PIGMENTS OF THE RETINA

II. Sea Robin, Sea Bass, and Scup

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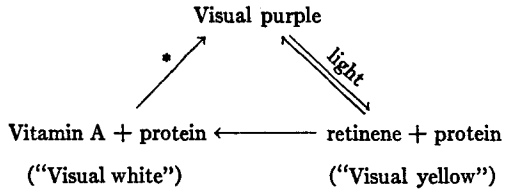
In the preceding paper of this series (Wald, 1935–36 *b*) the visual purple system of the bull frog was found to conform in part with the equations:



in which all but the reaction starred occur in the isolated retina as well as in the intact eye.

Identical processes are found in the retinas of the sea robin (*Prionotus carolinus*), the black sea bass (*Centropristes striatus*), and the scup or porgy (*Stenotomus chrysops*). In the pigment epithelium and choroid layer of these fishes pigments occur also which are either identical with or very closely related to those in frogs.

Köttgen and Abelsdorff (1896) found the absorption spectrum of visual purple from amphibia, owls, and mammals to possess a maximum at about 500 mμ; while that from eight species of fresh water fishes, though of the same general form, is displaced so that its maximum occurs at about 540 mμ. This spectral peculiarity, if general, should lend special interest to an examination of the visual system in

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fishes. However, visual purple solutions from the three marine species discussed in the present paper have been found to be almost identical spectroscopically with those of frogs, the maxima occurring in each case at about 500 mμ. The apparent discrepancy between these and Köttgen and Abelsdorff's results is being investigated further.

Retinas

Experiments performed in the examination of frog retinas have been repeated with fish tissues with identical results. The dark adapted retinas contain a small quantity of free vitamin A and a large amount of bound retinene. The latter is liberated by destroying the visual purple, either with light or with chloroform. Retinene liberated by light is subsequently converted to vitamin A by a thermal reaction, evidenced by the fading of the visual yellow retina to colorlessness.

I shall not describe the details of these experiments again, but instead record a simple procedure which has served to establish the nature of the visual purple system in a single experiment. To follow this one need only recall that both retinene and vitamin A yield blue colorations when mixed with antimony trichloride reagent, due in the case of retinene to an absorption band at 662–666 mμ, in that of vitamin A to one at 612–615 mμ.

Figs. 1 and 2 show the results of this type of experiment performed with scup and sea robin retinas, Fig. 3 a slight variant of this procedure with bass tissues.[1]

Right and left retinas of five dark adapted fishes (scup and sea robin, 8 to 10 inches long) were prepared separately. One set of five retinas was extracted thoroughly in the dark with about 12 cc. of benzine in 4 portions, shaking violently by machine for a total of 20 minutes. The extract brought into chloroform was colorless and when tested with antimony trichloride yielded blue solutions of which Curves *a* of Figs. 1 and 2 are the absorption spectra. The dark adapted retina therefore contains a small quantity of vitamin A alone.

The same tissues were bleached subsequently in daylight to an orange color ("visual yellow") and were immediately re-extracted

[1] All spectra shown in this paper were recorded automatically with a photoelectric spectrophotometer (Hardy, A. C., *J. Opt. Soc. America*, 1935, **25**, 305) at the Massachusetts Institute of Technology.

with benzine. The extract, brought into chloroform, was bright yellow. Tested with antimony trichloride it yielded Curves *b* of Figs. 1 and 2. The bleaching of visual purple to yellow therefore liberates a large quantity of retinene.

