portions of the procedure, are frozen-dried and extracted with petroleum ether to remove fat-soluble substances. The visual pigments are extracted from the residue with digitonin.

An important device for obtaining pure rhodopsin is to treat the retinas with alum, which renders most other proteins insoluble. This cannot be used here, for alum destroys iodopsin (Wald, 1937 *b*). Even our best preparations therefore contain other proteins than rhodopsin and iodopsin. The most troublesome is a trace of hemoglobin, which contributes a small Soret band to all our direct spectra.

Through the kindness of a local poulterer[4] we have periodically obtained large numbers of chicken heads, within ½ to 1 hour after the chickens had been killed. The killing was done indoors by artificial light. Fortunately chickens close their eyes immediately on execution. Shortly afterward, the heads are thrown into a box, and thereafter are kept dark. All these factors probably help to bring the retinas to the dark-adapted condition in which we find them on dissection.

The eyes are removed under dim red light, and opened by an equatorial cut. The front of the eyeball is lifted off, and the rear thrown into normal saline solution. The retina is lifted away with a spatula. Some pigment epithelium tends to come with it, but does not interfere with the further procedure.

The retinas are collected in a plastic centrifuge tube, and are stored dark at −15°C. Even if retinas are to be used at once, it has been found best to freeze them first. The tissue is partly thawed, transferred to a glass mortar, and ground thoroughly with a pestle at room temperature for about 5 minutes. The resulting brei is stirred into suspension in 0.9 per cent sodium chloride. This is centrifuged for 3 to 4 minutes at about 1200 R.P.M. to throw down the larger pieces of tissue. The supernatant suspension is decanted and recentrifuged in a Sorvall SP angle centrifuge at about 12,000 R.P.M. for 20 minutes. This sediments the fragments of the rods and cones. The supernatant is discarded.

The residue is resuspended in 50 per cent sucrose in 0.9 per cent NaCl, a mixture in which the rod and cone fragments float, and centrifuged at about 2500 R.P.M. for 5 minutes. The supernatant is decanted, and the sediment discarded. The supernatant is diluted about 5 times with 0.9 per cent NaCl, and is centrifuged at about 12,000 R.P.M. for 20 minutes. The supernatant is discarded.

The sediment is suspended in 0.9 per cent NaCl and rinsed into a 4 inch pyrex test tube. It is recentrifuged for 20 minutes at 12,000 R.P.M. The supernatant again is discarded.

The sediment is quickly frozen by plunging the test tube into a bath of dry ice in acetone. Then the cold test tube is placed in a larger tube, and its contents are lyophilized. The dry material is extracted by stirring and grinding in 3 portions of pe-

[4] We are greatly indebted to Phillips Poultry Supply, of East Boston, for repeated gifts of fresh chicken heads.

troleum ether (b.p. 20–40°C.), each time centrifuging lightly and discarding the extracts. The residue, from which the last remnants of petroleum ether are allowed to evaporate at room temperature, is stirred up in 2 per cent digitonin solution, and let stand at about 5°C. overnight in the dark. This suspension is centrifuged for 20 minutes at 12,000 R.P.M.; and if not yet clear is recentrifuged in the cold for 10 minutes at top speed in the Sorvall SS centrifuge. The resulting solution is used in our measurements.

The yields of iodopsin and rhodopsin provided by the three methods of preparation are compared in Table I. All the methods yield about the same proportions of the visual pigments, about 1.5 times as much rhodopsin as iodopsin. The yields from the various experiments have been made comparable by reducing all of them to extinction per retina per milliliter, *i.e.* the extinction measured in a 1 cm. layer, divided by the number of retinas employed, and multiplied by the volume of the extract in milliliters. The result is as though the visual pigments of a single retina had been extracted into 1 ml. and measured in a layer 1 cm. in depth.

On this basis the average yield of iodopsin by the first procedure is about 0.009, and of rhodopsin about 0.013. In the second procedure these yields rise to 0.015 for iodopsin and 0.023 for rhodopsin. In the third procedure the yields are low again—0.006 for iodopsin and 0.009 for rhodopsin—since in the isolation of the rod and cone fragments much material is lost. The relative purity of these preparations compensates for this waste; and this procedure yielded also solutions of high concentration, with extinctions of iodopsin as high as 0.5, and of rhodopsin as high as 0.8.

The Bleaching of Iodopsin and Rhodopsin in Solution

The absorption spectrum of a relatively clean preparation of the visual pigments of the chicken retina is shown in Fig. 4 (*a*). (Table I, preparation 13). The spectrum displays a broad main absorption band at about 500 mμ, owing to the visual pigments themselves, but distorted by being superimposed on a base line of extraneous absorption which rises into the ultraviolet. A smaller maximum at about 412 mμ marks the Soret band of a trace of hemoglobin.

This solution was exposed to an intense source of deep red light—the focused light of a 160 watt microscope lamp passing through a red Wratten 89 filter, and a 2 inch layer of water to remove heat radiation. There were 3 such exposures of 6 minutes each. The extinction was measured periodically at 560 mμ to follow the bleaching of iodopsin. When this was complete, the entire absorption spectrum was measured (curve *b*). The changes caused by red light are shown as the diagonally hatched area in Fig. 4. The extinction had fallen at wave lengths longer than 466 mμ, simultaneously rising below this wave length, marking the bleaching of iodopsin, and the simultaneous formation of a product absorbing in the near ultraviolet.

Bleaching of chicken retinal extract

////// red light

|||||| white light

Extinction

Wave length – mμ

FIG. 4. Bleaching of an aqueous digitonin extract of dark-adapted chicken retinas, in red and white light. The original extract, measured in the dark (*a*), was exposed to deep red light until this had no further effect. Its spectrum had changed to (*b*). On subsequent exposure to white light, its spectrum changed to (*c*) (25°C., pH 6.2). The hatched areas, representing the changes in extinction caused by red light (diagonal, iodopsin) and white light (vertical, rhodopsin) are plotted as difference spectra in Fig. 5.

The preparation was now exposed to intense white light—the original source with the red filter removed—and its spectrum was remeasured (curve *c*). The changes in white light are shown as the vertically hatched area in Fig. 4. The extinction fell at wave lengths longer than 431 mμ, simultaneously rising at shorter wave lengths. This marks the bleaching of rhodopsin, again with the formation of a product absorbing in the near ultraviolet.

Such a progression of curves as shown in Fig. 4 gives only a rough impression of the changes in red and white light. To bring out their meaning more clearly, these data are replotted in Fig. 5 in the form of difference spectra, showing only the changes in extinction which accompany bleaching. Usually such difference spectra are obtained by subtracting the spectrum of the product from that of the precursor; *e.g.*, *a-b* and *b-c* in Fig. 4. This would have given the difference spectra negative values at low wave lengths, where the extinction *rises* on bleaching (see for example Fig. 18 below). In Fig. 5 we have given all the changes positive values by always subtracting the lower from the upper curves; *i.e.*, the lower from the upper borders of the hatched areas in Fig. 4. It should be borne in mind therefore that in Fig. 5, as shown with arrows, the pair of curves at the right represents fall of extinction, *i.e.* bleaching—while the pair at the left represents the associated formation of product.

In Fig. 5, the solid circles show the changes which occurred in red light. The broad band at the right, maximal at 562 mμ, is the difference spectrum of iodopsin. The band at the left, maximal at 391 mμ and representing the product of bleaching, is the difference spectrum of retinene$_1$.

The open circles show the changes which accompany the subsequent bleaching of rhodopsin. The difference spectrum in the visible region is maximal at 509 mμ; and again the product formed in the ultraviolet is retinene$_1$.

It will be noted that the difference spectrum of retinene formed by the bleaching of rhodopsin lies at a lower wave length than that from iodopsin. This is a highly significant datum. It is known that the retinene which emerges from the bleaching of rhodopsin is predominantly the all-trans isomer. This is isomerized to a mixture of cis-trans isomers by simple exposure to light; but of course only such blue, violet, and ultraviolet light as retinene can absorb (Hubbard and Wald, 1952–53). The red light used to bleach iodopsin is not absorbed by retinene, and hence cannot isomerize it; it therefore maintains its original geometrical configuration. The white light used subsequently, however, not only bleaches rhodopsin but isomerizes all the retinene which has formed. It is this process that moves the retinene maximum from 391 to 385 mμ. The displacement of spectrum 5 to 6 mμ toward shorter wave lengths on isomerization is a unique property of the all-trans isomer (Hubbard, Gregerman, and Wald, 1952–53). It can be concluded that the bleaching of iodopsin, as of rhodopsin, yields all-trans retinene$_1$ as product.

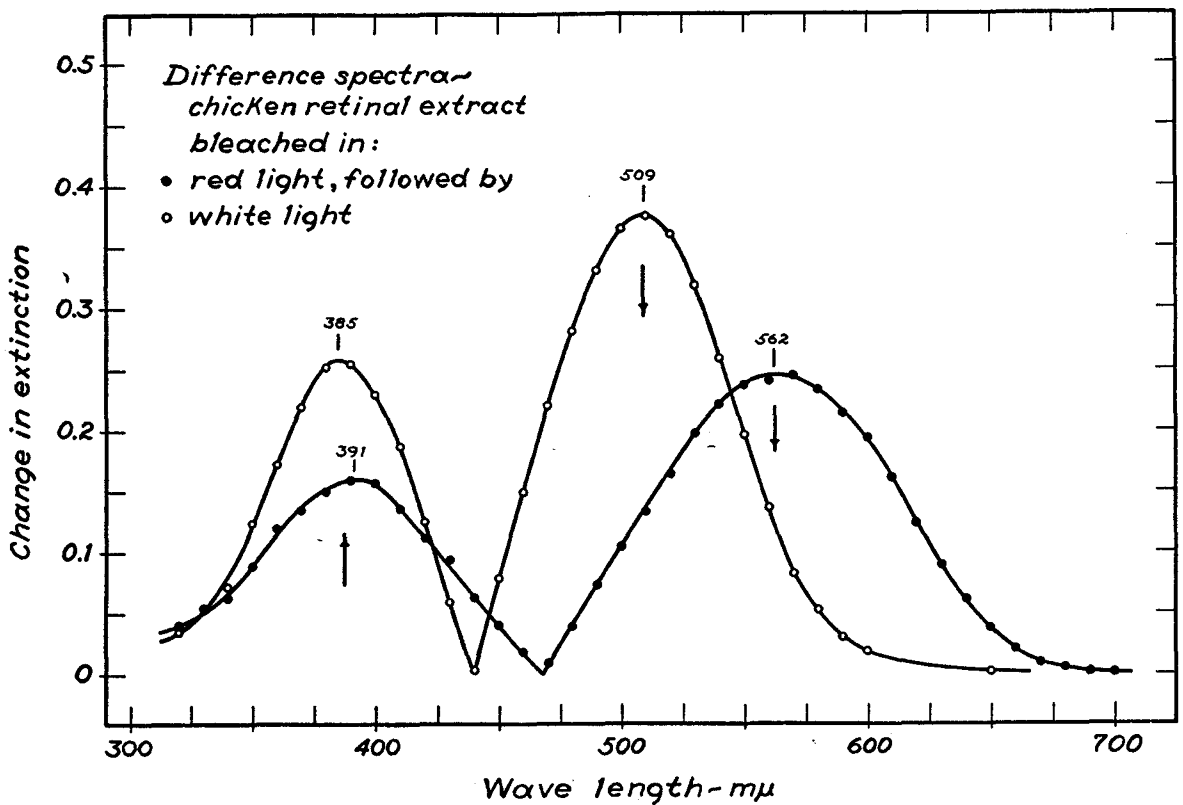


FIG. 5. The difference spectra of iodopsin and rhodopsin, from Fig. 4. The pair of curves at the right represent the bleaching of iodopsin (solid circles) and rhodopsin (open circles). Those at the left represent the simultaneous formation of retinene. The retinene formed by bleaching iodopsin in red light is the all-trans isomer (λ_{max} 391 mμ). The white light, used subsequently to bleach rhodopsin, also isomerizes all the retinene present to a mixture of cis-trans isomers (λ_{max} 385 mμ).

The Synthesis of Iodopsin

The synthesis of rhodopsin from retinene and opsin is a spontaneous reaction (Wald and Brown, 1950). A simple mixture of retinene and opsin, incubated in the dark, forms the visual pigment. This reaction however demands a specific cis isomer of retinene, called neoretinene *b*. The all-trans retinene formed by the bleaching of rhodopsin is inactive in its synthesis (Hubbard and Wald, 1952–53).

Iodopsin is involved in similar relations. It bleaches in red light irreversibly. The mixture of opsin and all-trans retinene which results shows no tendency to regenerate the visual pigment. Such a mixture therefore presents us with a relatively stable preparation of cone opsin, which can be tested by incubation with single cis-trans isomers of retinene. Fig. 6 shows the results of such an experiment.

Experiment.—A chicken retinal extract (Table I, preparation 14) was exposed to red light until its iodopsin was completely bleached. Five 0.4 ml. portions were placed in 5 microcells. To one cell, 0.1 ml. of digitonin solution was added; this was reserved as a blank for the spectrophotometric measurements. To each of the other cells, 0.1 ml. of a cis-trans isomer of retinene was added, in such concentration as to bring the final extinction of the added retinene to about 0.30. Each of these mixtures was let stand in the dark at about 23°C. for 10 minutes. Then its absorption spectrum was measured against that of the blank. Since the latter contained the entire retinal extract, including the products of bleaching iodopsin, the curves shown in Fig. 6 represent directly the absorption spectra of the added retinene isomers and whatever new products they had formed.

Fig. 6 shows that the addition of all-trans retinene to bleached iodopsin causes no regeneration of the visual pigment. The same is true of neoretinene *a*. In both cases at the end of the incubation the spectrum measured is virtually that of the added retinene. There is almost negligible new absorption in the iodopsin region.

The addition of neoretinene *b*, however, results in a large regeneration of iodopsin. After 10 minutes in the dark, most of the added retinene is gone, and iodopsin has appeared with an extinction at λ_{max} 562 mμ of 0.20. It should be noted that this experiment provides us with a true absorption spectrum of iodopsin, mixed with a residue of retinene—not a difference spectrum.

The iodopsin regenerated in this way is compared with that extracted from dark-adapted chicken retinas in Fig. 7. The pigment formed in Fig. 6 was bleached in red light, and the difference spectrum obtained was compared with that from the original preparation. To facilitate comparison, the maxima at 562 mμ were brought to the same height. It is clear that the iodopsin regenerated from neoretinene *b* is identical with the natural pigment.

If both the rhodopsin and iodopsin in a chicken retinal extract are bleached

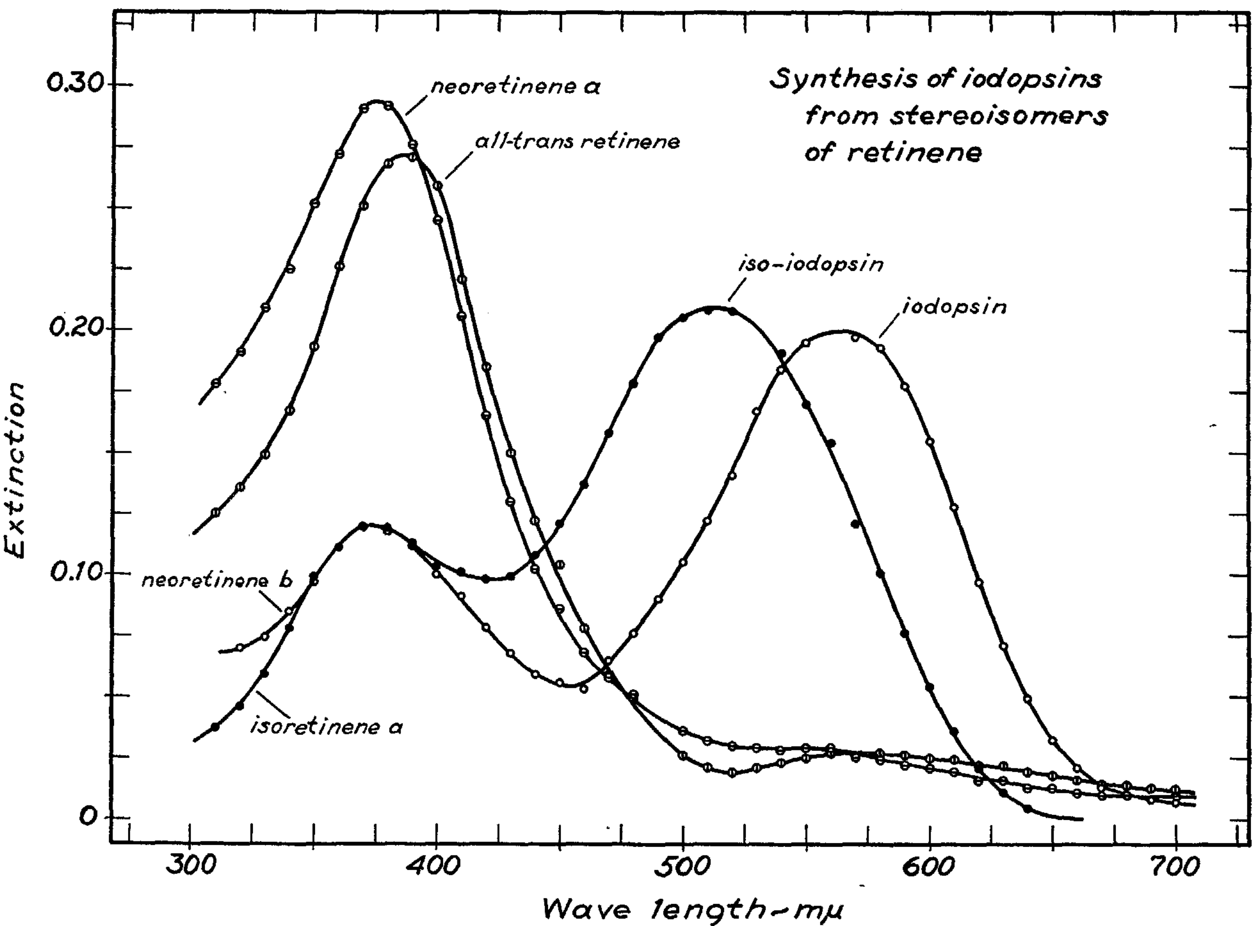


FIG. 6. Synthesis of iodopsin and iso-iodopsin in solution. In a chicken retinal extract, the iodopsin alone was bleached with deep red light to a mixture of all-trans retinene and photopsin. This product was incubated in the dark with 4 geometrical isomers of retinene. The absorption spectra were then measured against that of the red-bleached solution as blank. All-trans retinene and neoretinene *a* had remained almost as added. Neoretinene *b* had formed a large amount of iodopsin (λ_{max} 562 mμ). Isoretinene *a* had formed iso-iodopsin (λ_{max} 510 mμ). Both these photosensitive pigments are accompanied by residues of unchanged retinene, primarily

