A NEW PHOTOSENSITIVE PIGMENT OF THE EURYHALINE TELEOST, GILLICHTHYS MIRABILIS

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INTRODUCTION

In a recent review (1955) of his work on the visual and other carotenoid pigments of the vertebrate retina Wald has taken the position that there are two known visual pigments, rhodopsin and porphyropsin, which appear, either singly or in combination, to be involved in scotopic vision. It has been demonstrated that rhodopsins (wave length of maximum absorption near 500 mμ) are usually present in those marine fishes and terrestrial vertebrates with rod-containing retinae (summary by Krause, 1951). The fresh water fishes (Wald, 1939 *b*), on the other hand, possess porphyropsins (λ_{max} near 522 mμ) as the chief visual pigment of scotopic vision. It should be mentioned, however, that Dartnall (1952 *a*), to cite only a single paper, obtained evidence that photosensitive pigments of the fresh water tench and pike absorb light maximally at 533 mμ. This apparent variation in the spectral position of the retinal pigments of fresh water fishes is probably not completely understood. The photosensitive retinal pigment of the carp was examined by Crescitelli and Dartnall (1954), who found a λ_{max} at 523 mμ, confirming for this fresh water species Wald's general statement on the nature and occurrence of porphyropsin.

The euryhaline and migratory teleost fishes have been a source of special interest. Wald (1941) showed that spectral absorption curves of retinal extracts of such fishes (*Anguilla*, *Fundulus*, *Oncorhynchus*, *Salmo*, and *Salvelinus*) were intermediate between rhodopsin and porphyropsin. Because he obtained evidence that mixtures of the vitamins A_1 and A_2 occur in the retinae of these fishes, Wald inferred that both rhodopsin and porphyropsin are present in their retinae, in such proportions as to give the summed spectral

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absorption curves which he measured. There has been no direct analysis, however, of the visual pigments to test this plausible idea.

The investigation reported here was performed with retinal extracts of a euryhaline teleost, the longjaw goby or mudsucker, *Gillichthys mirabilis* Cooper. This species is a member of the family Gobiidae, a group of primarily marine fishes usually found in shallow coastal waters. Many of the species, however, are tolerant of reduced salt concentrations or may even be restricted to fresh water. *Gillichthys mirabilis* is common on tidal mudflats and salt marshes of the Pacific coast of North America from northern California to southern Baja California and in the Gulf of California. The mudsucker has a marked ability to endure for extended periods salt concentrations both above and below that of sea water (Weisel, 1948). The effect of varied dietary amounts of vitamin A on the quantity of visual pigment in the mudsucker retina was studied by Kampa (1953).

This paper describes experiments with retinal extracts of the euryhaline fish, *Gillichthys mirabilis*, and presents evidence that these extracts contain a single photosensitive pigment different from those of other animals so far examined by this method.

Materials and Methods

Adult mudsuckers (*Gillichthys mirabilis*, total length 10 to 15 cm.) were purchased from a local bait dealer. The fish were originally trapped in San Francisco Bay, California, and had been kept without feeding for an average period of 1 to 2 weeks in transport and in the dealer's holding tanks. The techniques used in this study have been described extensively elsewhere (Wald, 1938; Collins and Morton, 1950). All operations were performed in dim red light; 4 per cent potassium alum solution was used to harden the retinal tissue. Squirting alum solution between the retina and pigment epithelium with a hypodermic needle (Kampa, 1953) aided the dissection. The retinal tissue was centrifuged from the alum solution, washed twice in distilled water and twice in alkaline (pH 8.6) borate–KCl buffer, and extracted twice with 2 per cent aqueous digitonin solution in borate–KCl buffer (pH 8.4). The extracts were kept at 10°C. until used.