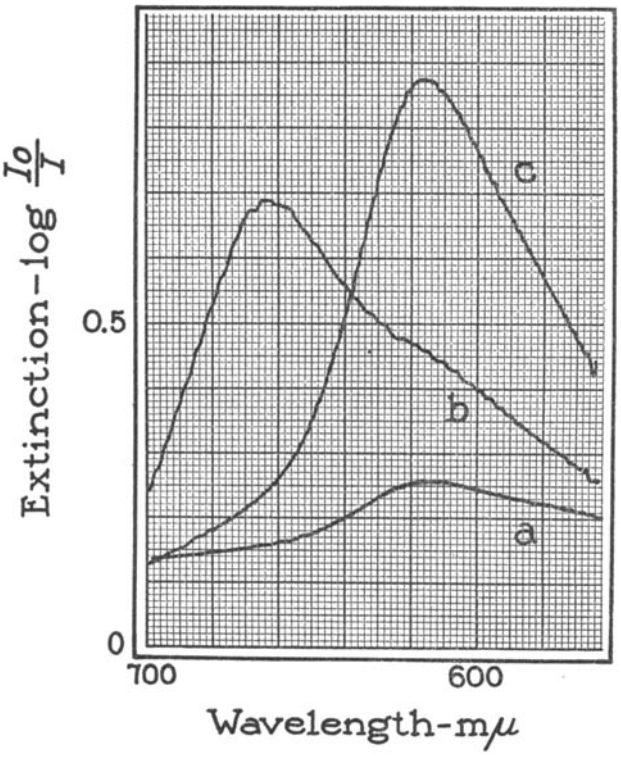

FIG. 1. Absorption spectra of the antimony trichloride reaction with benzine extracts of scup retinas. *a*, Dark adapted retinas; the extract contains a small quantity of vitamin A (615 mμ chromogen). *b*, The same retinas, re-extracted immediately after bleaching. A large quantity of retinene (664 mμ chromogen) has been liberated. *c*, Retinas from the same fishes, extracted 1 hour after bleaching. The free retinene has been converted to vitamin A.

The second set of five retinas was bleached in daylight and left at room temperature in moderate light for an hour. During this period the retinas faded from orange to colorlessness. They were extracted with benzine exactly as before. The extract, brought into chloroform,

was colorless, and when tested with antimony trichloride yielded Curves *c* of Figs. 1 and 2. The fading process converts retinene liberated in bleaching quantitatively to vitamin A.

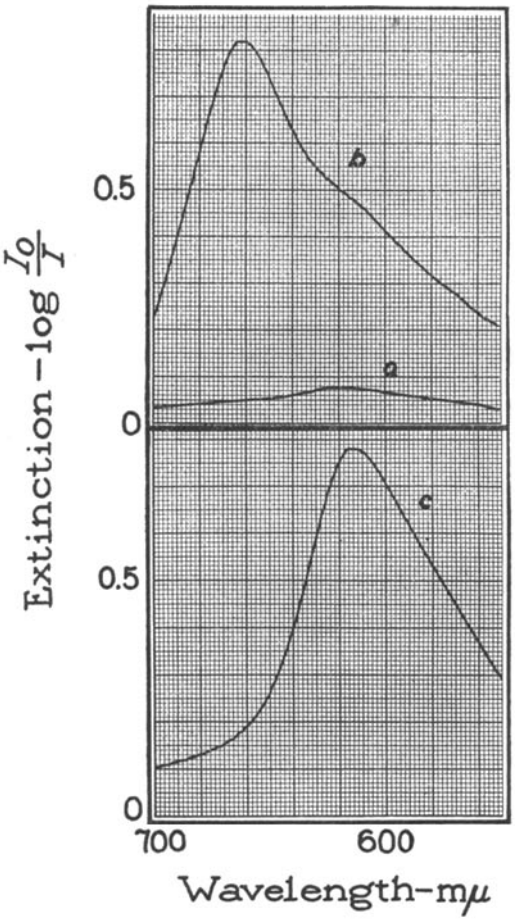

FIG. 2. Absorption spectra of the antimony trichloride reaction with benzine extracts of sea robin retinas. Compare with Fig. 1.

At the time of these experiments only 3 bass about 14 inches long were available. The retinas of two of these were used to prepare a visual purple extract. The residual tissue from this procedure still contained visual purple, which was destroyed by extracting with

chloroform. The extract was yellow, and with antimony trichloride yielded the upper curve of Fig. 3, showing the presence of retinene and a small amount of vitamin A. The retinas from the single re-

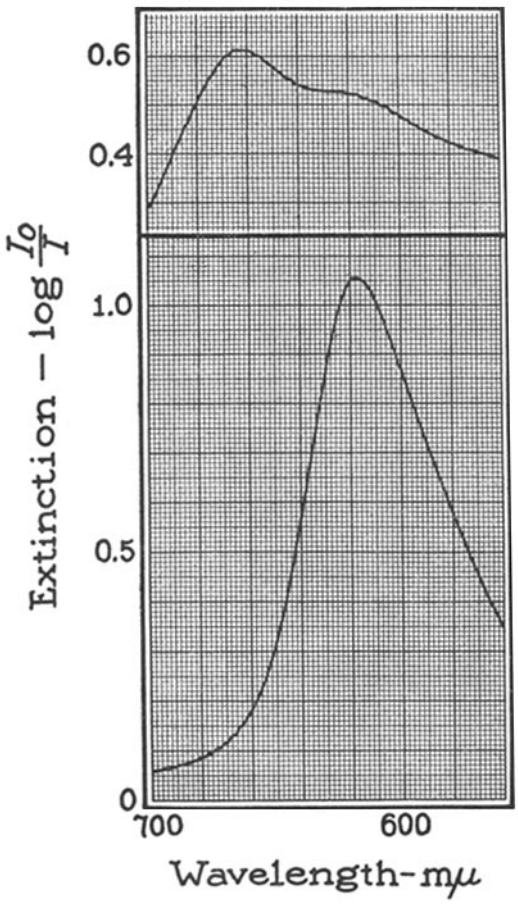

FIG. 3. Absorption spectra of the antimony trichloride reaction with extracts of sea bass retinas. *Upper curve*, chloroform extract of dark adapted retinas. *Lower curve*, benzine extract of bleached and faded retinas.