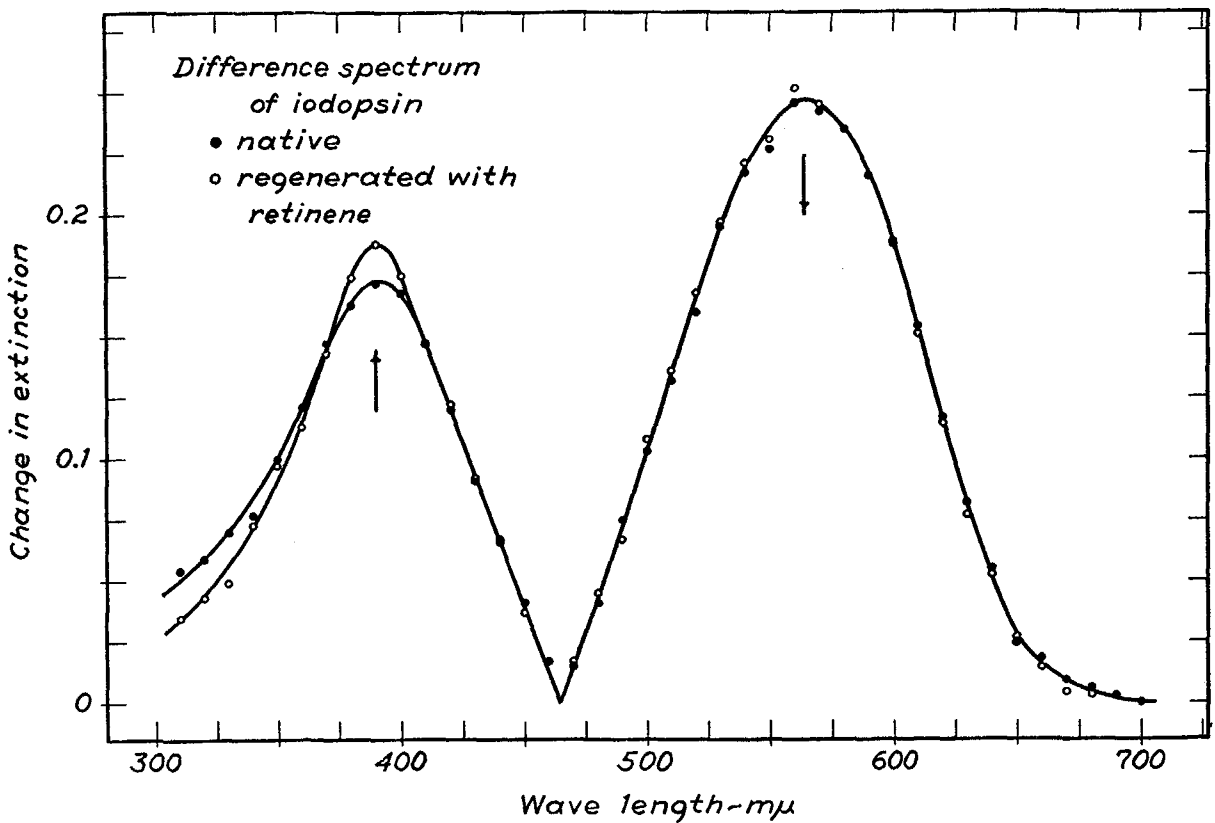


FIG. 7. Comparison of natural with regenerated iodopsin. The difference spectrum of iodopsin, extracted from dark-adapted chicken retinas (closed circles), compared with that of iodopsin synthesized by incubating the products of its bleaching (*i.e.*, photopsin and all-trans retinene) with neoretinene *b* (open circles).

in orange light, which does not isomerize retinene, the final product is a mixture of all-trans retinene and rod and cone opsins. On adding to this an excess of neoretinene *b* and incubating for 1 to 2 hours in the dark, one obtains a mixture of rhodopsin and iodopsin indistinguishable from that extracted from the dark-adapted retina.

Iodopsin, however, forms under these conditions with enormously greater speed than rhodopsin. Its synthesis is complete when that of rhodopsin has scarcely begun. As a result it is possible to synthesize both pigments separately. If one adds just enough neoretinene *b* to make the iodopsin, this forms very rapidly and virtually alone. Indeed this is the only way we have ever succeeded in preparing a solution which contains only iodopsin. Later, on adding more neoretinene *b*, one obtains rhodopsin. Such an experiment is shown in Fig. 8.

Experiment.—The same preparation was used in this experiment as in those of Figs. 6 and 7. 0.4 ml. portions of the retinal extract were introduced into 2 microcells, and bleached for 2 minutes in a non-isomerizing orange light—the focused light of a 160 watt microscope lamp passing through a Corning 3484 filter and 2 inches of water. This bleached both rhodopsin and iodopsin, leaving the all-trans retinene which emerged unaltered. To one solution, 0.1 ml. of digitonin solution was added to serve as blank. To the other, 0.1 ml. of neoretinene *b* in digitonin was added, bringing its extinction at 380 mμ to 0.30. By previous trial it had been found that this is just enough retinene to form the iodopsin. The extinction at 560 mμ was measured as soon as possible, and again after 5 and 10 minutes, to follow the iodopsin synthesis (pH 6.2; about 25°C.). The result is shown in Fig. 21 below (solid circles). Within 45 seconds, the time it took to make the first reading, the synthesis of iodopsin was already 92 per cent complete. Within 2 to 3 minutes it was complete.

The absorption spectrum of the product, measured against the bleached extract as blank, is shown in Fig. 8 with solid circles. It is a direct absorption spectrum of iodopsin, comparable with that shown in Fig. 6. Its λ_{max} is at 561 mμ.

After these measurements were completed, a small excess of neoretinene *b* was added to the same solution. The synthesis of rhodopsin was followed with periodic measurements at 500 mμ (*cf.* Fig. 21, open circles). After 30 minutes' incubation, the absorption spectrum of this solution was measured against the wholly bleached extract as blank; and from the result the absorption spectrum of the iodopsin formed previously in this solution was subtracted. The resulting absorption spectrum of rhodopsin is shown with open circles in Fig. 8.

It is apparent that neoretinene *b* is the precursor of iodopsin as of rhodopsin. Iodopsin is synthesized with so much greater speed than rhodopsin that it is possible to form these pigments successively in a mixture. This is the only way we have yet found to prepare a solution containing iodopsin alone.

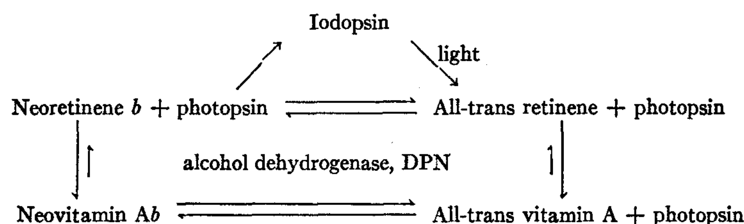
Recapitulation: the Iodopsin Cycle

The forms of retinene—and hence of vitamin A—involved in the synthesis and bleaching of iodopsin are identical with those which take part in the syn-

FIG. 8. Successive syntheses of iodopsin and rhodopsin in solution. A chicken retinal extract was wholly bleached with an orange non-isomerizing light to a mixture of all-trans retinene, and rod and cone opsins. To this mixture was added just enough neoretinene *b* to regenerate iodopsin alone; iodopsin forms so much more rapidly than rhodopsin that its synthesis is complete when that of rhodopsin has scarcely begun (*cf.* Fig. 21 below). The absorption spectrum of the product, formed within a few minutes in the dark, is shown with solid circles. Then a small excess of neoretinene *b* was added, and the mixture was reincubated in the dark for 30 minutes. This yielded rhodopsin (open circles).

thesis and bleaching of rhodopsin. The difference between these visual pigments must therefore have its source in their protein components, their opsins. The opsins of rod or scotopic vision may be called *scotopsins*; those of cone or photopic vision, *photopsins*. In these terms iodopsin bleaches to a mixture of photopsin and all-trans retinene, and is resynthesized from a mixture of photopsin and neoretinene *b*. When to these components are added the products

of their interaction with the alcohol dehydrogenase system, the iodopsin cycle takes the form:



Iso-Iodopsin

The opsin of rod vision, scotopsin, incubated with neoretinene *b* yields rhodopsin, with λ_{max} 500 mμ. Incubated with another cis isomer of retinene, isoretinene *a*, it yields a similar light-sensitive pigment, the absorption spectrum of which is displaced about 13 mμ toward shorter wave lengths (λ_{max} 487 mμ). This is called iso-rhodopsin (Hubbard and Wald, 1952–53).

In the same way, as shown in Fig. 6, the incubation of photopsin with isoretinene *a* yields iso-iodopsin. The λ_{max} of this pigment lies at about 510 mμ, about 52 mμ shorter than the iodopsin maximum. The source of this large displacement is an interesting problem. The absorption maxima of neoretinene *b* and isoretinene *a* lie within 1 to 2 mμ of each other. Some characteristic of the linkage between these retinene isomers and photopsin, which depends upon their cis-trans configuration, is responsible for this very large shift of spectrum. We have here a strong indication that in the visual pigments the binding between retinene and opsin involves not only end groups, but very intimately the geometry of the hydrocarbon chain.

In the experiment shown in Fig. 6, neoretinene *b* and isoretinene *a* had been added in slight excess, so that the amounts of iodopsin and iso-iodopsin formed were limited by the amount of available photopsin. Since the latter was the same in both cases, the slightly higher extinction of iso-iodopsin compared with iodopsin in Fig. 6 is probably significant. Rhodopsin and iso-rhodopsin exhibit a similar relationship; in a comparable experiment they were found to have extinctions in the ratio 1:1.07 (Hubbard and Wald, 1952–53). In Fig. 6 the extinctions of iodopsin and iso-iodopsin are in the ratio 1:1.05.

Kinetics of Bleaching and Synthesis

Bliss (1945–46 *a*) showed that the bleaching of iodopsin in solution follows the kinetics of a first-order reaction, relatively independent of the temperature, and proportional in rate to the light intensity. These are the conventional properties of a photochemical reaction, and had been demonstrated earlier in the bleaching of rhodopsin, by Hecht (1920–21 *a*, *b*; 1923–24).

Actually one observes such properties only when rhodopsin is bleached in sufficiently dim lights to make the light reaction rate-limiting. In bright light and other special circumstances it can be shown that the bleaching of rhodopsin is in fact a complex process, initiated by light, but completed by ordinary

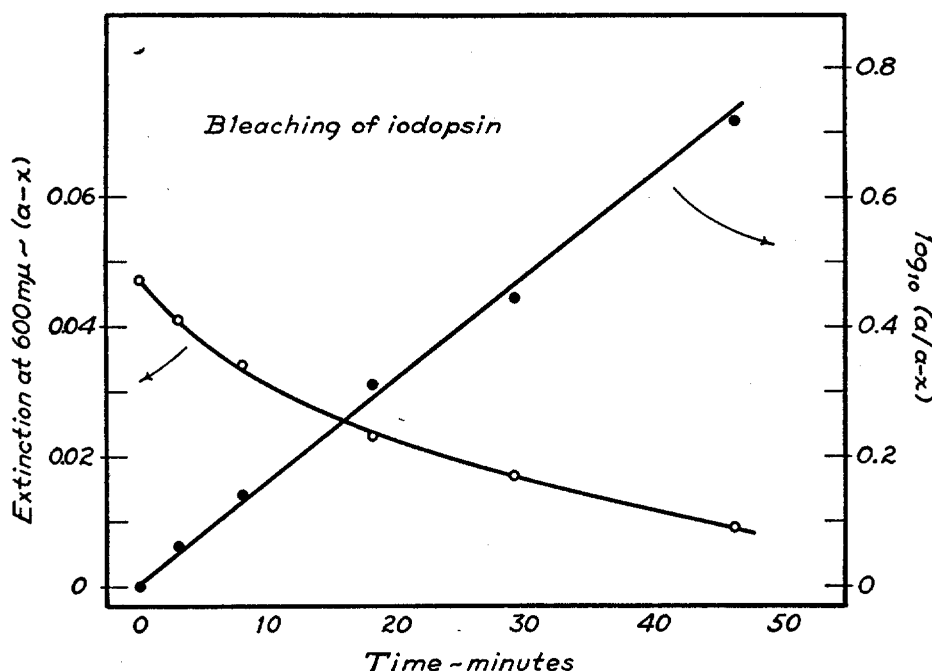


FIG. 9. Kinetics of the bleaching of iodopsin. An extract of chicken retinas was exposed to deep red light, and its extinction at 600 mμ was measured periodically to follow the bleaching of iodopsin (open circles). The extinction measured at 86 minutes, when all the iodopsin had bleached, represented the impurities present; this was subtracted from all the other measurements. The open circles represent therefore the extinction, and hence the concentration, of iodopsin alone ($a - x$). The initial concentration, a, is represented by the initial extinction. In these terms, log ($a/a - x$), plotted as solid circles, is seen to be linear with time, showing that the bleaching of iodopsin under these conditions is a first-order reaction.

"dark"; *i.e.*, thermal reactions (Wald, 1937 *a*; Lythgoe, 1937; Wald, Durell, and St. George, 1950). Very likely the same is true of iodopsin.

Fig. 9 shows an experiment on the bleaching of iodopsin in solution which confirms Bliss's conclusion that this is a first-order process. A chicken retinal extract (Table I, preparation 6) at pH 6.93 was exposed to the dim red light of a 60 watt photographic ruby lamp passing through a red Corning 2403 filter and a Jena BG 10 heat filter. This combination transmits only wave lengths

longer than 640 mμ. Readings of the extinction were made periodically at 600 mμ, to follow the bleaching of iodopsin. A final reading at 86 minutes, when all the iodopsin had been bleached, provides a correction for extraneous absorption. This final extinction was subtracted from all the others to yield values of the declining extinction of iodopsin alone. These are plotted as open circles in Fig. 9.

The equation of a first-order reaction is

$$kt = \log_{10}[a/(a - x)]$$

in which k is the velocity constant; t the time; a the initial concentration of reactant; and $a - x$ the concentration of reactant remaining at time t (*i.e.*, x

TABLE II

Kinetics of Bleaching of Iodopsin in Solution. pH 6.93

Irradiation at constant intensity with red light of wave lengths longer than 640 mμ. Extinctions (E_t) measured at 600 mμ. The final extinction, measured at 86 minutes, representing the absorption of impurities alone, was subtracted from all the others. The initial concentration of iodopsin, a, is represented by the initial extinction, E_0 = 0.047.

Time	$(a - x)$ E_t	$a/(a - x)$ (E_0/E_t)	$\log a/(a - x)$ $(\log E_0/E_t)$	k
min.				
0	0.047	1	0.00	—
3	0.041	1.15	0.061	0.020
8	0.034	1.38	0.140	0.017
18	0.023	2.04	0.310	0.017
29	0.017	2.77	0.443	0.015
46	0.009	5.22	0.718	0.016
Average				0.017

is the concentration of product). For our purposes, concentrations may be represented throughout by the extinctions to which they are proportional. In these terms a is represented by the initial extinction, E_0; and $(a - x)$ by the extinction of iodopsin at any time during the course of bleaching, E_t. The quantity, $\log a/(a - x)$, then becomes $\log E_0/E_t$. According to the above equation, this quantity plotted against time should yield a straight line of slope k. This is shown with solid circles in Fig. 9. The data and calculations are presented in Table II.

It may be concluded that under ordinary circumstances in which the light reaction is limiting, the rate of bleaching of iodopsin, as of rhodopsin, is proportional simply to the rate at which these pigments absorb quanta of light; and hence the amount of iodopsin or rhodopsin bleached is the same proportion of the total number of quanta absorbed. In the case of rhodopsin this

proportionality factor lies between 0.5 and 1.0; *i.e.*, this is the quantum efficiency of bleaching (Wald and Brown, 1953–54). The quantum efficiency of bleaching iodopsin, though still undetermined, is probably not very different.

It has recently been shown that the synthesis of rhodopsin in solution from neoretinene *b* and scotopsin is a second-order process, provided that both reactants are present initially in roughly equivalent concentrations (Hubbard and Wald, 1952–53).

The synthesis of iodopsin in solution is too rapid at room temperature to be measured accurately (*cf.* Fig. 21). This process has the high temperature coefficient typical of thermal reactions. We have therefore lowered the temperature to 10°C. to slow it, and so permit us to study the rates of synthesis of both iodopsin and rhodopsin in a chicken preparation. The results of this experiment are shown in Figs. 10 and 11.