A variant of this technique, described by Saito (1938) and Collins, Love, and Morton (1952), normally yields preparations freer from contaminants. After washing, the retinal material was shaken with 40 per cent sucrose solution, in which fragments of the outer segments of the visual cells became suspended. Centrifugation permitted separation from the retinal residue; dilution with distilled water and recentrifugation brought down the outer segments. Both outer segments and retinal residue were extracted with digitonin and examined spectrophotometrically.

After centrifugation the retinal extracts were analyzed in a Beckman model DU spectrophotometer with photomultiplier attachment. As a blank, 2 per cent digitonin solution was used; the temperature was regulated at 20 ± 1°C. Optical density of the extracts was measured from 700 to 340 mμ at 10 mμ intervals. For bleaching a Bausch and Lomb grating monochromator with interference filters

placed in the exit light path provided light of the desired wave length. Both the monochromator exit slit width (3.3 mμ spectral dispersion per mm. width) and length of bleaching time were varied to control the amount of bleaching which oc-

TABLE I

Preparation and Spectral Characteristics of Retinal Extracts

Extracts are of *Gillichthys mirabilis* unless otherwise indicated.

Extract aliquot	No. of animals	Date of analysis	Extract age	Type of preparation	Density 700 mμ	Maximum density	Minimum density	Ratio	Maximum	Minimum
		1955	*days*						*mμ*	*mμ*
1A	20	July 1	4	Whole retina	0.032	0.356	0.260	0.73	507	430
1B	20	July 5	8	" "	0.006	0.236	0.161	0.68	507	430
1C	20	July 7	10	" "	0.007	0.308	0.213	0.69	506	430
2A	20	Aug. 2	14	Whole retina	0.013	0.273	0.204	0.75	503	430
2B	20	Dec. 6	140	" "	0.000	0.243	0.195	0.80	504	430
2C	20	Dec. 14	148	" "	0.002	0.195	0.155	0.80	504	430
3A	22	July 25	5	Outer segment	0.003	0.090	0.038	0.42	510	420
3B	22	July 27	7	" "	0.000	0.081	0.033	0.41	510	420
3C	22	Aug. 8	19	Residue	0.002	0.152	0.095	0.63	507	430
4A	40	Aug. 4	9	Outer segment	0.002	0.263	0.133	0.51	510	420
4B	40	Nov. 23	120	" "	0.002	0.240	0.155	0.65	508	440
4C	40	Nov. 26	123	" "	0.000	0.203	0.131	0.65	508	430
4D	40	Dec. 3	130	Residue	0.000	0.260	0.249	0.96	490	450
4E	40	Dec. 10	137	"	0.001	0.288	0.276	0.96	490	450
4F	40	Dec. 16	143	"	0.001	0.229	0.217	0.95	488	450
Rana catesbiana		Feb. 26, 1956	4	Outer segment	0.000	0.272	0.100	0.37	502	390
Cyprinus carpio		" " "	12	" "	0.002	0.198	0.160	0.81	515	450
Rana and Cyprinus		" 27, "	—	" "	0.015	0.231	0.196	0.85	503	450

curred at any wave length. Following exposure to light the extracts were replaced in the spectrophotometer and the absorption spectrum measured as before. In this way the effects on each extract of several bleaching wave lengths were studied successively.

RESULTS

1. Spectral Absorption Curves of Mudsucker Retinal Extracts.—Some of the properties of different unbleached aliquots of four extracts prepared from mudsucker retinae are listed in Table I. The absorption maxima which were

obtained were generally intermediate between the λ_{max} of rhodopsin and of porphyropsin. The range of the wave length of maximum absorption (λ_{max}) of the mudsucker extracts from 488 to 510 mμ indicates either that the λ_{max} is not an unbiased indicator of the wave length of maximum absorption of the mudsucker retinal pigment or that different pigments (or the same pigments in different proportions) were present in these extracts. Experiments