maining fish were bleached in daylight and allowed to fade for an hour. They were then extracted with benzine. The colorless extract, tested with antimony trichloride, yielded the lower curve of Fig. 3, showing,

as in the other forms, the conversion of retinene liberated by light to vitamin A.

Maximal quantities of vitamin A occur in retinas which have been bleached and allowed to fade completely *in vitro*. These quantities, estimated roughly by a colorimetric method (Wald, 1935–36 *a*) are: sea robin, 1.4; scup, 2.8; and bass, 4.7 γ per retina. The retinal dimensions in the specimens used increased in the same order, so that it is possible that the quantity of vitamin A per unit weight of retina is approximately constant in all three species.

Acid-Base Effects upon the Visual Yellow Retina.—Characteristic responses of the visual yellow retina to acids and alkalies have been observed in frogs and fishes and examined in some detail in the sea robin. Chase (1935–36) has shown that a yellow product of the bleaching of visual purple in solution behaves as an acid-base indicator, turning colorless in alkaline solutions. This observation is closely related to those to be described. The behavior of the retinal pigments *in situ*, however, is much more complicated than in solution, and the precise connections between the two situations are still to be elucidated. At present certain features in the acid-base behavior of the sea robin retina may be indicated.

The bleached, visual yellow retina is of a distinctly orange color. When made sufficiently alkaline, it turns practically colorless; if made strongly acid, bright yellow. The change from yellow to colorless is freely reversible, the tissue behaving as an acid-base indicator. However, I have never succeeded in bringing such retinas back to the original orange color of visual yellow. Some irreversible change therefore accompanies these abnormal pH's.

The bright yellow, comparatively photostable material which is formed when the retina is treated with strong acids has long been employed as a test for visual purple (Boll, 1877; van Genderen Stort, 1887). In reality it is a test for retinene or visual yellow. It is yielded by dark adapted and visual yellow retinas, but not by retinas which have been bleached and allowed to fade completely; that is, in which the retinene has been converted to vitamin A. Since the acid yellow color is considerably deeper than that of neutral visual yellow, it reveals the presence of retinene in retinas which when neutral may appear quite colorless. In this way the last traces of retinene in the fading retina may be distinguished.

Neutral visual yellow retinas, cooled to 0°C., retain their color for hours, even in bright daylight. Such retinas may be titrated on ice with alkalies to determine the pH at which the color change occurs. In 0.035N NaOH or 0.35N NH_3 the orange retina loses its color within about 10 minutes at 0°C. in daylight. In more strongly alkaline solutions or at higher temperatures the reaction is more rapid. These time intervals are not needed primarily for penetration of the reagent, for a dark adapted retina soaked in 0.23N NH_3 for ½ hour in the dark before being exposed to light on ice, first turns orange and then still requires about 12 minutes to fade to colorlessness. This period is apparently principally occupied by the irreversible reaction which transforms visual yellow to the yellow-colorless pH indicator.

The pH at which the visual yellow retina is decolorized is well outside the physiological range. The concentrations of alkali cited above as approximately minimal correspond to pH's of about 11–12. In such solutions the tissue disintegrates rapidly. Dark adapted retinas, soaked for 10 minutes in phosphate buffers at pH 6.0 and 8.0 before being exposed to light, exhibited no detectable differences either in color before and after bleaching or in rates of bleaching, fading, and regeneration. A retina at pH 9.0 behaved similarly. The acid-base changes discussed above are therefore decidedly abnormal.

In ammoniacal retinas (0.23N NH_3) the colorless derivatives of visual yellow are not removed to form vitamin A, nor do they regenerate visual purple in the dark. Even after 3 hours in the light at room temperature their presence is revealed by the strong yellow color which develops when the retina is acidified. On the other hand the reversion of visual yellow itself to purple is greatly accelerated in 0.23N NH_3. Ammoniacal retinas, returned to the dark immediately after bleaching, before the visual yellow has been appreciably decolorized by the alkali, regenerate considerable amounts of visual purple within ½ hour even at 0°C., though at this temperature neutral retinas do not change appreciably within a comparable period. Curiously, the ammoniacal visual yellow retina regenerates more visual purple at 0°C. than at room temperature. It seems as though two reactions compete for the removal of visual yellow—reversion to purple, and the irreversible formation of the pH indicator; and that the former is relatively favored at low temperatures.