An extract of dark-adapted chicken retinas was exposed to an orange, non-isomerizing light until all its iodopsin and rhodopsin had bleached. This took 10 minutes' irradiation with the focused light of a 160 watt microscope lamp passing through a Corning 3484 filter and 2 inches of water.

The bleached solution (0.5 ml.) in a microcell was cooled to 10°C. in the cell compartment of a Beckman spectrophotometer through which cold water was circulated. Then 0.06 ml. of neoretinene *b* in digitonin solution was added, to bring its final extinction in the reaction mixture to about 0.3 at 385 mμ (pH 6.48). The rise of extinction was followed in the dark at 560 mμ (iodopsin) and 500 mμ (rhodopsin). These measurements are shown at the left in Fig. 10. At 10°C. the synthesis of iodopsin is completed within 3 minutes, while that of rhodopsin is measurably incomplete at 18 hours. At this time the solution was bleached, first in red light, then in white light, as described above (*cf.* Figs. 4 and 5), to obtain the difference spectra of the iodopsin and rhodopsin which had formed. These are shown at the right in Fig. 10.

The equation of a second-order reaction is

$$kt = \frac{2.303}{(a-b)} \log_{10} \frac{b(a-x)}{a(b-x)}$$

in which k is the velocity constant, t the time, a and b the initial concentrations of the reactants, and x the concentration of products. That is, $(a - x)$ and $(b - x)$ represent the declining concentrations of the reactants through the course of the reaction.

This equation can be rewritten in the form:

$$t = \underbrace{\frac{2.303}{k(a-b)} \log_{10} \frac{b}{a}}_{\text{intercept}} + \underbrace{\frac{2.303}{k(a-b)}}_{\text{slope}} \log_{10} \frac{(a-x)}{(b-x)}$$

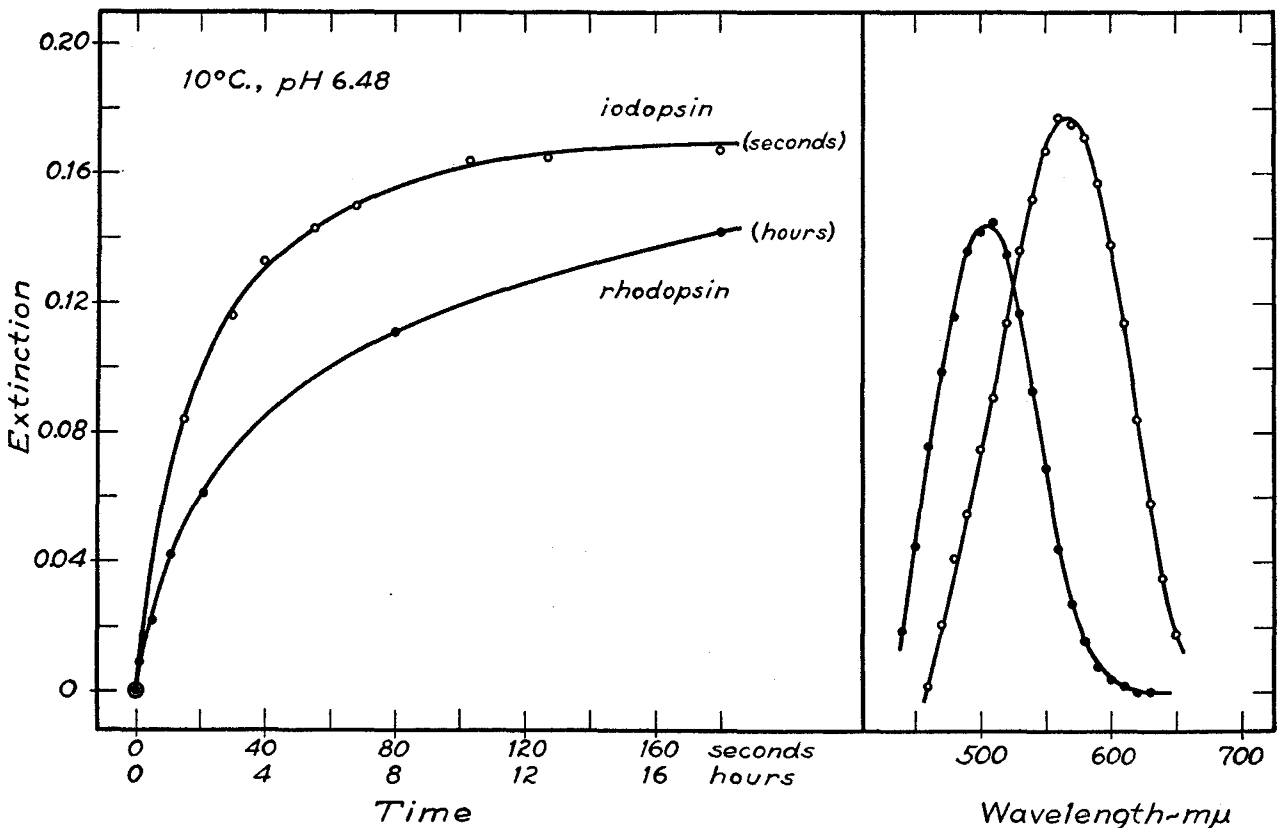


FIG. 10. The synthesis of iodopsin and rhodopsin at 10°C. An extract of chicken retinas was bleached in an orange, non-isomerizing light to a mixture of all-trans retinene and rod and cone opsins. Neoretinene *b* was added, and the formation of iodopsin and rhodopsin followed in the dark by measuring the extinctions at 560 and 500 mμ respectively. These are shown at the left, iodopsin plotted to a time scale of seconds, rhodopsin to one of hours. At the end of the experiment, the difference spectra of the pigments were measured by bleaching successively with red and orange lights; these are shown at the right. The kinetic analysis of these data is shown in Fig. 11.

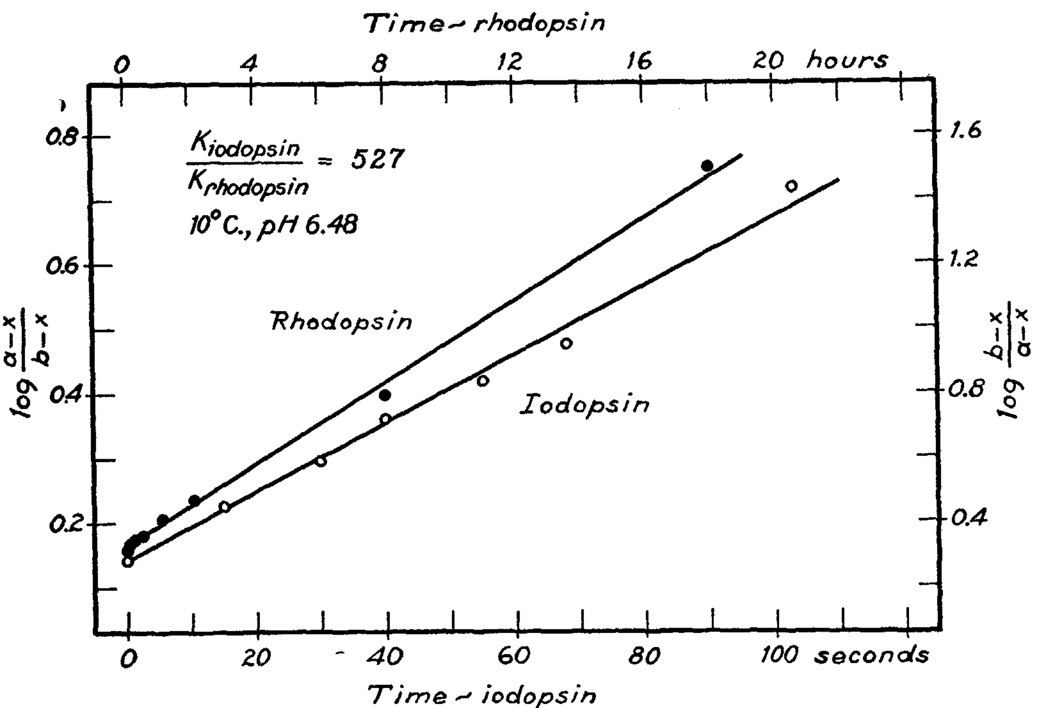


FIG. 11. Kinetics of synthesis of iodopsin and rhodopsin in solution, from Fig. 10. When the data are plotted as shown here, their linearity is evidence that the syntheses follow the course of a second-order (*i.e.*, bimolecular) reaction. From the slopes of these lines, the velocity constant for iodopsin synthesis is computed to be 527 times that for rhodopsin synthesis.

This is the equation of a straight line, when log $[(a - x)/(b - x)]$ is plotted against time. The first term on the right is the intercept constant, the first part of the second term the slope constant.

The data of Fig. 10 are plotted in this form in Fig. 11, and are presented numerically in Tables III and IV. The data for both iodopsin and rhodopsin yield adequate straight lines. It can be concluded that the syntheses of these pigments in solution from neoretinene *b* and the appropriate opsins are second-order—indeed bimolecular. From the slopes of the lines, substituted in the above equation, k can be computed for both reactions. It emerges that under these identical conditions the rate of iodopsin synthesis is 527 times the rate of rhodopsin synthesis.

The computations involved in Fig. 11 and Tables III and IV require some further explanation. As in the analysis of bleaching, we represent all concentrations by extinctions, to which they are proportional. Also we state all extinctions in terms of the extinction of visual pigment—rhodopsin or iodopsin—to which they are equivalent. Thus the concentration of scotopsin is stated in terms of the extinction of rhodopsin from which it has been formed or to which it gives rise. Similarly photopsin is measured by its equivalent extinction of iodopsin; and retinene by its equivalent extinction of either pigment. In this way all the extinctions employed in the calculation represent molar equivalents.

The original retinal extract used in this experiment contained a mixture of iodopsin and rhodopsin, which, corrected for the later dilution with retinene solution, had the following extinctions:

$$\text{Iodopsin: } K_{560} = 0.191$$

$$\text{Rhodopsin: } K_{500} = 0.226$$

At the end of the experiment, as shown in Fig. 10, we had regenerated a mixture of iodopsin and rhodopsin with the extinctions:

$$\text{Iodopsin: } K_{560} = 0.170$$

$$\text{Rhodopsin: } K_{500} = 0.157$$

That is, we had got back 89 per cent of the original iodopsin, but only 69 per cent of the rhodopsin. We take this to mean that we had added enough neoretinene *b* to regenerate all the iodopsin, but not all the rhodopsin.

We therefore estimate concentrations as follows:—

Initial concentration of photopsin, equivalent to the K_{560} of iodopsin formed = 0.170.

Initial concentration of scotopsin, equivalent to the K_{500} of rhodopsin present originally = 0.226.

Initial concentration of neoretinene *b*, equivalent to the K_{560} of iodopsin formed + K_{500} of rhodopsin formed = 0.170 + 0.157 = 0.327. This is the neoretinene *b* available for the synthesis of iodopsin.

TABLE III

The Synthesis of Iodopsin in Solution from Photopsin and Neoretinene b. 10°C., pH 6.48

a = initial concentration of photopsin = K_{560} iodopsin to be formed = 0.170.

b = initial concentration neoretinene b = K_{560} iodopsin + K_{500} rhodopsin to be formed = 0.170 + 0.157 = 0.327.

x = K_{560} iodopsin formed at time t. For convenience we have calculated $\log \frac{b-x}{a-x} = -\log \frac{a-x}{b-x}$.

Time t	Iodopsin (K_{560}) x	$a - x$	$b - x$	$\frac{b-x}{a-x}$	$\log \frac{b-x}{a-x}$
sec.					
0	0	0.170	0.327	1.924	0.283
15	0.084	0.086	0.243	2.826	0.452
30	0.116	0.054	0.211	3.907	0.592
40	0.133	0.037	0.194	5.243	0.719
55	0.143	0.027	0.184	6.815	0.834
68	0.150	0.020	0.177	8.850	0.947
103	0.164	0.006	0.163	27.17	1.435
127	0.165	0.005	0.162	32.40	1.511
180	0.167	0.003	0.160	53.33	1.727
∞	0.170				

TABLE IV

The Synthesis of Rhodopsin in Solution from Scotopsin and Neoretinene b. 10°C., pH 6.48

a = initial concentration of scotopsin = K_{500} of rhodopsin present originally = 0.226.

b = initial concentration neoretinene b = K_{500} of rhodopsin to be formed = 0.157.

x = K_{500} of rhodopsin formed at time t.

Time t	Rhodopsin (K_{500}) x	$a - x$	$b - x$	$\frac{a-x}{b-x}$	$\log \frac{a-x}{b-x}$
hrs.					
0	0	0.226	0.157	1.439	0.158
0.1	0.009	0.217	0.148	1.466	0.167
0.25	0.017	0.209	0.140	1.493	0.173
0.50	0.022	0.204	0.135	1.511	0.179
1.1	0.042	0.184	0.115	1.600	0.204
2.1	0.061	0.165	0.096	1.719	0.235
8.0	0.111	0.115	0.046	2.500	0.398
18.0	0.142	0.084	0.015	5.600	0.748
∞	0.157				

Concentration of neoretinene b remaining for the synthesis of rhodopsin, equivalent to the K_{500} of rhodopsin formed = 0.157.

The slope of the iodopsin line in Fig. 11 is −92.0. By the above second-order equation, this equals $2.303/k(a - b) = -2.303/0.157k$. k therefore equals 0.159 when time is measured in seconds, or 0.159 × 60 = 9.54, with time in minutes.

Similarly the slope of the rhodopsin line in Fig. 11 is 1843, with time in minutes; k is therefore 0.0181. The ratio of velocity constants for the synthesis of iodopsin and rhodopsin under these conditions is 9.54/0.0181 = 527.

The pH Stability of Iodopsin and Rhodopsin

In the course of our experiments it became clear that iodopsin is much less stable than rhodopsin in solutions which depart appreciably from neutrality. We therefore made a special series of measurements to examine the pH sta-

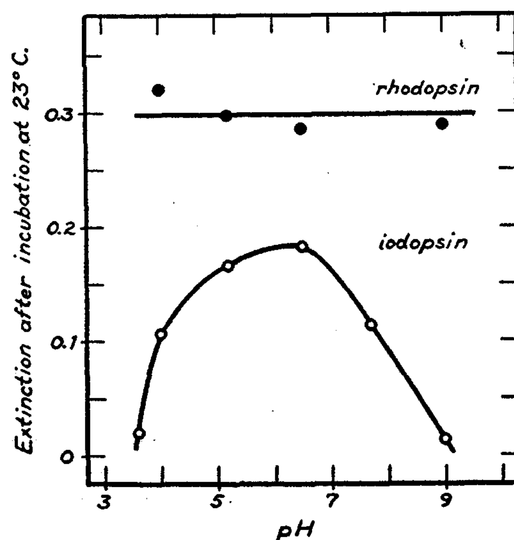


FIG. 12. Stability of chicken iodopsin and rhodopsin at various pH's. The figure shows the extinctions of iodopsin and rhodopsin remaining after 60 minutes' incubation at 23°C. Rhodopsin was stable between pH 4–9; but iodopsin decayed rapidly to both sides of pH 5–7.

bility of both pigments in a single preparation. The results are shown in Fig. 12 and Table V.

Two experiments were performed with a single extract of dark-adapted chicken retinas. The results were essentially similar in both, but the second experiment was technically more satisfactory than the first, and is described here.

The retinal extract was brought to various pH's by mixing 0.4 ml. with 0.1 ml. of buffered digitonin solution. The solutions were left dark for 30 minutes, and were centrifuged in the dark for 15 minutes to remove turbidities which had formed, particularly at acid pH's. The absorption spectrum was measured in the dark, and again after exposure to red and to white light, to obtain the difference spectra of the iodopsin and rhodopsin which remained. The total

period of incubation at various pH's was 60 minutes (about 25°C.). The pH was measured always at the end of the experiment.

Chicken rhodopsin, like that of frogs and cattle, remains stable under these circumstances between pH 4 and 9. Iodopsin, however, has a much more restricted stability range. Between pH 5 and 7 there is little fall of extinction within an hour at room temperature; but at pH's to either side the extinction falls rapidly. At pH 3.6 and 9 almost no measurable iodopsin remains at the end of an hour. The maximum stability is close to pH 6.5.

TABLE V

pH-Stability of Iodopsin and Rhodopsin

A mixture of iodopsin and rhodopsin in a chicken retinal extract was incubated a total of 60 minutes in the dark at room temperature at various pH's. Each solution was then bleached, first in red, then in white light to measure the remaining content of visual pigments. This is shown in columns 3 and 4 below.

pH	Buffer	Pigment remaining after 1 hr. at 25°C.	
		Iodopsin K_{560}	Rhodopsin K_{500}
3.6	Acetate	0.020	—
4.0	Acetate	0.106	0.320
5.2	Phosphate	0.166	0.297
6.5	Phosphate	0.182	0.286
7.7	Phosphate	0.114	—
9.0	Borate	0.014	0.289

Iodopsin Poisons

Certain substances which combine spontaneously with either retinene or opsin poison the synthesis of *rhodopsin*. So for example the synthesis is inhibited completely by about 1.0 M formaldehyde and about 0.1 M hydroxylamine (Wald and Brown, 1950). Formaldehyde competes with retinene for the amino and sulfhydryl groups of opsin with which both aldehydes can couple; while hydroxylamine competes with opsin by combining with retinene to form retinene oxime. The synthesis of rhodopsin depends also upon sulfhydryl groups of opsin, and is blocked completely by 10^{-4} M *p*-chloromercuribenzoate (Wald and Brown, 1951–52). The concentrations of these poisons which block rhodopsin synthesis do not appreciably attack the completed molecule.