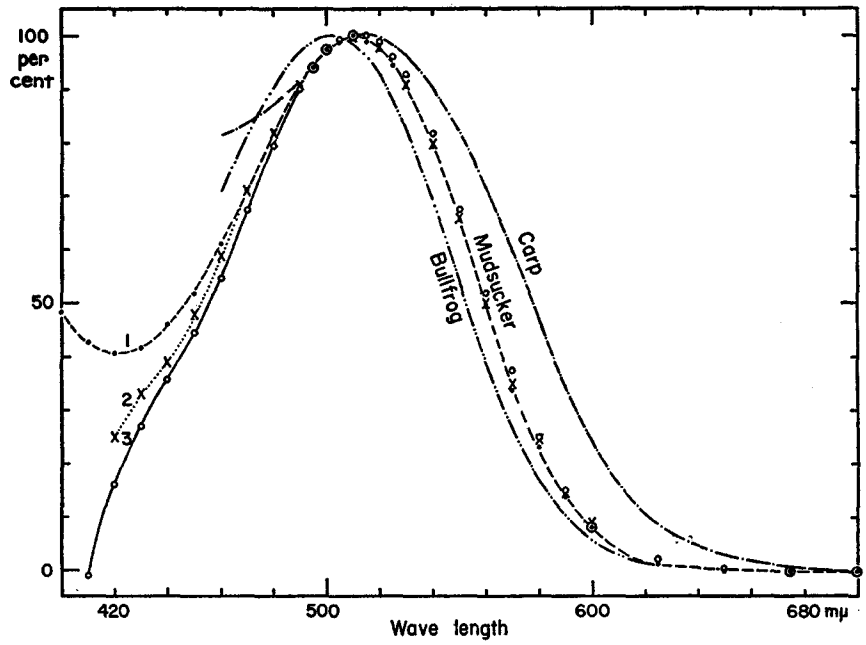

FIG. 1. Curve 1, absorption spectrum of a relatively pure retinal extract of the mudsucker (D_{min}/D_{max} = 0.42); curve 2, constructed from Dartnall's nomogram, assuming a maximum at 512 mμ; curve 3, hydroxylamine experiment, difference spectrum after exposure of mudsucker extract 4C to red light (λ = 606 mμ). Absorption spectra of retinal extracts of the bullfrog and carp are given for comparison.

in which the extracts were bleached with monochromatic light resolved this problem (see section 2 below). A comparison of the ratio of the optical densities of the preparations at their points of minimum and maximum light absorption (D_{min}/D_{max} ratio) and the λ_{max} of the extracts listed in Table I shows that the lower the ratio, the longer was the wave length of maximum absorption. Preparations with lower D_{min}/D_{max} ratios contained smaller proportions of violet-absorbing, photostable substances; their spectral absorption curves, therefore, better represent the actual spectral absorption of the photosensitive pigment(s) present.

The spectral absorption curve of the unbleached extract aliquot 3A, an outer segment preparation with the relatively low D_{min}/D_{max} ratio 0.42, is shown as curve 1 of Fig. 1. The experimental points from which this curve was constructed are plotted as percentages of the maximum (λ = 510 mμ). If a single photosensitive pigment were present in the mudsucker retinal extracts, this curve should fairly well represent its spectral absorption down to about 460 mμ. For comparison spectral absorption curves of bullfrog (*Rana catesbiana*) rhodopsin and carp (*Cyprinus carpio*) porphyropsin, obtained in this work, are also included in Fig. 1. These curves differ at all wave lengths from the spectral absorption of the mudsucker extract, and not merely at λ_{max}. For interpretation of the spectral absorption curves of the mudsucker extracts it was necessary to demonstrate the presence of photosensitive pigment in these extracts and to determine whether more than one component was present in this pigment.