The accelerating effect of NH_3 upon the reversion process explains Kühne's observation, which I have confirmed, that the ammoniacal retina bleaches more slowly than the neutral tissue (Kühne, 1878). This difference cannot be ascribed to induced photostability in the visual purple itself; for ammoniacal visual purple *solutions*—in which appreciable reversion does not occur—bleach much more *quickly* than neutral solutions. This is true also of solutions buffered at pH 9.3 (Chase, 1935–36), so that it is a general alkaline effect and not one restricted specifically to ammonia.

Pigmented Layers

The combined pigment epithelia and choroid layers of these marine fishes contain vitamin A, flavine, and an unidentified xanthophyll.

Flavine was first found in fish pigment epithelia by von Euler and Adler (1934); nothing in the present investigation adds materially to the description of it given by these authors. The spectrum of flavine from bass pigmented tissue is shown in Fig. 4.

Fish xanthophyll is spectroscopically different from that found in frogs, and so will be described in some detail. It occurs in the tissues as an ester: when partitioned between 90 per cent methanol and benzine, it accumulates in the benzine layer before and in the methanol layer after saponification. It is readily extracted from strongly alkaline aqueous alcohol with benzine. The free pigment is strongly adsorbed on a column of calcium carbonate, forming a golden layer. These are general properties of the hydroxycarotenoids or xanthophylls.

In CS_2 the pigment possesses absorption maxima at 439, 470–472, and 500–501 mμ. A scup preparation diverged slightly from this, showing bands at 439, 476, and 502 mμ; but this spectrum was unusually diffuse as though the pigment had deteriorated in solution. The spectrum of free xanthophyll from bass is shown in Fig. 5.

In general form these spectra resemble those of the dihydroxycarotenoids, $C_{40}H_{56}O_2$. The band positions, however, are closest to those of violaxanthin and taraxanthin, isomers of composition $C_{40}H_{56}O_4$. Violaxanthin yields a blue color when treated in ether with 25 per cent HCl. A single trial of this test with a sea robin extract was negative, possibly due to low concentration of the pigment.

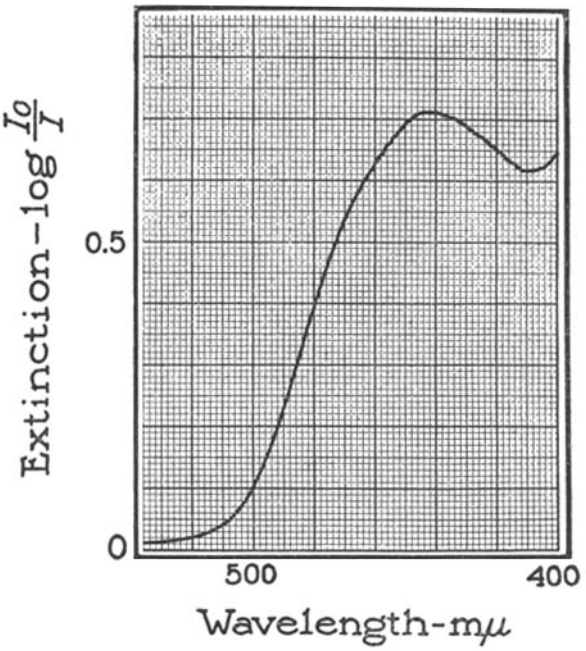

FIG. 4. Spectrum of an aqueous solution of flavine from sea bass pigmented layers.