In general such poisons not only inhibit the synthesis of *iodopsin*, but bleach this molecule in solution. We had an early indication that iodopsin is much more sensitive to reagents than rhodopsin in the observation that alum, which plays an important part in the purification of rhodopsin, destroys iodopsin (Wald, 1937 *b*). We have seen also that iodopsin is much more susceptible than rhodopsin to acids and bases.

p-Chloromercuribenzoate.—The sulfhydryl poison, *p*-chloromercuribenzoate (PCMB), blocks the synthesis of iodopsin, and in the same range of concentration destroys the completed pigment.

The destruction of iodopsin by PCMB is shown in Fig. 13. 0.1 ml. of various dilutions of PCMB and 0.1 ml. of phosphate buffer were added to 0.3 ml. portions of

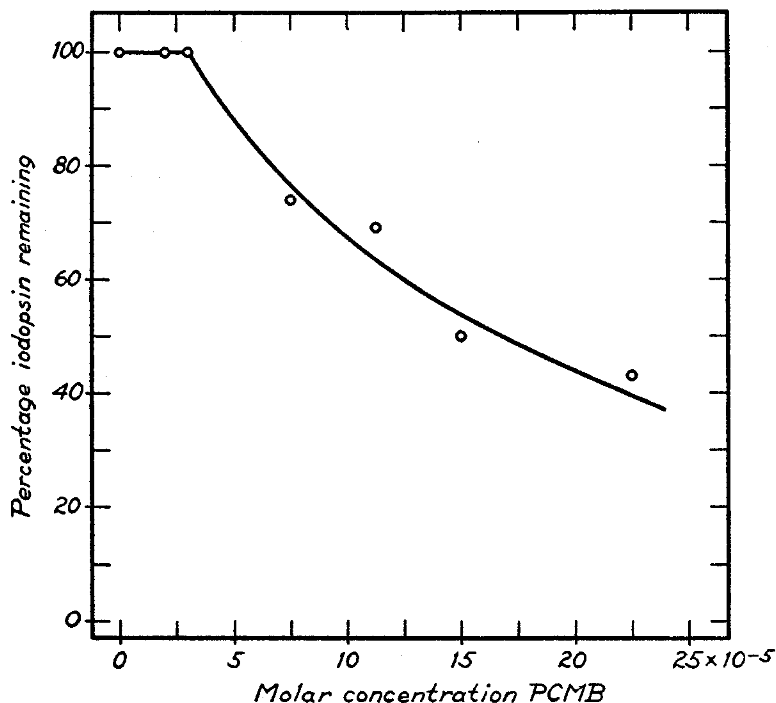


Fig. 13. The destruction of iodopsin by *p*-chloromercuribenzoate (PCMB). A chicken retinal extract was left for an hour in the dark in the presence of various concentrations of PCMB (23°C., pH 7). At the end of this period, the iodopsin remaining was determined by bleaching in deep red light, and measuring the fall of extinction at 560 mμ. The results are expressed as percentages of the iodopsin in a control solution to which no PCMB had been added.

a chicken retinal extract (pH 7). These mixtures were incubated in the dark at about 23°C. for a total of 60 minutes. Then the iodopsin was bleached by 20 minutes' exposure to red light, to determine how much had survived the incubation.

Following this bleaching, 0.1 ml. of neoretinene *b* in digitonin solution was added to each mixture, and the solutions were incubated in the dark for 10 minutes. Then they were again bleached completely in red light, to determine how much new iodopsin had regenerated. This is shown as a function of the PCMB concentration in Fig. 14.

Fig. 13 shows that iodopsin, left in the dark for an hour at 23°C., is stable in the presence of PCMB up to a concentration of about 3×10^{-5} M. This represents about 10 times the molar concentration of iodopsin in the reaction mixtures, in terms of retinene equivalents. Above this concentration of PCMB,

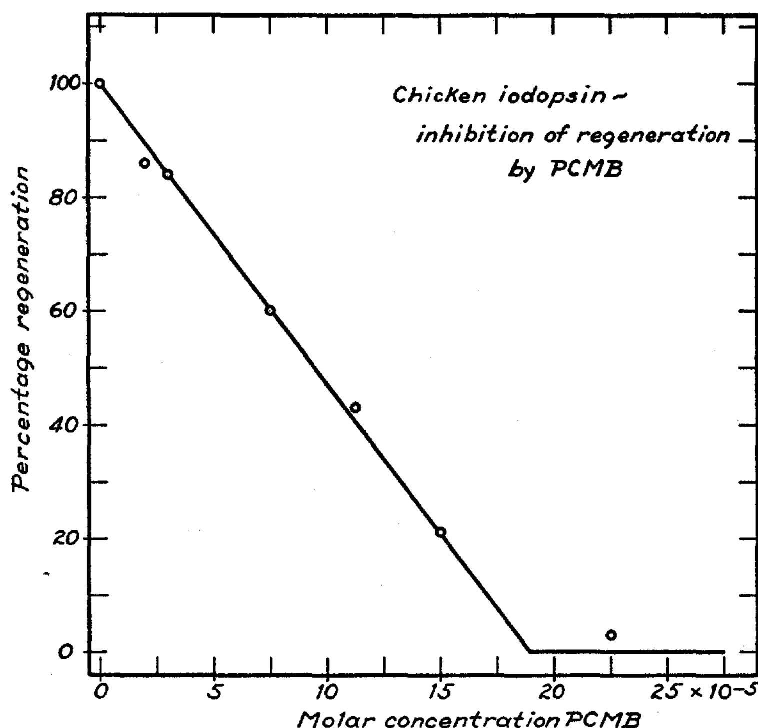


FIG. 14. Inhibition of iodopsin synthesis by *p*-chloromercuribenzoate (PCMB). The residues from Fig. 13 had been bleached for 20 minutes in red light. Then neoretinene *b* was added to each solution, and it was incubated in the dark for 10 minutes. The iodopsin which formed was determined by measuring the fall in extinction at 560 mμ on exposing the final solutions to deep red light. This is expressed as percentage of the synthesis of iodopsin in a control solution to which no PCMB had been added.

the iodopsin is bleached more and more quickly, and would have been wholly destroyed at concentrations of the order of 10^{-3} M.

Fig. 14 shows that PCMB in any amount inhibits to some degree the synthesis of iodopsin, as it does of rhodopsin. The synthesis is blocked completely at about 1.8×10^{-4} M PCMB. This is a much higher concentration of the poison than is needed to block rhodopsin synthesis (Wald and Brown, 1951–52). It is about 50 times the molar equivalent of the retinene involved in the

reaction; *i.e.*, about 50 molecules of PCMB are required here to prevent 1 molecule of neoretinene *b* from combining with photopsin to form iodopsin. This may not be a fair appraisal of the situation, however, for the retinal extract was heavily contaminated with other substances than the visual pigments, and these might have combined with much of the PCMB that had been added.

Though the first action of PCMB upon a protein may be simply to combine with sulfhydryl groups, this is frequently followed by deep-seated changes comparable with denaturation. The first action can be reversed by removing the mercurial with other sulfhydryl-containing molecules such as glutathione; the second may be irreversible. Thus the inhibition of rhodopsin synthesis with PCMB could be reversed about 30 per cent by adding glutathione within 15 minutes (Wald and Brown, 1951–52). Similar experiments with iodopsin yielded no measurable reversal of PCMB poisoning at all. One sees from this as from Fig. 13 that the action of PCMB on iodopsin goes beyond simple combination with sulfhydryl groups, and involves a fundamental attack upon the structure of the molecule.

Hydroxylamine.—This reagent has a peculiar effect on iodopsin, in a sense just the opposite of its effect on rhodopsin. Hydroxylamine blocks rhodopsin synthesis in concentrations which do not attack the completed molecule; conversely it destroys iodopsin in concentrations which do not prevent its synthesis. These relationships are demonstrated in the following experiment, the results of which are shown in Figs. 15 and 16.

In each of 5 microcells, 0.4 ml. of a chicken retinal extract (Table I, preparation 18) was mixed with either 0.1 ml. of digitonin solution to serve as control; or with 0.1 ml. of various concentrations of neutral hydroxylamine. Changes in the concentration of iodopsin were followed by measuring the extinction at 560 mμ (Fig. 15).

The solutions were first incubated in the dark for 1 hour at room temperature. During this period the control solution maintained its original extinction; but all the solutions containing hydroxylamine bleached to some degree. The way in which the extent of bleaching increased with the concentration of hydroxylamine is shown in Fig. 16.

At the end of this period the solutions were exposed for 20 minutes to the deep red light of a 160 watt microscope lamp passing through a Wratten 89 filter. This bleached the iodopsin completely.

Then neoretinene *b* in digitonin solution was added to all the cells in a final concentration of about 3×10^{-5} M. Though all these solutions contained hydroxylamine in great excess, iodopsin was synthesized in all of them with great speed and in about the original amount. (The extinctions shown at the right in Fig. 15 are not corrected for dilution by the retinene solution added; to make them comparable with those at the left they should be multiplied by 6/5.)

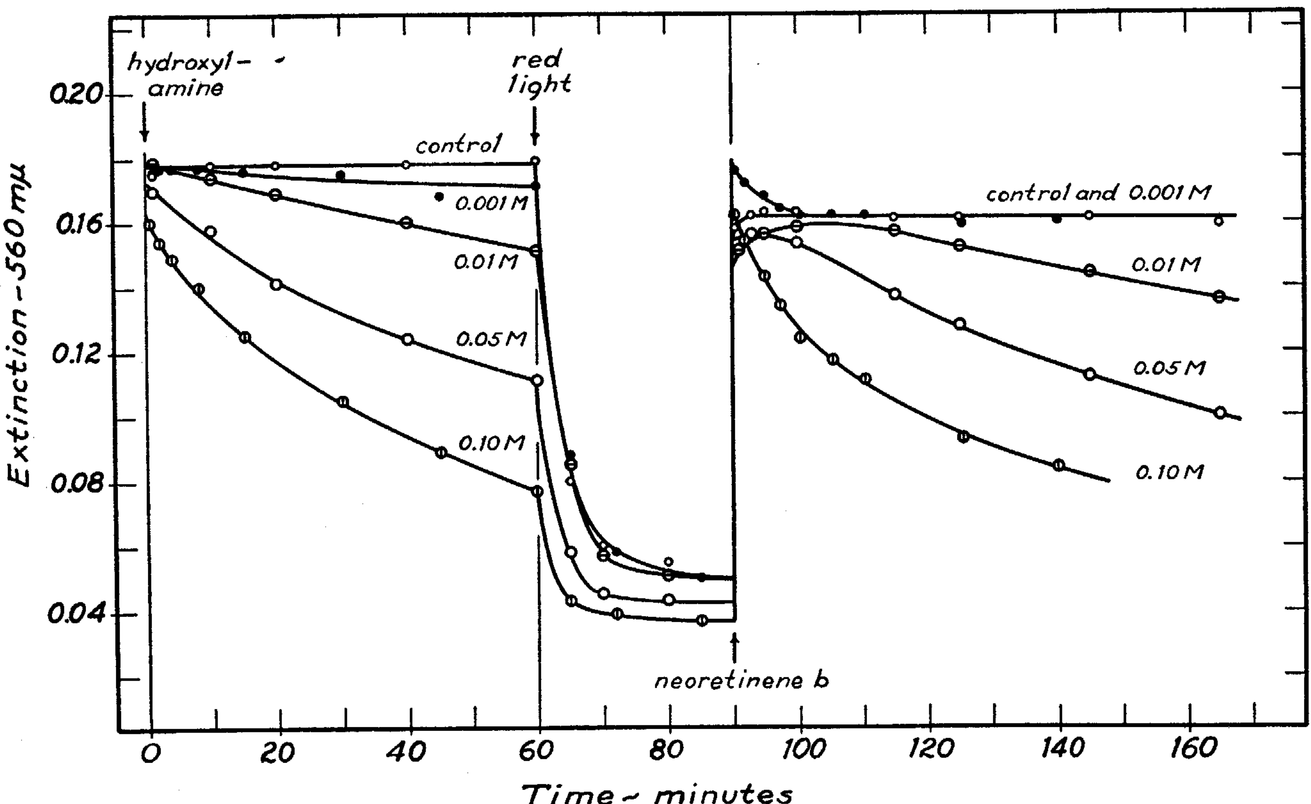


FIG. 15. Destruction and resynthesis of iodopsin in the presence of hydroxylamine. Iodopsin solutions were mixed with hydroxylamine in the concentrations shown, and left in the dark for 60 minutes. The concentrations of iodopsin, measured by the extinction at 560 mμ, declined at various rates, depending on the hydroxylamine concentration. Then the solutions were exposed to deep red light for 20 minutes to bleach their remaining iodopsin. Neoretinene *b* was added in equal amount to all the solutions. In spite of the presence of hydroxylamine in large excess, iodopsin regenerated in all of them, with great speed and in about the original amount (the extinctions at the right are not corrected for the small dilution with neoretinene *b* solution). During a subsequent hour in the dark, the extinctions of iodopsin declined again as before (pH 6.5, 23°C.).

Thereafter in the dark the extinctions of iodopsin fell again, much as before (Fig. 15).

This experiment shows that hydroxylamine, though it destroys iodopsin, does not block its synthesis. We interpret this result as follows. It will be recalled that neoretinene *b* combines very rapidly with photopsin to form iodopsin, about 500 times as quickly as it combines with scotopsin to form rhodopsin.

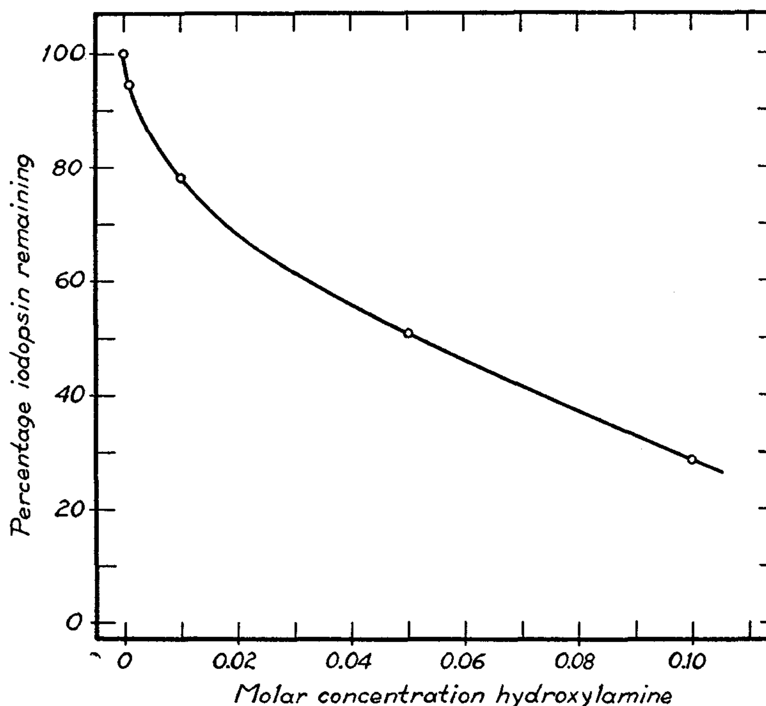


FIG. 16. Destruction of iodopsin by hydroxylamine. Percentages of iodopsin remaining after 1 hour in the dark in the presence of various concentrations of hydroxylamine (pH 6.5, 23°C.).

Hydroxylamine poisons the synthesis of rhodopsin, because it competes successfully with scotopsin for neoretinene *b*. Hydroxylamine cannot compete successfully, however, with photopsin, which reacts so rapidly with neoretinene *b* as to capture virtually all of it. Hydroxylamine is therefore not an efficient poison for the synthesis of iodopsin.

Here iodopsin's immunity ends; for rhodopsin, once formed, is stable to hydroxylamine, while iodopsin is slowly attacked. If the attack were reversible, it could make little headway against the rapid resynthesis of iodopsin. In fact, however, iodopsin bleaches irreversibly, since one of its products is inactive,

all-trans retinene. Though photopsin competes successfully with hydroxylamine for neoretinene *b*, it cannot compete at all for all-trans retinene. Hence neoretinene *b*, offered to a mixture of photopsin and hydroxylamine, begins by combining with the first, but ends attached to the second: it begins by forming iodopsin, but ends as all-trans retinene oxime.

This sequence of reactions can be viewed in another way. Photopsin here is acting as an enzyme—a retinene isomerase—which catalyzes the isomerization of retinene from the cis form, neoretinene *b*, to the all-trans configuration. In this process iodopsin plays the role of enzyme-substrate complex. The same point can be made of the normal functioning of iodopsin in the visual cycle; but there light is required to break the complex, while here it is broken by hydroxylamine in a "dark" reaction.

The Absorption Spectrum of Iodopsin

Since iodopsin has never been prepared free from other pigments, we have had to be content heretofore with its difference spectrum, obtained as described above. Useful as this is, it is inadequate for many purposes for which one would like to know the true absorption spectrum. Fortunately the experiments described above permit one to compute this in two independent ways, which yield very nearly the same result.

1. The first procedure is illustrated in Fig. 17 and Table VI. Curve *A*, taken from Fig. 6, represents the absorption spectrum of iodopsin mixed with a residue of neoretinene *b*. By subtracting from this the absorption spectrum of neoretinene *b*, one should obtain the spectrum of pure iodopsin.

We possess the absorption spectrum of neoretinene *b* in aqueous digitonin solution. The problem is to determine its extinction in the mixture represented by curve *A*. To estimate this, we first assume tentatively that the absorption spectrum of iodopsin resembles that of rhodopsin in having a minimum in the violet, the extinction of which is about 0.2 of the maximum at 562 mμ. We begin therefore by choosing an extinction of neoretinene *b* which, subtracted from that of the mixture, yields this result.