2. Homogeneity of the Mudsucker Photosensitive Pigment.—That the retinal extracts which were examined contained light-sensitive pigments is shown in Table II, in which the optical density losses following exposure to light are recorded. To show whether one or more components were bleached in these experiments required an analysis using a technique of partial bleaching. This is the method employed by Crescitelli and Dartnall (1954) in their work on the carp. The retinal extract is exposed to monochromatic light and bleached in stages. If the change in optical density of the extract's spectral absorption (difference spectrum) is plotted after each exposure, a shift in the wave length of maximum change indicates the bleaching successively of more than one photosensitive component. A constant λ_{max} of the successive difference spectra means, on the other hand, that only a single, homogeneous pigment has been bleached. In the present work care was taken in the exposure of extracts to light of shorter wave lengths, reported to isomerize the products of bleaching (Hubbard and Wald, 1952; Hubbard, Gregerman, and Wald, 1953). The spectrophotometric analyses were performed with the retinal extracts at pH 8.4 to shift the absorption maximum of the products of bleaching as far as possible from the λ_{max} of the visual pigment. This minimized distortion of the difference spectrum due to the appearance of violet-absorbing products of bleaching.

The results of all bleaching experiments performed with aliquots of mudsucker extracts 1 through 4 are listed in Table II. Difference spectra were obtained as a result of exposure to light at a number of wave lengths from 660 to 390 mμ. The difference spectra are almost the same, the λ_{max} ranging from 512 to 517 mμ with an average near 514 mμ. After bleaching of this pigment with colored light was nearly complete, exposure to white light was sometimes used to determine whether any other previously unbleached component might still be present in the extracts. A difference spectrum charac-

TABLE II

Bleaching Experiments

Total change is the loss in optical density at 515 mμ due to all bleaches. When an extract was bleached several times, the results are tabulated with the first on top and the successive ones below.

Extract aliquot	Hydroxylamine	Total change	Bleaching wave length	Wave length of maximum difference	Maximum density loss
			mμ	*mμ*	
1A	No	0.320	660	517	0.089
			580	514	0.231
1B	No	0.215	660	513	0.092
			580	512	0.123
1C	(No)*	0.280	660	514	0.171
			580	514	0.109
2A	No	0.223	640	515	0.104
			606	515	0.119
2B	No	0.214	606	516	0.119
			640	514	0.055
			W‡	Isom.§	—
2C	Yes‖	0.171	390¶	512	0.147
			606	512	0.024
3A**	No	0.076	606	515	0.076
3B**	(No)	0.073	606	513	0.073
3C	No	0.121	640	513	0.056
			606	515	0.065
4A**	No	0.245	640	515	0.060
			640	Indefinite‡‡	0.040
			606	515	0.079
			606	512	0.067
4B**	No	0.198	640	515	0.060
			640	515	0.036
			606	515	0.050
			606	515	0.052
			W	Isom.	—
4C**	Yes	0.190	606	512	0.181
			W	—	—
4D	Yes	0.217	640	512	0.174
			606	512	0.037
			W	—	—
4E	No	0.233	640	515	0.074
			606	515	0.145
			W	Isom.	—
4F	Yes	0.176	390	512	0.051
			606	512	0.126

TABLE II—*Concluded*

Extract aliquot	Hydroxylamine	Total change	Bleaching wave length	Wave length of maximum difference	Maximum density loss
			mμ	mμ	
Rana and *Cyprinus***	No	0.170	640	525	0.043
			640	515	0.026
			606	510	0.033
			606	504	0.047
			W	Isom.	—

* Only 0.02 ml. of 0.1 M NH_2OH was added; this was too small a quantity to have any marked effect.

‡ W means that a tungsten filament bulb (100 watts) at a distance of 6 inches was used as the bleaching source.

§ Isomerization of the bleaching products resulted in a small change; negligible additional bleaching of pigment occurred. See Fig. 3.

‖ 0.1 ml. of 0.1 M NH_2OH was added to both extract and blank.

¶ No interference filter was available for the wave length 390 mμ.