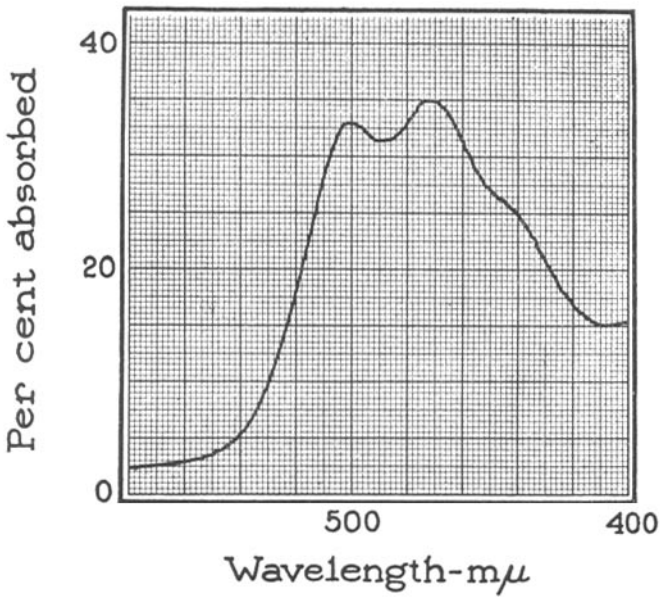

FIG. 5. Spectrum of free xanthophyll in CS_2 from sea bass pigmented layers. The non-saponifiable portion of the crude extract had been partitioned between benzine and 90 per cent methanol. The methanol fraction is shown.

The distribution of the fish pigments between benzine and various concentrations of methanol is identical in all three species. No appreciable quantity of pigment leaves the benzine for 70 per cent methanol, a minor proportion does so for 80 per cent methanol, and practically all of it goes into 90 per cent methanol. This behavior is typical, and, indeed, roughly specific for the dihydroxycarotenoids.

The relative solubilities of xanthophylls in polar solvents like methanol compared with non-polar solvents like benzine rise rapidly with the number of oxygen atoms in the molecule. This relation is shown in Table I, compiled from various portions of Zechmeister's monograph (1934); the behavior of the $C_{40}H_{56}$ carotenes is included. The terms epiphasic and hypophasic refer respectively to pigment solubility in the upper, benzine, and in the lower, alcoholic layers.

TABLE I

Carotenoid	Partitioned between benzine and —	Behavior
$C_{40}H_{56}$	90 per cent methanol	Almost wholly epiphasic
$C_{40}H_{56}O$	95 per cent methanol	Partly hypophasic
$C_{40}H_{56}O_2$	90 per cent methanol	Almost wholly hypophasic
	70 per cent methanol	Almost wholly epiphasic
$C_{40}H_{56}O_3$	" " "	Slightly (1/9) hypophasic
$C_{40}H_{56}O_4$	" " "	More (1/6) hypophasic
$C_{40}H_{56}O_6$	" " "	Almost wholly hypophasic

The fish pigment therefore resembles the $C_{40}H_{56}O_2$ xanthophylls. Its spectrum, however, is almost uniformly displaced 4–7 mμ below that of lutein, the most similar known xanthophyll of this composition. More specific identification of the fish pigment, only very small quantities of which were available in impure condition, is at present impossible.

Cunningham and MacMunn (1893) and Lönnberg (1933–34) have found carotenoids in the skins, fins, and other tissues of a large number of species of marine fishes. The species investigated by Lönnberg form two groups, one in which the carotenoids resemble lutein spectroscopically, and an equally large group in which the spectra are displaced 4–6 mμ toward shorter wavelengths. It is not improbable that the second type of pigment is identical with that found in the present investigation.

Sumner and Fox (1933) have found that the carotenoid pigments of superficial and deep structures in four species of marine fishes are exclusively of the xanthophyll series. It seems that, as in the frog, these pigments are generally distributed about the organism, and that their presence in the pigmented layers of the eye is probably of no special significance.

Rough estimates of the quantities of xanthophyll, vitamin A, and flavine in the pigmented tissues are presented in Table II. These were measured with a Pulfrich photometer in the manner already described (Wald, 1935–36*a*, *b*). In the case of xanthophyll, the same factor for converting photometer readings into absolute units was used as in the frog, since Kuhn and Brockmann (1932) have shown

TABLE II

Fish	No. of eyes	Xanthophyll per eye	Vitamin A per eye	Flavine per eye
		γ	γ	γ
Sea robin	14	1.0	Trace	9
Scup	20	0.56	0.8	18
Sea bass	6	3.0	5	28

that a number of xanthophylls have approximately the same depth of color.

SUMMARY

1. Visual purple from the sea robin, sea bass, and scup is almost identical spectroscopically with that from frogs. The interrelations of this pigment with vitamin A and retinene are also the same as in the frog.

2. In strong acids or at pH > 11, the visual yellow of sea robin retinas is converted irreversibly into a pH indicator, yellow in acid and almost colorless in alkaline solution. Unlike neutral visual yellow, the indicator is not removed to form either vitamin A or visual purple. In the ammoniacal retina the reversion of visual yellow itself to purple is accelerated.

3. The combined pigment epithelium and choroid layer in these fishes contain vitamin A, flavine, and an unidentified xanthophyll.

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