This provides a tentative spectrum of iodopsin. We know that iodopsin bleaches in red light to a mixture of photopsin and all-trans retinene; and we have the absorption spectrum of such a mixture (*cf.* Fig. 18 *B*). If we subtract this from the tentative spectrum of iodopsin itself, the result should be the difference spectrum of iodopsin (*cf.* Figs. 5 and 7). The latter possesses a number of specific properties, all of which must be satisfied: (*a*) the opposed changes of extinction at 562 and 391 mμ should be in the correct ratio, about 1.50; (*b*) the computed difference spectrum should have the correct shape; and (*c*) it must assume the value zero at the wave length at which iodopsin and the retinene formed from it have the same extinction, at which therefore there is no change of extinction on bleaching (the isosbestic point). In iodopsin bleached at pH 6.5 this is at about 465 mμ.

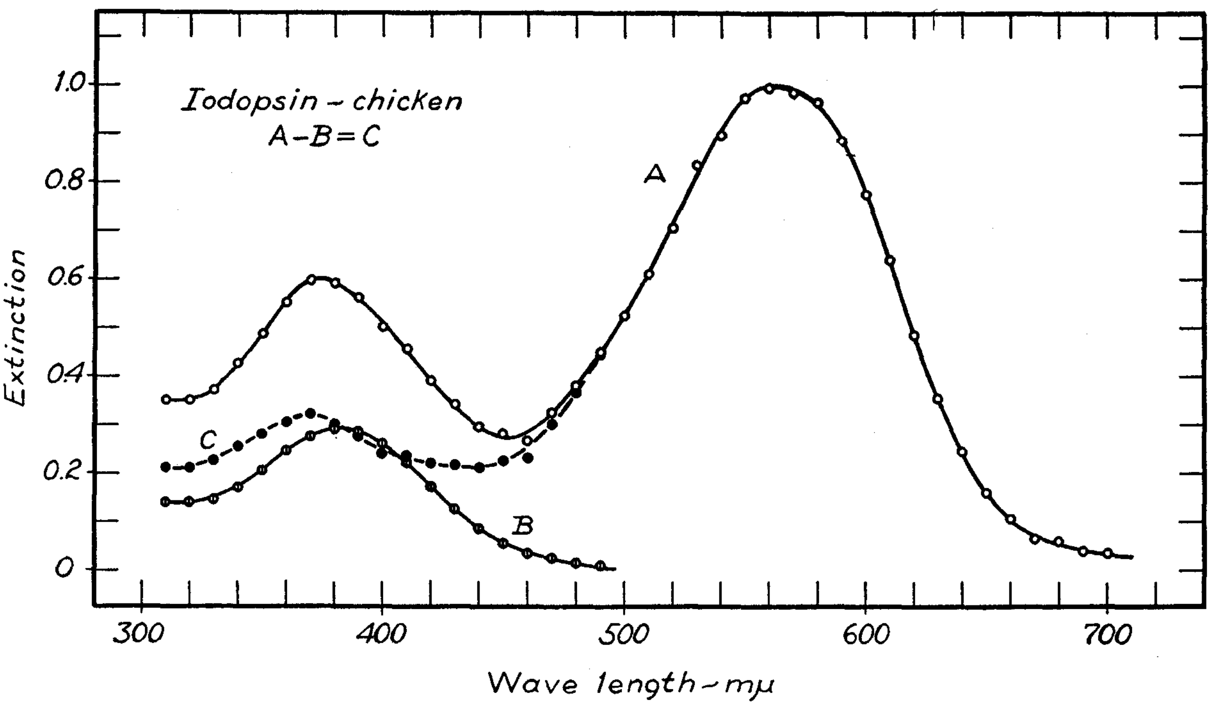


FIG. 17. The absorption spectrum of iodopsin (method 1). Curve *A* is the preparation of iodopsin synthesized in Fig. 6. In addition to iodopsin it contains a residue of neoretinene *b*. *B* is the spectrum of neoretinene *b* in digitonin solution. When this is subtracted from *A*, the spectrum of iodopsin results (*C*).

TABLE VI

The Absorption Spectrum of Iodopsin

Wave length	Extinction in 2 per cent digitonin		
	(*A*) Iodopsin + neoretinene *b*	(*B*) Neoretinene *b*	(*C*) Iodopsin
mμ			
310	0.350	0.140	0.210
320	0.350	0.140	0.210
330	0.370	0.145	0.225
340	0.425	0.170	0.255
350	0.485	0.205	0.280
360	0.550	0.245	0.305
370	0.595	0.275	0.320
380	0.590	0.290	0.300
390	0.560	0.285	0.275
400	0.500	0.260	0.240
410	0.455	0.220	0.235
420	0.390	0.170	0.220
430	0.340	0.125	0.215
440	0.295	0.085	0.210
450	0.280	0.055	0.225
460	0.265	0.035	0.230
470	0.325	0.025	0.300
480	0.380	0.015	0.365
490	0.450	0.010	0.440
500	0.525	—	0.525
510	0.610		0.610
520	0.705		0.705
530	0.835		0.835
540	0.895		0.895
550	0.975		0.975
560	0.995		0.995
570	0.985		0.985
580	0.965		0.965
590	0.885		0.885
600	0.775		0.775
610	0.640		0.640
620	0.485		0.485
630	0.355		0.355
640	0.245		0.245
650	0.160		0.160
660	0.105		0.105
670	0.065		0.065
680	0.060		0.060
690	0.040		0.040
700	0.035		0.035

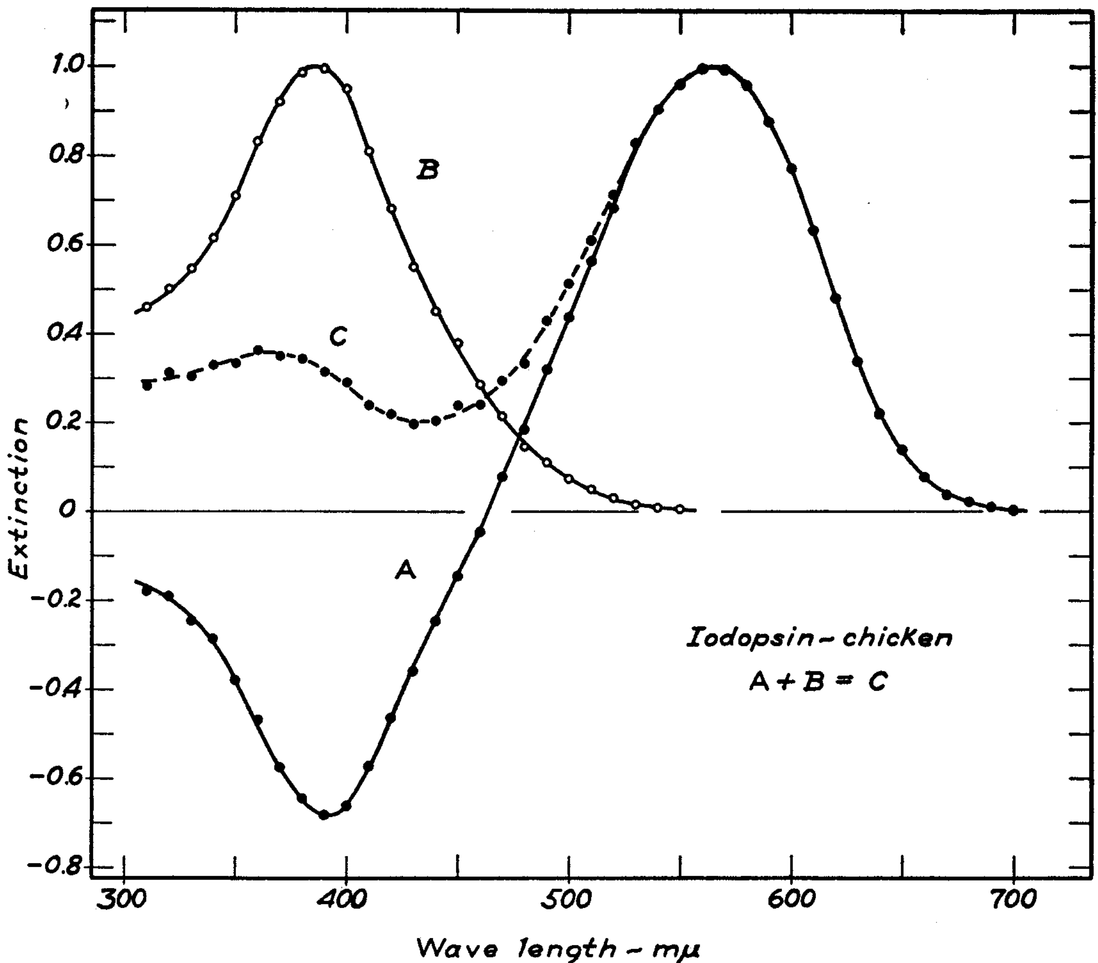


FIG. 18. The absorption spectrum of iodopsin (method 2). Curve *A* is the difference spectrum of iodopsin. By definition this is the difference between the absorption spectra of iodopsin and the products of its bleaching (all-trans retinene and photopsin). Curve *B* is the absorption spectrum of all-trans retinene in a chicken retinal extract containing photopsin. The sum of *A* and *B* is therefore the absorption spectrum of iodopsin (*C*).

TABLE VII

The Absorption Spectrum of Iodopsin

Wave length	Extinction in 2 per cent digitonin				
	(*A*) Difference spectrum: Iodopsin (average of 3)	(*B*) All-trans retinene in retinal extract	(*C*) Iodopsin (A + B)	Iodopsin (Table VI)	Iodopsin (Average)
mμ					
310	−0.180	0.460	0.280	0.210	0.258
320	−0.190	0.500	0.310	0.210	0.287
330	−0.246	0.545	0.299	0.225	0.283
340	−0.286	0.615	0.329	0.255	0.311
350	−0.379	0.710	0.331	0.280	0.319
360	−0.468	0.830	0.362	0.305	0.349
370	−0.574	0.920	0.346	0.320	0.342
380	−0.645	0.985	0.340	0.300	0.332
390	−0.683	0.995	0.312	0.275	0.305
400	−0.663	0.950	0.287	0.240	0.277
410	−0.573	0.810	0.237	0.235	0.238
420	−0.465	0.680	0.215	0.220	0.218
430	−0.359	0.550	0.191	0.215	0.201
440	−0.247	0.450	0.203	0.210	0.204
450	−0.145	0.380	0.235	0.225	0.234
460	−0.046	0.285	0.239	0.230	0.238
470	0.078	0.215	0.293	0.300	0.295
480	0.185	0.145	0.330	0.365	0.340
490	0.319	0.110	0.429	0.440	0.433
500	0.437	0.075	0.512	0.525	0.517
510	0.562	0.050	0.612	0.610	0.611
520	0.683	0.030	0.713	0.705	0.711
530	0.819	0.015	0.834	0.835	0.833
540	0.904	0.010	0.914	0.895	0.908
550	0.961	0.005	0.966	0.975	0.967
560	0.996	—	0.996	0.995	0.996
570	0.994		0.994	0.985	0.992
580	0.960		0.960	0.965	0.962
590	0.878		0.878	0.885	0.880
600	0.773		0.773	0.775	0.774
610	0.634		0.634	0.640	0.636
620	0.481		0.481	0.495	0.484
630	0.339		0.339	0.350	0.342
640	0.221		0.221	0.240	0.226
650	0.139		0.139	0.150	0.142
660	0.078		0.078	0.080	0.079
670	0.037		0.037	0.035	0.036
680	0.020		0.020	0.020	0.022
690	0.008		0.008	0.010	0.010
700	0.004		0.004	0.000	0.001

Our problem then was to set an extinction of neoretinene *b* which, subtracted from curve *A* in Fig. 17, would end in satisfying all these conditions. This is curve *B* of the figure. The difference, $A - B = C$, represents the true absorption spectrum of iodopsin. Above 500 mμ its shape is identical with that of curve *A*, and is unequivocal. At shorter wave lengths its shape is in some measure of doubt, and here it is drawn as a broken line. It possesses a small subsidiary maximum at about 370 mμ, apparently analogous to the β-band in the spectrum of rhodopsin.

2. Fig. 18 and Table VII illustrate the second method of computing the absorption spectrum of iodopsin.

When iodopsin is bleached in red light, its difference spectrum is defined by the formula:

$$\text{Iodopsin} - (\text{photopsin} + \text{all-trans retinene}) = \text{difference spectrum}$$

The difference spectrum records only the changes which result from bleaching. It is independent of any colored impurities which may be present, provided they are light-stable. It would represent an absolute property of iodopsin, invariant in form, were it not for one circumstance. The all-trans retinene liberated in bleaching does not remain free. It couples spontaneously with amino groups in opsins and other molecules to yield complexes which vary in absorption with pH (Ball *et al.*, 1949). At all pH's the absorption spectra of the complexes vary somewhat from that of free retinene. All the molecules present which can couple with retinene may affect its absorption, and hence the difference spectrum of iodopsin. These effects are small, but significant.

Were it not for this, one could obtain the absorption spectrum of iodopsin over most of its range by simply adding to any difference spectrum the absorption spectrum of all-trans retinene. As it is, one requires for this computation the absorption spectrum of the mixture of complexes which all-trans retinene forms in a bleached retinal extract, at the same pH at which the difference spectrum was measured.

Such a spectrum is shown in curve *B* of Fig. 18. A chicken retinal extract was exposed to red light to bleach its iodopsin, and its absorption spectrum was measured. Then a small amount of all-trans retinene solution was added, and the absorption spectrum remeasured. The difference between these spectra is the desired absorption spectrum of all-trans retinene in bleached chicken retinal extract.

The next problem involves the extinction to assign to this in making it represent the product of bleaching iodopsin. We know that rhodopsin bleaches in neutral solution to yield nearly an equal extinction of all-trans retinene. We assume tentatively that the same is true of iodopsin, and so make the extinction of all-trans retinene at about 390 mμ equal to the extinction of the difference spectrum of iodopsin at 562 mμ. This is not exact; but any very different assumption leads to an absurd result.

Nothing remains but to add the resulting spectrum of all-trans retinene to the difference spectrum of iodopsin. We have done this with three exemplary difference spectra, each obtained from a different retinal preparation. The results agreed very well with one another. The average of all three difference spectra is shown as *A* in Fig. 18 and Table VII; and *C* is the average absorption spectrum of iodopsin obtained by adding this to *B*.

At wave lengths longer than 530 mμ, the computed absorption spectrum is virtually identical with the difference spectrum, and therefore is unequivocal. Below this wave length, where there is some possibility of error, the spectrum is drawn in Fig. 18 with a broken line. A minimum appears at 435 mμ, at which the extinction is 0.20 of that at 562 mμ. It will be recalled that this is the ratio of minimum to maximum in the spectrum of rhodopsin, and that it provided one of the criteria used to establish the spectrum of iodopsin by the previous method. Once again also, a small maximum appears at about 370 mμ, analogous to the β-band of rhodopsin.

The results obtained by both methods of computing the absorption spectrum of iodopsin are compared in Fig. 19. The divergence of the curves at low wave lengths expresses the degree of doubt which still obtains in this region of the spectrum. The results of all the computations are averaged in the last column of Table VII.

In summary, the absorption spectrum of iodopsin possesses the following characteristics: an α-band, covering most of the visible spectrum, maximal at 562 mμ; a minimum at about 435 mμ, of extinction 0.2 times that at 562 mμ; and a small β-band at about 370 mμ of extinction 0.32 to 0.35 times that at 562 mμ. The protein photopsin must also have a specific absorption associated with it at lower wave lengths (the γ-band); but we have no way of estimating this at present.

An important result of this study has been to show that unit extinction of iodopsin, like unit extinction of rhodopsin, yields approximately unit extinction of all-trans retinene on bleaching. This means that per equivalent of retinene, iodopsin and rhodopsin have nearly the same extinction. It was recently shown that the molar extinction of cattle rhodopsin is 40,600 (Wald and Brown, 1953–54). Iodopsin possesses nearly the same molar extinction per retinene equivalent.