** Outer segment preparations; others were extracts of the residues or of whole retinae (see Table I).

‡‡ The peak of this small difference was flat and not clearly marked.

teristic of the bleaching of a visual pigment was in no case obtained after such a terminal exposure to white light. Curve 5-6 of Fig. 3 B shows the typical result: a low, ill-defined, often more or less two-peaked change. This effect was also observed following a final white light bleach of an artificial mixture of bullfrog rhodopsin and carp porphyropsin (Fig. 4 B, curve 5-6). The use of hydroxylamine, which combines with retinene to form the oxime, largely prevents such effects (Hubbard and Wald, 1952; Wald and Brown, 1953). Curve H of Fig. 3 B shows the change due to terminal exposure to white light of another aliquot (4C) of the same extract, but one to which hydroxylamine had been added. The suppression in the hydroxylamine-treated aliquot of the white light effect makes it clear that no additional visual pigment had been bleached by the white light. Partial bleaching ef extract aliquots 2C and 4F (to which hydroxylamine had been added) with violet light of 390 mμ gave a difference spectrum maximum of 512 mμ. This figure was constant for the hydroxylamine difference spectrum and is also evidence for the lack of other light-sensitive pigments in the mudsucker extracts.

Graphic evidence for the homogeneity of the mudsucker photosensitive pigment is contained in Fig. 2, which presents averaged data for the difference spectra obtained after all bleaches (in extracts to which hydroxylamine was not added), plotted as percentages of the maxima. The broken line represents the grand mean of the difference spectra obtained after all 24 bleaches, and the points the means of the difference spectra obtained after exposure to each

selected wave length from 580 to 660 mμ. The averaged results of bleaches at these individual wave lengths are seen to match closely, especially at λ_{max} and in their longer wave length segments. This uniformity indicates that the photosensitive pigment present in these retinal extracts was a single, homogeneous pigment.

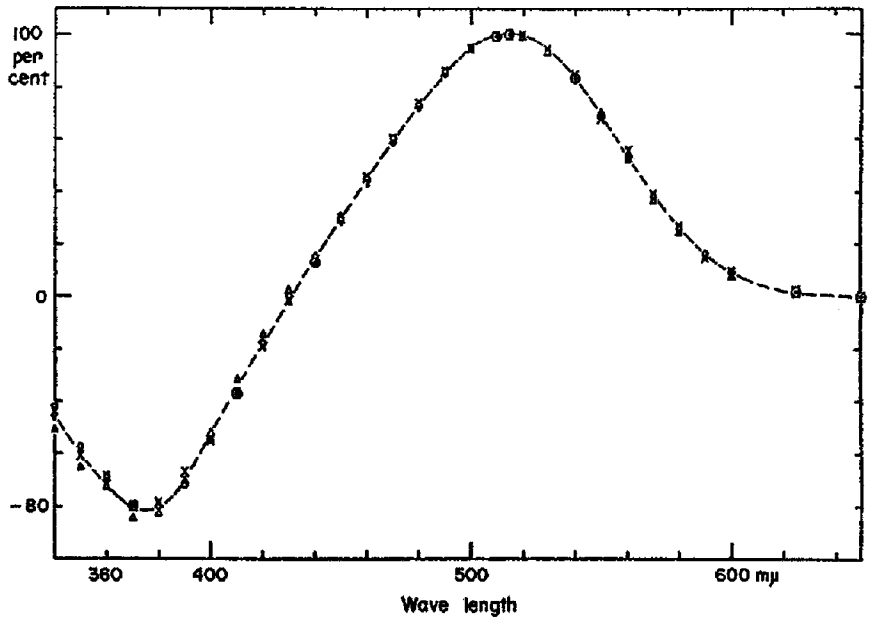

FIG. 2. Averaged difference spectra scaled with maxima set equal to 100 per cent. Broken line, grand average of all bleaches. ×'s, average of 3 bleaches at 660 mμ (maximum total change 0.330 optical density units); open circles, average of 8 bleaches at 640 mμ (change 0.483); points, average of 10 bleaches at 606 mμ (change 0.844); triangles, average of 3 bleaches at 580 mμ (change 0.462).