Physiological Correlations

Some of the fundamental properties of vision have their source in chemical and physical properties of the retinal pigments. Three such relationships have now to be considered: (*a*) that between the concentrations of visual pigment in wholly dark-adapted rods and cones, and the sensitivities of rod and cone vision; (*b*) the kinetics of synthesis of rhodopsin and iodopsin, and rod and cone dark adaptation; and (*c*) the absorption spectra of rhodopsin and iodopsin, and the spectral sensitivities of the rods and cones. A fourth correlation—the de-

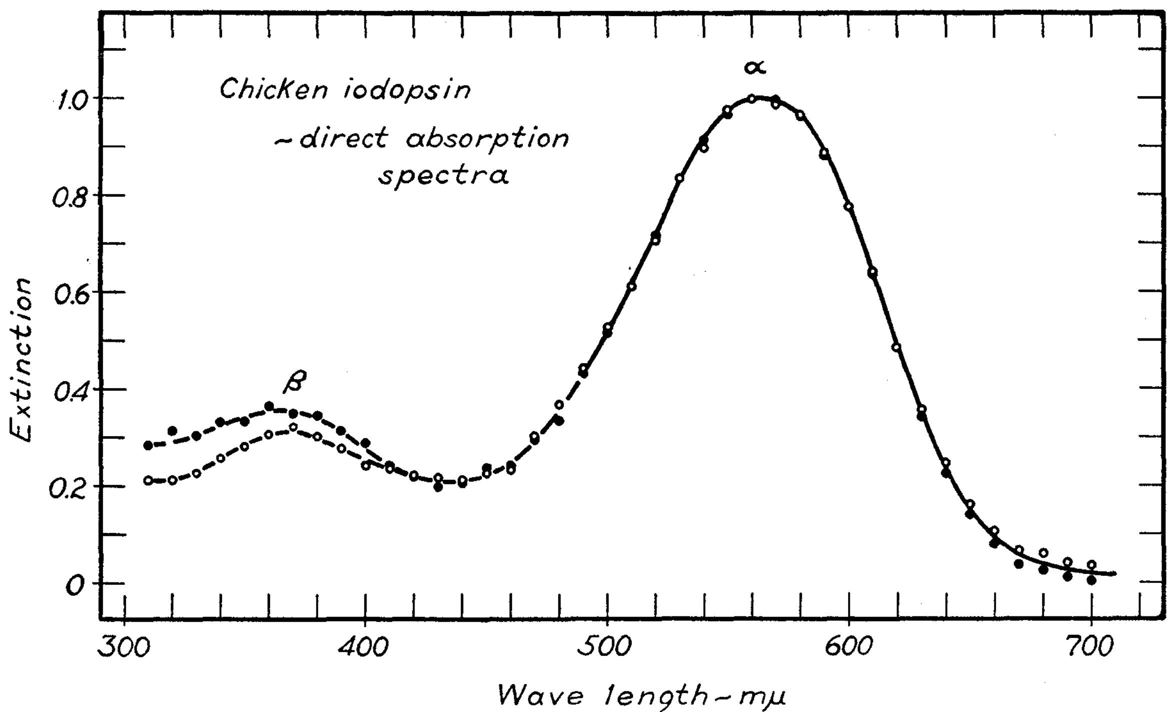


FIG. 19. The absorption spectrum of iodopsin, obtained by two independent methods (curves *C* of Figs. 17 and 18). At wave lengths longer than 500 mμ the absorption spectrum has been measured directly, and is unequivocal. At shorter wave lengths it is the result of computation; the discrepancy between the curves indicates the degree to which the spectrum is still in doubt. Both curves show the same general characteristics: an α-band maximal at 562 mμ, and a small β-band in the near ultraviolet at about 370 mμ.

pendence of rod and cone vision upon vitamin A, the common precursor of rhodopsin and iodopsin—can better be discussed elsewhere.

(*a*) *Pigment Concentration and Visual Sensitivity.*—Dark-adapted rods are much more sensitive to light than cones; they contain also much more photosensitive pigment. What is the connection between these properties?

In general one expects that, *other conditions being equal*, the sensitivity of a receptor, when wholly dark-adapted, should be determined by its capacity to absorb light. The basic quantity with which we are concerned is the *percentage absorption* of visual pigment in the receptors, for this gives us directly the probability that an incident quantum will be absorbed. The *concentration* of visual pigment is important only as it affects the percentage absorption. When Beer's law is applicable, the extinction is proportional to concentration; and percentage absorption is proportional to both, up to about 10 per cent absorption. At higher absorptions, the percentage absorption rises more slowly than the extinction or concentration. On occasion still another factor intervenes—the molecular orientation of visual pigment in the receptor. Schmidt (1938) has observed that in frog rods, rhodopsin is so oriented as to make the rod dichroic. With plane-polarized light passing transversely through it, the rod appears red in one orientation, and colorless in the perpendicular orientation. In light passing down the axis of the rod, the normal direction in the retina, it appears red in all planes of polarization. Because of this orientation, rhodopsin in the rod—perhaps also in cones—absorbs light which enters axially more strongly than if the same amount of rhodopsin were distributed at random, as in free solution. Through this factor its axial extinction for *unpolarized* light may be increased as much as 1.5 times (Commoner and Lipkin, 1949).

To answer our question rigorously, therefore, we should compare the true percentage absorption of visual pigment in the rods and cones, measured in the direction of their long axes, with the sensitivity to light of the individual dark-adapted cells. There is no reason why the comparison should not ultimately be made upon this basis; but for the present we must be content with much less.

Our maximum yield of visual pigments from the chicken retina (Table I, preparations 5 and 6), in terms of the extinction at λ_{max}, per retina, reduced to 1 $cm.^3$ of solution and measured in 1 cm. depth, was:

Rhodopsin (500 mμ): 0.023

Iodopsin (560 mμ): 0.015

The area of the chicken retina is about 2.5 $cm.^2$ The extinctions of these pigments, therefore, if spread in an even layer over the retinal surface, would be:

Rhodopsin: 0.0092

Iodopsin: 0.0060

Of course these pigments are not distributed evenly over the retina, but are concentrated at discrete points, the outer segments of the rods and cones.

We are indebted to Professor Russell Carpenter of Tufts College for a preliminary count of the numbers of rods and cones in a central area of the chicken retina. A section of retina 6 μ thick and 2.64 mm. long, starting from the disc—the point of entry of the optic nerve—was found to contain 1227 cones and 151 rods, a ratio of 8.1 cones : 1 rod. Professor Carpenter states that more peripheral regions of the retina contain relatively fewer rods.

If we assume that the retinal surface is covered approximately 9/10 with cones and 1/10 with rods, the extinctions of iodopsin and rhodopsin in these structures should be:

Average extinction of rhodopsin in a rod: 0.092
Average extinction of iodopsin in a cone: 0.0067

The corresponding percentages absorbed are 19 and 1.5. That is, the absorption of visual pigment in a rod may on the average be about 13 times that in a cone. This estimate is valid only in order of magnitude.

With this we should like to compare the ratio of sensitivities of single dark-adapted rods and cones. Lacking such data, we must use instead the sensitivities of rod and cone vision in the whole animal. Honigmann (1921) has measured the visual sensitivity of mature chickens after 8 hours of dark adaptation. He gives this the value 253 at 520 mμ, which we can take to be the arbitrary measure of the maximal rod sensitivity. The cone sensitivity, measured within 1 minute in the dark following 5 minutes' light adaptation, was 6.9 at 580 mμ. The ratio of rod to cone sensitivity on this basis is 37. The cones, however, were relatively light-adapted. Honigmann measured the dark adaptation of the chicken at 660 mμ, where it depends primarily on cones, and found the visual sensitivity to increase 1.6 times during 27 minutes in darkness. If this factor is applied as a correction to the above measurement of cone sensitivity it becomes $6.9 \times 1.6 = 11$. On this basis the ratio of rod to cone sensitivity is 23.[5]

These estimates permit us to conclude only that the ratio of sensitivities of rod and cone vision in the intact animal (*ca.* 23) is of the same order as the ratio of absorptions of visual pigments in the rods and cones (*ca.* 13). Improvement of the data along the lines discussed above should clarify this relationship further.

[5] Using an ingenious conditioning procedure, Blough (1954) has made accurate measurements of dark adaptation in mature pigeons. The course of dark adaptation in this animal—plotted as log sensitivity against time in the dark—presents the familiar division into an initial rapid cone, followed by a slow terminal rod segment. The cone plateau lies about 1.7 log units above the rod plateau; that is, the dark-adapted rod apparatus is about 50 times as sensitive as the dark-adapted cone apparatus.

The visual sensitivity must be affected by many other factors than the absorption of visual pigment; yet in the dark-adapted receptors this simple physicochemical property appears to a first approximation to set the levels of sensitivity, and hence to determine whether the receptor shall function in the animal as an organ primarily of day or night vision.

(*b*) *Dark Adaptation and Synthesis of Visual Pigments.*—For many years it has been assumed that light and dark adaptation reflect the bleaching and resynthesis of visual pigments in the rods and cones. To test this view, one needs to know the relationship between the visual threshold, or its reciprocal the sensitivity, and the concentration of visual pigment in the receptors.

We have seen that in wholly dark-adapted receptors, the sensitivity is approximately proportional to the concentration of visual pigment. This says nothing, however, about the way in which sensitivity and pigment concentration are related in the course of visual adaptation—witness the fact that the rods and cones reverse their relative positions during adaptation: the rods, though much *more* sensitive than cones in the dark-adapted eye, are much *less* sensitive than cones in the light-adapted eye.

Recently a theory of the mechanism of the visual threshold and adaptation has been proposed which leads to a relation between pigment concentration and sensitivity (Wald, 1954). The receptor is assumed to be compartmented, each compartment responding in an all-or-nothing way on absorbing a first quantum of light; *i.e.*, on the bleaching of a first molecule of visual pigment. If the receptor contains n_0 compartments, and n_x respond, its threshold (I_t) rises above the wholly dark-adapted threshold (I_0) by the factor:

$$\frac{I_t}{I_0} = \frac{n_0}{n_0 - n_x} \tag{1}$$

That is, the rise of visual threshold is equal to the ratio between the total number of compartments and the number left undischarged.

If in a receptor the number of molecules of visual pigment bleached by light is x, the mean number bleached per compartment is x/n_0. According to the Poisson law (Fisher, 1925),[6] the fraction of compartments which have *no* molecules bleached, and hence have not responded, is

$$\frac{n_0 - n_x}{n_0} = e^{-x/n_0} \tag{2}$$

[6] If a number can take the values 0, 1, 2 . . . x, and is distributed according to the Poisson series, then the frequency with which the values occur is given by the series:

$$e^{-m}\left(1, m, \frac{m^2}{2!}, \;\cdots\cdots\; \frac{m^x}{x!}\right)$$

The Poisson series does not apply rigorously to our problem, for one condition of its applicability is that all trials be independent of one another, and here they are not:

From (1), the rise of threshold is the reciprocal of this:

$$\frac{I_t}{I_0} = e^{x/n_0}$$

$$x = 2.303\ n_0 \log \frac{I_t}{I_0} \tag{3}$$

Since n_0 and I_0 are constants, this equation describes a linear relationship between the number of molecules of visual pigment bleached in the receptors and the logarithm of the visual threshold.

If the initial number of molecules of visual pigment in the dark-adapted receptors is x_0, the number remaining unbleached—and hence the concentration, C—is $(x_0 - x)$:

$$C = (x_0 - x) = x_0 - 2.303\ n_0 \log \frac{I_t}{I_0}$$

$$C = (x_0 + 2.303\ n_0 \log I_0) - 2.303\ n_0 \log I_t$$

Since the term $(x_0 + 2.303\ n_0 \log I_0)$ is a constant (a), and $2.303\ n_0$ also a constant (b), this equation reduces to the form:

$$C = a - b \log I_t$$

—the equation of a straight line of slope b and intercept a. It states that apart from the intercept constant, the concentration of visual pigment in the receptors is proportional to $-\log$ threshold; *i.e.*, to log sensitivity.

Applied to the problem of dark adaptation, this relation implies a close correspondence between the rise of concentration of rhodopsin and iodopsin and the rise of log sensitivity in the rods and cones.

We have long known that in the human eye dark adaptation is much more rapid in cones than in rods. In the cones it is virtually complete within about 4 to 5 minutes, while in the rods it occupies about 45 minutes. We do not yet

those compartments of the rods and cones which face the light are most likely to absorb incident quanta, and interfere to a degree with the absorption of quanta by compartments which lie behind them. Similarly the bleaching of visual pigment in the forward compartments allows more light to penetrate to those further back. The discrepancies introduced in this way are very small for cones, and for most rods; and the Poisson series offers an adequate first approach to our problem. The class of compartment we are most interested in has absorbed *no* quanta, and hence is undischarged. Its frequency is e^{-m}, in which m is the mean number of quanta absorbed per compartment; *i.e.*, the total number of quanta absorbed divided by the total number of compartments, x/n_0. In the present treatment, we have equated the number of quanta absorbed to the number of molecules of visual pigment "bleached," including in this term all intermediate as well as end products of bleaching (*cf.* Wald, Durell and St. George, 1950).

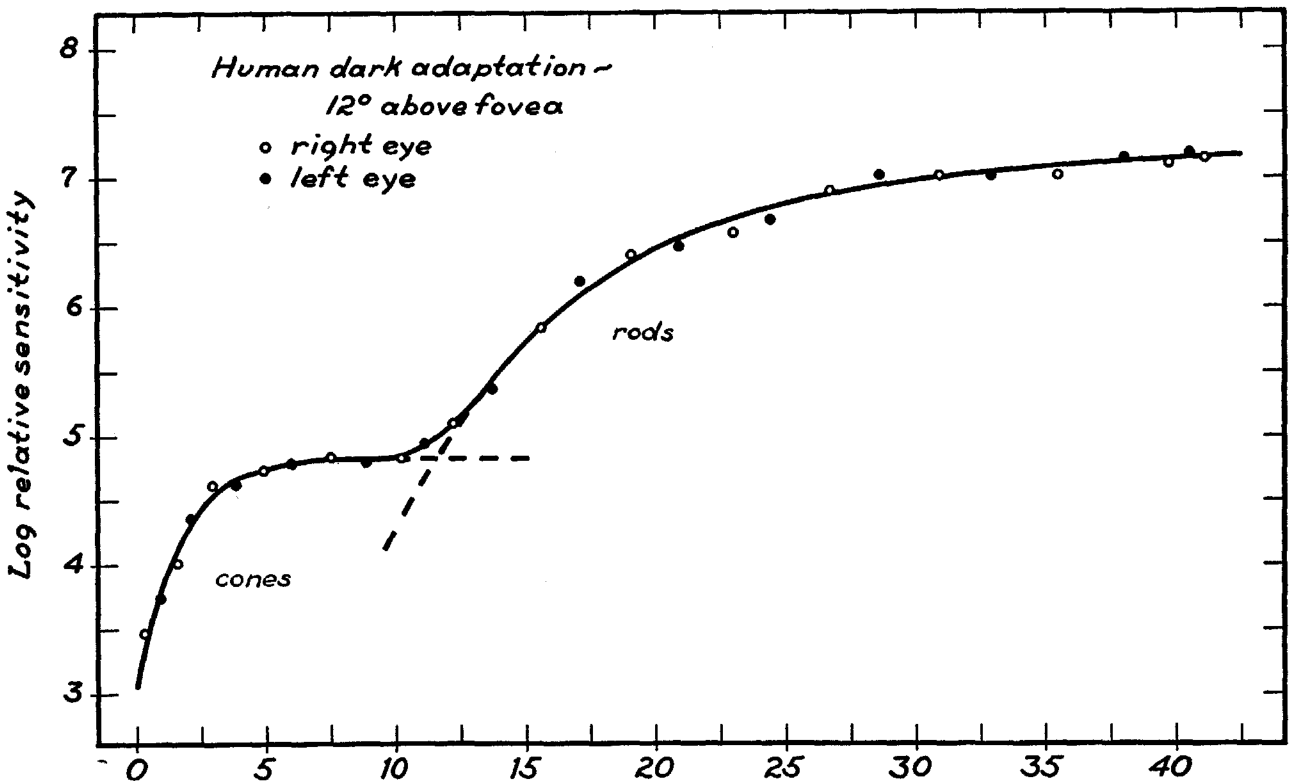


FIG. 20. Dark adaptation of the human eye, measured in a peripheral area which contains both rods and cones. The dark adaptation of the cones is completed within about 5 minutes, that of the rods within about 45 minutes.

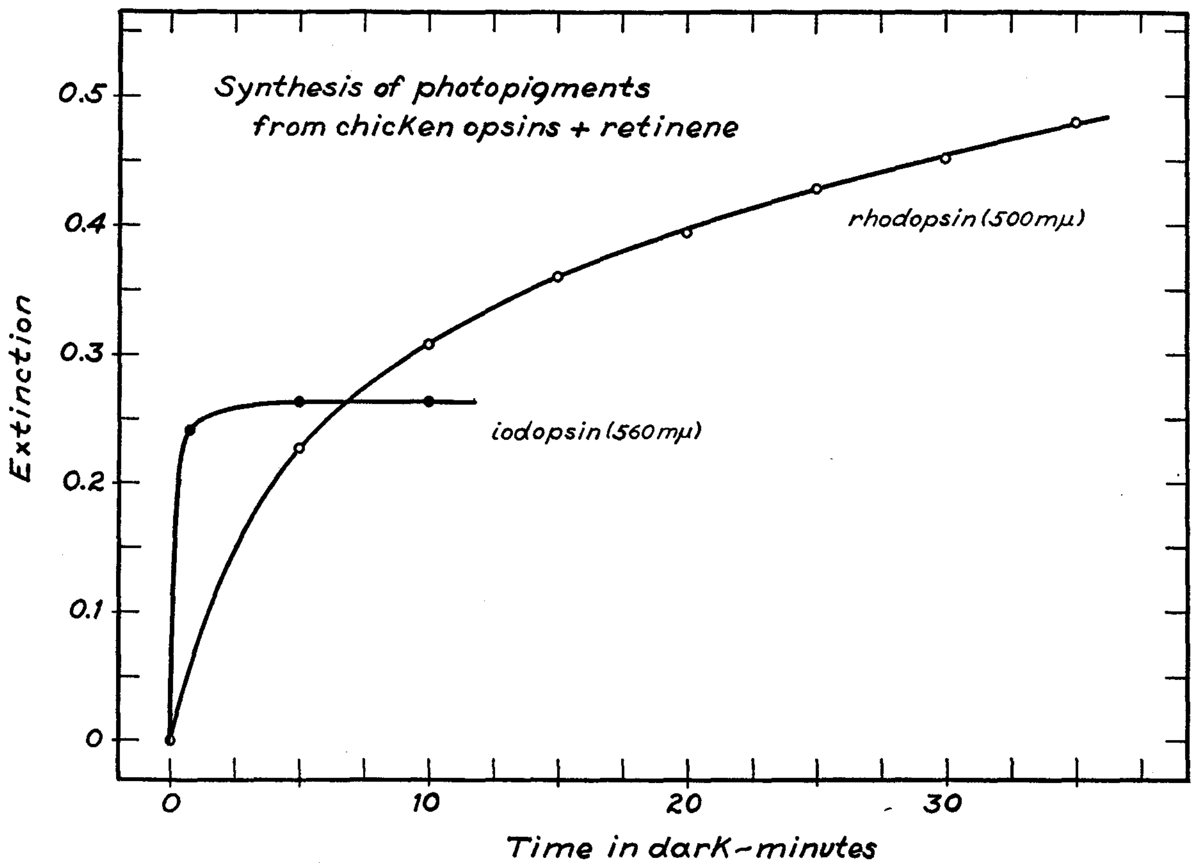


FIG. 21. Synthesis of iodopsin and rhodopsin in solution from a mixture of chicken opsins and neoretinene *b*. This is the same experiment shown in Fig. 8, but with the rhodopsin extinctions multiplied by 1.3. At 23°C., the temperature of these measurements, iodopsin synthesis is complete within 2 to 3 minutes, whereas rhodopsin synthesis still continues after 35 minutes.

possess comparable data for the chicken; but Blough (1954) has recently shown that in the pigeon dark adaptation follows a course very similar to that in man (see footnote 5 above).

The dark adaptation of a peripheral area of the human retina is shown in Fig. 20. To facilitate comparison with the synthesis of visual pigments, it is plotted in terms of −log threshold or log sensitivity—the reverse of the usual way of plotting such data. It is divided into the familiar two segments, an initial rapid rise of sensitivity due to the cones, followed by a long, slow rise due to the rods.

Fig. 21 shows the synthesis of chicken iodopsin and rhodopsin in solution from a mixture of neoretinene *b* and cone and rod opsins (25°C.). This is the very experiment shown in Fig. 8, except that all rhodopsin extinctions have been multiplied by 1.3. It is clear that to a first approximation, the course of synthesis of chicken iodopsin and rhodopsin in solution resembles the rise of log sensitivity in cone and rod vision.

It would be fruitless to attempt to push this comparison further with the present data. It involves measurements from widely different species, made at different temperatures; and we know that the synthesis of visual pigments *in vivo* includes more reactions than the combination of opsins with neoretinene *b* (Hubbard and Wald, 1952–53; Wald, 1953 *b*).

These disparities, however, hardly detract from the general conclusion that a striking parallelism exists between the course of dark adaptation, rod and cone, and the synthesis of visual pigments; and that the connection between these functions relates the concentration of visual pigment in the receptors with the *logarithm* of the visual sensitivity.

(*c*) *Absorption Spectra and Spectral Sensitivity.*—It is well recognized that the spectral sensitivity of vision has its ultimate source in the absorption spectra of visual pigments. When corrections have been made for distortions introduced by colored ocular structures, a high degree of correspondence is expected between these functions. Such correspondence has been demonstrated between the spectral sensitivity of human rod vision and the absorption spectrum of rhodopsin (Wald, 1951). With the discovery of iodopsin, this type of comparison could be extended to the cones, and to the shift of sensitivity which accompanies the change from rod to cone vision—the Purkinje phenomenon (Wald, 1937 *b*). Recently also the absorption spectrum of cyanopsin, made by combining retinene$_2$ with chicken photopsin, has been shown to agree in form with the spectral sensitivity of cone vision in the tench and tortoise (Wald, Brown, and Smith, 1953).

In making this type of comparison, the data of spectral sensitivity must be corrected for distortions caused by colored ocular structures; and should be quantized, *i.e.*, rendered in terms of the reciprocals of the numbers of quanta of incident light at each wave length needed to evoke a constant response.

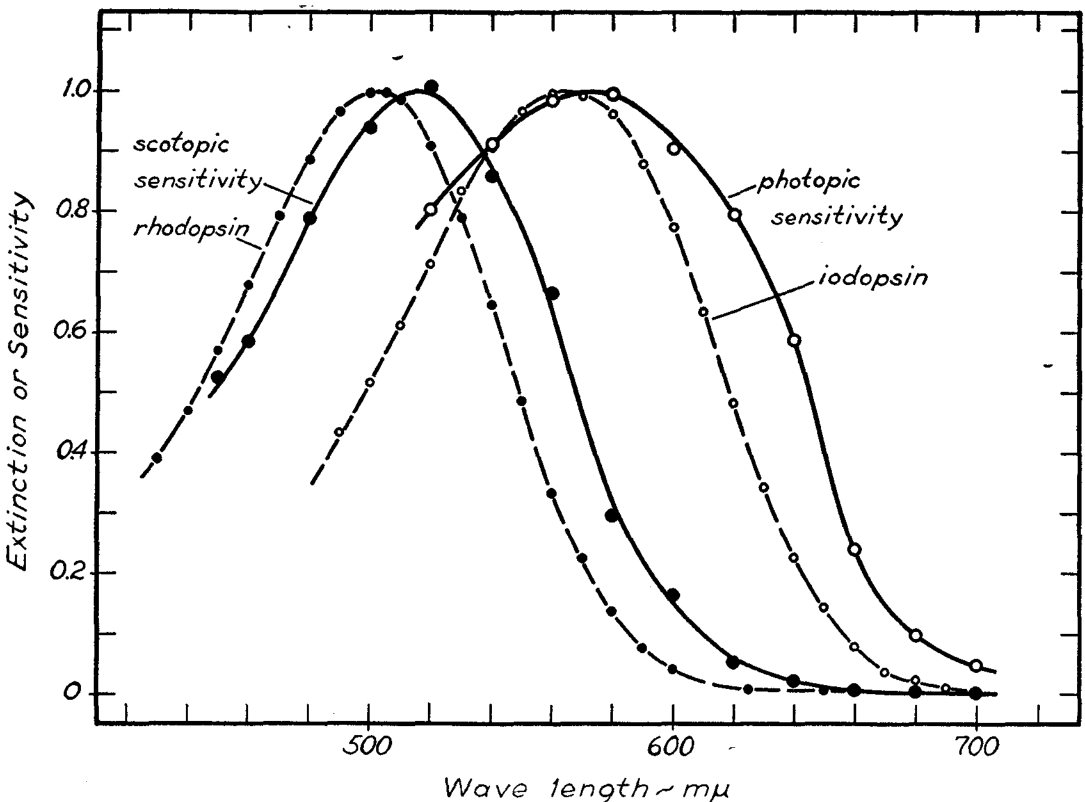


FIG. 22. The absorption spectra of rhodopsin and iodopsin and the Purkinje phenomenon in the chicken. The solid lines show Honigmann's measurements (1921) of the spectral sensitivity of mature chickens, in the dark- and light-adapted condition. The broken lines show the absorption spectra of chicken rhodopsin and iodopsin. The displacement of visual sensitivity toward the red on light adaptation—the Purkinje phenomenon—is mimicked by the displacement of absorption spectrum from the rhodopsin of the rods to the iodopsin of the cones.

The spectra of the visual pigments should be stated in terms of percentage absorption (*cf.* Wald, 1937–38). Extinction and percentage absorption, however, are almost exactly proportional to each other up to 10 per cent absorption; and depart only slightly from proportionality even up to 20 per cent absorption. All known cones and most rods have absorptions well below this value. In the figures which follow, therefore, we have plotted the absorption spectra of the visual pigments as extinction. This introduces appreciable error only in the comparison with frog rod vision (Fig. 24).

The absorption spectra of chicken rhodopsin and iodopsin are shown in Fig. 22 with broken lines. The iodopsin spectrum is the average taken from the last column of Table VII. We have found the difference spectrum of chicken rhodopsin to be virtually identical with that from the bullfrog, and so have used the absorption spectrum of bullfrog rhodopsin—the average of a number of our best preparations—to represent chicken rhodopsin.

The solid lines in Fig. 22 are Honigmann's measurements of the spectral sensitivity of dark- and light-adapted chickens; *i.e.*, of chicken rod and cone vision (1921). Mature birds were presented with lines of rice grains, in a darkened cabinet. One line of grains at a time was illuminated with light of a single wave length, and the intensity raised until the chickens just pecked grains from this line alone. The reciprocal of the energy needed at each wave length to induce this constant response is the spectral sensitivity. The scotopic sensitivity was measured after 8 hours in darkness, the photopic sensitivity within 1 minute in the dark following 5 minutes' light adaptation. The measurements have been quantized by dividing the relative sensitivity by the wave length. All the curves have been brought to the same arbitrary height to facilitate comparison.

The scotopic sensitivity is maximal at about 515 mμ, the photopic at about 575 mμ. This displacement of visual sensitivity toward the red as the eye passes from dim to bright light is the Purkinje phenomenon. The absorption spectra of rhodopsin and iodopsin display a parallel displacement. They show also significant correspondences in shape with the sensitivity curves; for example, the curves representing rhodopsin and scotopic sensitivity are narrower than those representing iodopsin and photopic sensitivity.[7]

This degree of correspondence is satisfactory to a first approximation. It is

[7] Pletnjev (1937) has reported an interesting series of experiments which appear to depend upon the Purkinje phenomenon in young chicks. Offered two lights at moderate intensities in a choice box, the chicks go toward the brighter light. If a red light was equated with a blue at moderate intensities, after high light adaptation the chicks sought the red side, after dark adaptation, the blue. This is conventional Purkinje behavior. Chicks kept 5 to 10 days on a vitamin A-free diet inclined more toward the red than controls, presumably because the deficient diet had decreased the sensitivity of their rod vision relatively more than that of their cones.

as far as one can go with Honigmann's measurements. To carry the comparison further at present it must be transferred to the more accurate measurements of Granit (1942 *c*) and Donner (1953) on the closely related retina of the pigeon. To make these measurements, the lens and cornea were removed from the eye, and microelectrodes inserted into the retina. The pigeons were either dark-adapted 1 to 2 hours following the operation, or were light-adapted in white light. At each wave length, the energy needed to evoke a constant electrical response was measured. The reciprocal of the energy—the sensitivity—was quantized by dividing by the wave length.

Fig. 23 shows the comparison of these measurements with the absorption spectra of rhodopsin and iodopsin. The scotopic sensitivity curve agrees very well with the absorption spectrum of rhodopsin. The photopic curve is displaced about 20 mμ toward the red from the spectrum of iodopsin. In part at least this displacement is caused by the filtering action of the brightly colored oil globules found in the cones of pigeon and chicken retinas in the position of color filters (Wald and Zussman, 1938). Yet the displacement seems larger than such collections of colorless, light yellow, golden, and red globules as a chicken retina possesses could well account for. Only the red globules would possess sufficiently dense, long wave length absorption to explain it.

This consideration has particular relevance for the pigeon. On isolating a pigeon retina one sees at once that though three quadrants of it are light yellow in color, the fourth, a sharply limited segment from the center to one edge, is deep orange-red. This is the so called "red field," comprising the dorso-temporal quadrant of the retina, which receives the images of objects at which the bird pecks (*cf.* Waelchli, 1883). In this region, deep red globules predominate, and are probably sufficiently dense to account for the entire displacement of photopic sensitivity shown in Fig. 23. Unfortunately, neither Granit nor Donner state whether their microelectrodes had been placed in the red or in the much larger yellow field of the retina; so this factor must for the present remain unresolved. It is of course possible also that the visual pigment of pigeon cones lies at longer wave lengths than chicken iodopsin.

How widely distributed is iodopsin in cone vision? Some idea of this can be gained from Fig. 24. Here the absorption spectra of chicken rhodopsin and iodopsin are compared with the spectral sensitivities of rod and cone vision in the frog, snake, guinea pig, and cat, as measured electrophysiologically by Granit and his coworkers. The scotopic data from frog, cat, and guinea pig agree very well with the absorption spectrum of rhodopsin. They would agree even better in the case of the frog, had the comparison been made on the basis of the percentage absorption rather than the extinction of rhodopsin. The photopic sensitivities agree so well with the absorption spectrum of iodopsin that it seems probable that this is the major pigment of cone vision in the frog, snake, and cat.

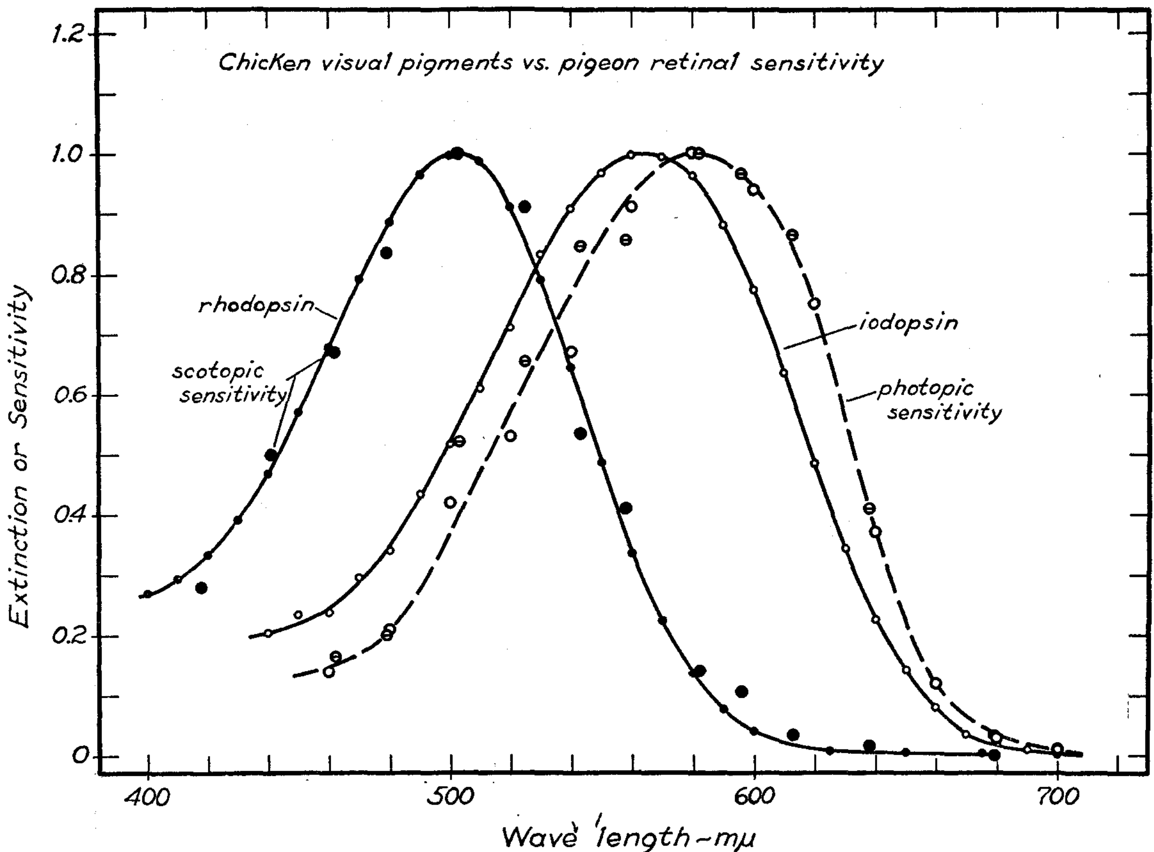


FIG. 23. Absorption spectra of chicken rhodopsin and iodopsin, compared with the spectral sensitivities of dark- and light-adapted pigeons. The spectral sensitivities were measured electrophysiologically, and are plotted in terms of the reciprocals of the numbers of quanta needed to evoke a constant electrical response. The scotopic data are from Donner (1953), the photopic data from the same source (barred circles), and from Granit (1942 *c*; open circles). The scotopic sensitivity agrees very well with the absorption spectrum of rhodopsin. The photopic sensitivity is displaced about 20 mμ toward the red from the absorption spectrum of iodopsin, due at least in part to the filtering action of the colored oil globules of pigeon cones.

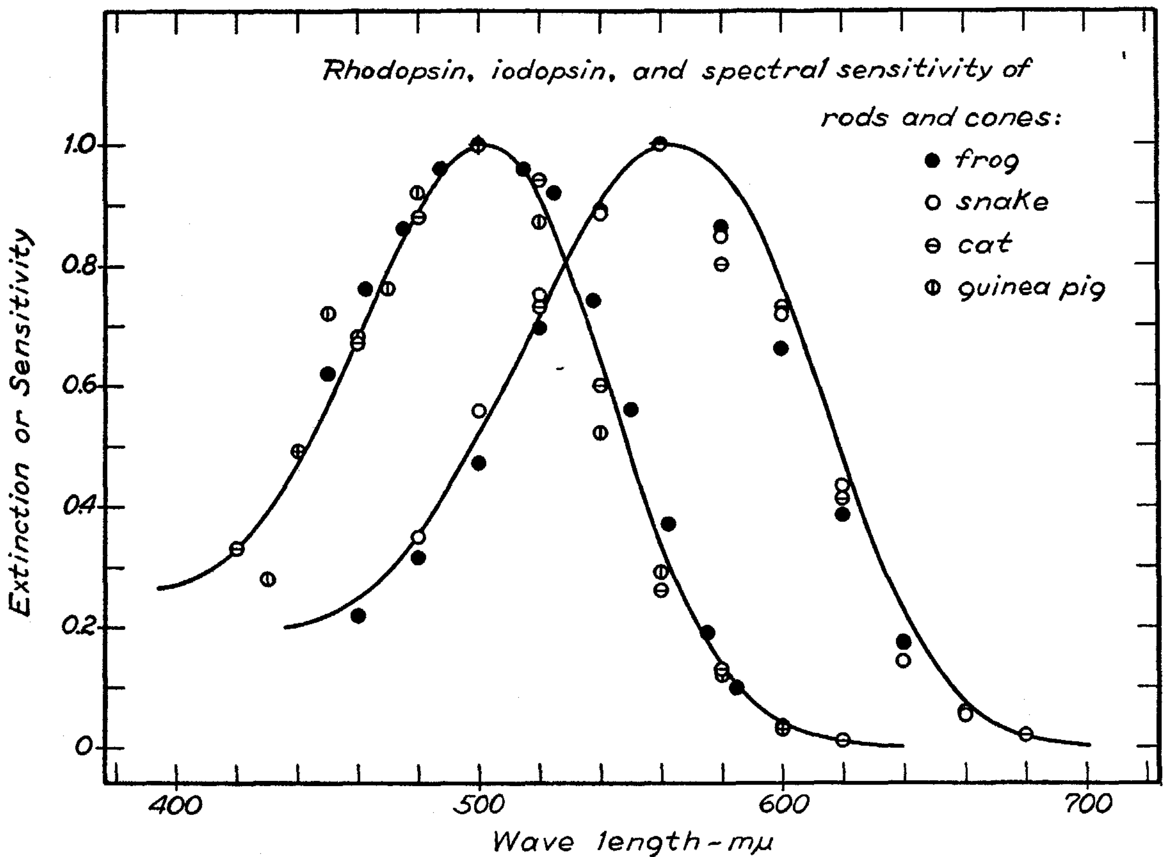


FIG. 24. The absorption spectra of chicken rhodopsin and iodopsin, compared with the scotopic and photopic sensitivities of various animals. The lines show the absorption spectra of the visual pigments, the points electrophysiological measurements of spectral sensitivity (quantized). Scotopic data, frog (Granit, 1947, p. 292); cat (Donner and Granit, 1949); guinea pig (Granit, 1942 *b*). Photopic measurements, frog (Granit, 1942 *a*); snake (Granit, 1943 *a*); cat (Granit, 1943 *b*).

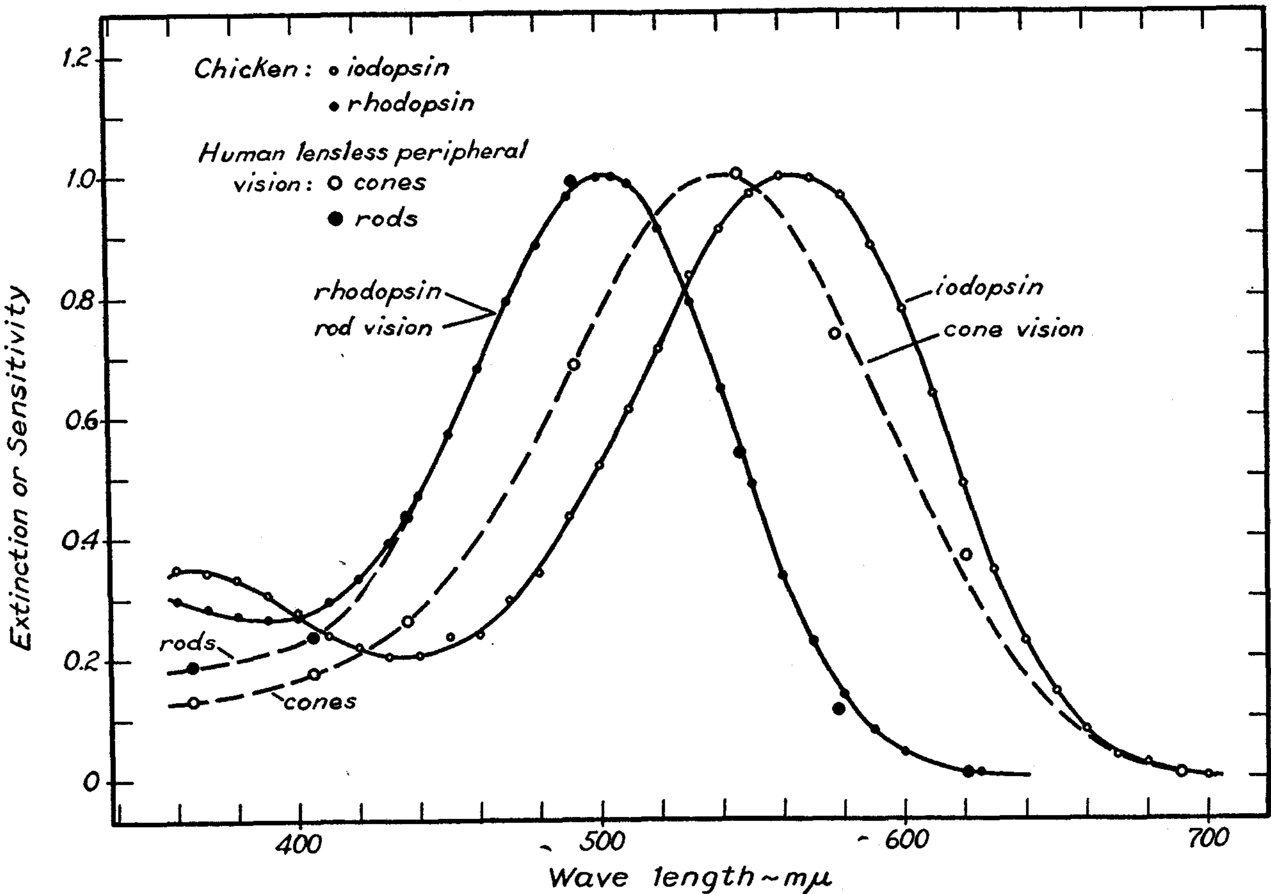


FIG. 25. Absorption spectra of chicken rhodopsin and iodopsin compared with the spectral sensitivity of human rod and cone vision. The spectral sensitivity measurements were made in a peripheral field in the aphakic (lensless) eye, to avoid distortions caused by the yellow pigmentations of the lens and macula lutea. The measurements represent as close an approximation to the sensitivities of the naked rods and cones as can be achieved in the living eye (*cf.* Wald, 1945 *b*, 1949). The scotopic (rod) sensitivity agrees with the absorption spectrum of rhodopsin over most of its course. The photopic (cone) sensitivity is displaced some 20 mμ toward the blue from the absorption spectrum of iodopsin; it represents the resultant of the spectral sensitivities of at least three groups of cones, concerned with color vision.

Fig. 24 shows that in favorable cases, in which colored ocular structures do not introduce serious distortions, the Purkinje phenomenon emerges directly and quantitatively from the absorption spectra of rhodopsin and iodopsin. This relationship, already foreshadowed in the chicken and pigeon, yet somewhat obscured in them by the presence of the cone oil globules, appears in all clarity here. It is reasonably sure that in all these eyes the Purkinje phenomenon involves nothing more than the transfer of vision from dependence upon rhodopsin in dim light, to dependence upon iodopsin in bright light.

Iodopsin, however, is not the only pigment of cone vision. In fresh-water fishes and turtles, in which cone vision is based upon vitamin A_2 rather than A_1, iodopsin is replaced by the blue photosensitive pigment, cyanopsin (Wald, Brown, and Smith, 1953). Even in those animals which use vitamin A_1 in both cone and rod vision, iodopsin must be only one of many cone pigments. Those birds and turtles which possess systems of colored oil globules in their cones may achieve color vision with a single photosensitive pigment, either iodopsin or cyanopsin; but in the absence of such color filters, color vision demands that the cones possess a variety of different photosensitive pigments. In human vision, for example, which is normally trichromatic, it is commonly assumed that the cones contain at least three different photosensitive pigments, possessing different absorption spectra, and hence different spectral sensitivities.

Fig. 25 compares the absorption spectra of chicken rhodopsin and iodopsin with the spectral sensitivities of human rod and cone vision. The spectral sensitivity measurements were made in the periphery of the aphakic (lensless) eye, to avoid distortions otherwise introduced by the yellow pigmentations of the human lens and macula lutea (*cf.* Wald, 1945 *b*, 1949). The scotopic sensitivity is in good agreement with the absorption spectrum of rhodopsin; but the photopic sensitivity is displaced about 20 mμ toward the *blue* from iodopsin. This is not astonishing, for the human photopic sensitivity appears to be the resultant of the spectral sensitivities of at least three classes of cone. According to Stiles's analysis (1949), these possess maxima at about 440, 550, and 590 mμ (*cf.* also Auerbach and Wald, 1954). The middle member of this trio may possibly represent iodopsin or a closely related pigment; but in the human cones this cooperates with at least two other cone pigments to provide the mechanism of color differentiation.

SUMMARY

The iodopsin system found in the cones of the chicken retina is identical with the rhodopsin system in its carotenoids. It differs only in the protein—the opsin—with which carotenoid combines. The cone protein may be called *photopsin* to distinguish it from the *scotopsins* of the rods.

Iodopsin bleaches in the light to a mixture of photopsin and all-trans retinene$_1$. The latter is reduced by alcohol dehydrogenase and cozymase to all-

trans vitamin A_1. Iodopsin is resynthesized from photopsin and a *cis* isomer of vitamin A, neovitamin A*b*, or the corresponding neoretinene *b*, the same isomer that forms rhodopsin. The synthesis of iodopsin from photopsin and neoretinene *b* is a spontaneous reaction. A second *cis* retinene, isoretinene *a*, forms iso-iodopsin (λ_{max} 510 mμ).

The bleaching of iodopsin in moderate light is a first-order reaction (Bliss). The synthesis of iodopsin from neoretinene *b* and opsin is second-order, like that of rhodopsin, but is very much more rapid. At 10°C. the velocity constant for iodopsin synthesis is 527 times that for rhodopsin synthesis.

Whereas rhodopsin is reasonably stable in solution from pH 4–9, iodopsin is stable only at pH 5–7, and decays rapidly at more acid or alkaline reactions.

The sulfhydryl poison, *p*-chloromercuribenzoate, blocks the synthesis of iodopsin, as of rhodopsin. It also bleaches iodopsin in concentrations which do not attack rhodopsin.

Hydroxylamine also bleaches iodopsin, yet does not poison its synthesis. Hydroxylamine acts by competing with the opsins for retinene. It competes successfully with chicken, cattle, or frog scotopsin, and hence blocks rhodopsin synthesis; but it is less efficient than photopsin in trapping retinene, and hence does not block iodopsin synthesis.

Though iodopsin has not yet been prepared in pure form, its absorption spectrum has been computed by two independent procedures. This exhibits an α-band with λ_{max} 562 mμ, a minimum at about 435 mμ, and a small β-band in the near ultraviolet at about 370 mμ.

The low concentration of iodopsin in the cones explains to a first approximation their high threshold, and hence their status as organs of daylight vision.

The relatively rapid synthesis of iodopsin compared with rhodopsin parallels the relatively rapid dark adaptation of cones compared with rods. A theoretical relation is derived which links the logarithm of the visual sensitivity with the concentration of visual pigment in the rods and cones. Plotted in these terms, the course of rod and cone dark adaptation resembles closely the synthesis of rhodopsin and iodopsin in solution.

The spectral sensitivities of rod and cone vision, and hence the Purkinje phenomenon, have their source in the absorption spectra of rhodopsin and iodopsin. In the chicken, for which only rough spectral sensitivity measurements are available, this relation can be demonstrated only approximately. In the pigeon the scotopic sensitivity matches the spectrum of rhodopsin; but the photopic sensitivity is displaced toward the red, largely or wholly through the filtering action of the colored oil globules in the pigeon cones. In cats, guinea pigs, snakes, and frogs, in which no such colored ocular structures intervene, the scotopic and photopic sensitivities match quantitatively the absorption spectra of rhodopsin and iodopsin. In man the scotopic sensitivity matches the absorption spectrum of rhodopsin; but the photopic sensitivity, when not distorted by the yellow pigmentations of the lens and macula lutea,

lies at shorter wave lengths than iodopsin. This discrepancy is expected, for the human photopic sensitivity represents a composite of at least three classes of cone concerned with color vision.

REFERENCES

Auerbach, E., and Wald, G., Identification of a violet receptor in human color vision, *Science*, 1954, **120**, 401.

Ball, S., Collins, F. D., Dalvi, P. D., and Morton, R. A., Reactions of retinene$_1$ with amino compounds, *Biochem. J.*, 1949, **45**, 304.

Bliss, A. F., The chemistry of daylight vision, *J. Gen. Physiol.*, 1945–46 *a*, **29**, 277.

Bliss, A. F., Photolytic lipids from visual pigments, *J. Gen. Physiol.*, 1945–46 *b*, **29**, 299.

Blough, Donald S., Dark adaptation in the pigeon, *Doctoral dissertation*, Harvard University, 1954.

Commoner, B., and Lipkin, D., The application of the Beer-Lambert law to optically anisotropic systems, *Science*, 1949, **110**, 41.

Donner, R. O., The spectral sensitivity of the pigeon's retinal elements, *J. Physiol.*, 1953, **122**, 524.

Donner, K. O., and Granit, R., Scotopic dominator and state of visual purple in the retina, *Acta Physiol. Scand.*, 1949, **17**, 161.

Fisher, R. A., Statistical Methods for Research Workers, Edinburgh, Oliver and Boyd, 1925, 57.

Granit, R., Colour receptors of the frog's retina, *Acta Physiol. Scand.*, 1942 *a*, **3**, 137.

Granit, R., Spectral properties of the visual receptor elements of the guinea pig, *Acta Physiol. Scand.*, 1942 *b*, **3**, 318.

Granit, R., The photopic spectrum of the pigeon, *Acta Physiol. Scand.*, 1942 *c*, **4**, 118.

Granit, R., "Red" and "green" receptors in the retina of *Tropidonotus*, *Acta Physiol. Scand.*, 1943 *a*, **5**, 108.

Granit, R., The spectral properties of the visual receptors of the cat, *Acta Physiol. Scand.*, 1943 *b*, **5**, 219.

Granit, R., Sensory Mechanisms of the retina, London, Oxford University Press, 1947, 292.

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

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

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

Hecht, S., and Mandelbaum, J., Rod-cone adaptation and vitamin A, *Science*, 1938, **88**, 219.

Honigmann, H., Untersuchungen über Lichtempfindlichkeit und Adaptierung des Vogelauges, *Arch. ges. Physiol.*, 1921, **189**, 1.

Hubbard, R., Gregerman, R. I., and Wald, G., Geometrical isomers of retinene, *J. Gen. Physiol.*, 1952–53, **36**, 415.

Hubbard, R., and Wald, G., Cis-trans isomers of vitamin A and retinene in the rhodopsin system, *J. Gen. Physiol.*, 1952–53, **36**, 269.

Kühne, W., Chemische Vorgänge in der Netzhaut, *in* Handbuch der Physiologie, (L. Hermann, editor), Leipzig, F. C. W. Vogel, 1879, **3**, pt. 1, 312.

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

Pletnjev, A. W., Vitamin A, die Sehfunktion und Richtungsbewegungen von Küken, *Z. Vitaminforsch.*, 1937, **6**, 140.

St. George, R. C. C., The interplay of light and heat in bleaching rhodopsin, *J. Gen. Physiol.*, 1951–52, **35**, 495.

Schmidt, W. J., Polarisationsoptische Analyse eines Eiweiss-Lipoid-Systems, erläutert am Aussenglied der Sehzellen, *Kolloid-Z.*, 1938, **85**, 137.

Stiles, W. S., The determination of the spectral sensitivities of the retinal mechanisms by sensory methods, *Nederl. Tijdschr. Natuurk.*, 1949, **15**, 125.

Waelchli, G., Zur Topographie der gefärbten Kugeln der Vogelnetzhaut, *Arch. Ophth.*, Leipzig, 1883, **29**, Abt. 3, 205.

Wald, G., Bleaching of visual purple in solution, *Nature*, 1937 *a*, **139**, 587.

Wald, G., Photo-labile pigments of the chicken retina, *Nature*, 1937 *b*, **140**, 545.

Wald, G., On rhodopsin in solution, *J. Gen. Physiol.*, 1937–38, **21**, 795.

Wald, G., The spectral sensitivity of the human eye, I. A spectral adaptometer, *J. Opt. Soc. America*, 1945 *a*, **35**, 187.

Wald, G., Human vision and the spectrum, *Science*, 1945 *b*, **101**, 653.

Wald, G., The photochemistry of vision, *Documenta Ophthalmologica*, 1949, **3**, 94.

Wald, G., The photochemical basis of rod vision, *J. Opt. Soc. America*, 1951, **41**, 949.

Wald, G., Vision, *Fed. Proc.*, 1953 *a*, **12**, 606.

Wald, G., The biochemistry of vision, *Ann. Rev. Biochem.*, 1953 *b*, **22**, 497.

Wald, G., On the mechanism of the visual threshold and visual adaptation, *Science*, 1954, **119**, 887.

Wald, G., and Brown, P. K., The synthesis of rhodopsin from retinene$_1$, *Proc. Nat. Acad. Sc.*, 1950, **36**, 84.

Wald, G., and Brown, P. K., The role of sulfhydryl groups in the bleaching and synthesis of rhodopsin, *J. Gen. Physiol.*, 1951–52, **35**, 797.

Wald, G., and Brown, P. K., The molar extinction of rhodopsin, *J. Gen. Physiol.*, 1953–54, **37**, 189.

Wald, G., Brown, P. K., and Smith, P. H., Iodopsin, *Fed. Proc.*, 1952 *a*, **11**, 304; Communications, *2nd Internat. Biochem. Congr. Paris*, 1952 *b*, 214.

Wald, G., Brown, P. K., and Smith, P. H., Cyanopsin, a new pigment of cone vision, *Science*, 1953, **118**, 505.

Wald, G., Durell, J., and St. George, R. C. C., The light reaction in the bleaching of rhodopsin, *Science*, 1950, **111**, 179.

Wald, G., Jeghers, H., and Arminio, J., An experiment in human dietary night-blindness, *Am. J. Physiol.*, 1938, **123**, 732.

Wald, G., and Zussman, H., Carotenoids of the chicken retina, *J. Biol. Chem.*, 1938, **122**, 449.