3. Comparison of the Mudsucker Extracts with a Mixture of Rhodopsin and Porphyropsin.—As a critical test of the homogeneity of the mudsucker retinal extract, two bleaches were performed at 640 mμ and two at 606 mμ, under conditions believed to differentiate clearly a mixture of rhodopsin and porphyropsin. The results of this experiment are given in Fig. 3. Fig. 3 A shows the actual spectral absorption curves measured initially and following each exposure to light and Fig. 3 B the successive difference spectra due to each exposure. These difference spectra are almost identical, showing a maximal change at 515 mμ; a final exposure to white light produced only the anomalous curve 5-6, already described. Curve H of Fig. 3 B shows how this white light effect was almost eliminated by the addition of hydroxylamine to another aliquot (4C) of the same solution, which had previously been bleached

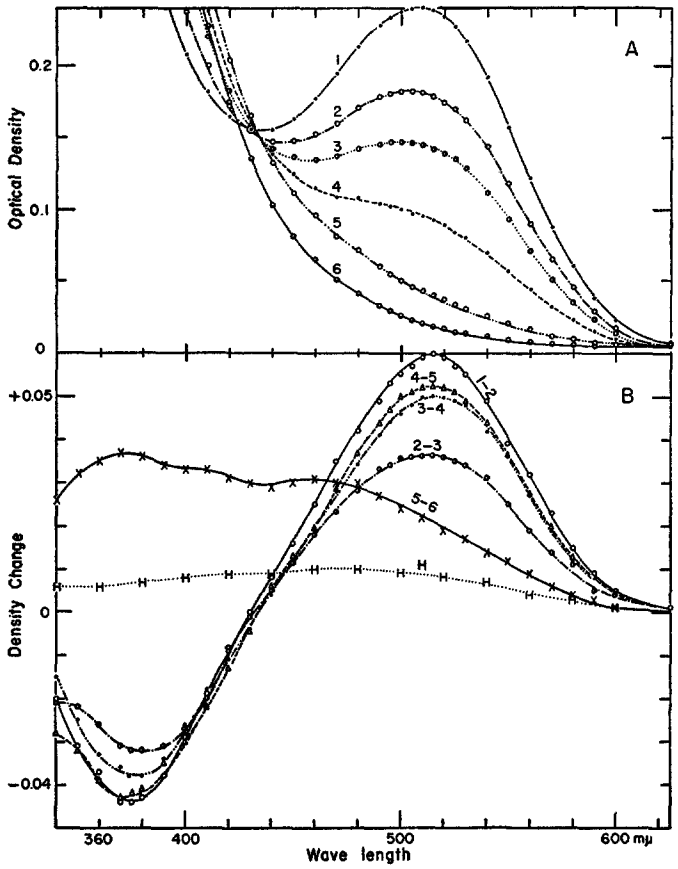

FIG. 3 A. A selective bleaching experiment (aliquot 4B). Curve 1, absorption spectrum of unbleached extract; curves 2 and 3, absorption spectra measured after successive 1 hour bleaches with 640 mμ light (monochromator exit 5 mm.); curves 4 and 5, absorption spectra measured after successive bleaches of 10 and 30 minutes, respectively, with 606 mμ light (exit 5 mm.); curve 6, absorption spectrum measured after 10 minute exposure to white light (100 watt tungsten bulb).

FIG. 3 B. Difference spectra of experiment with aliquot 4B. Positive changes (upward) indicate loss of density; negative changes (downward) indicate gain in density. Curves 1–2 and 2–3 are the results of the two 640 mμ bleaches; 3–4 and 4–5 are due to the exposures to 606 mμ light. Curve 5–6 is the final change due to exposure to white light. Curve H is the difference obtained with a final white light bleach in another aliquot (4C) of the same extract, but one to which hydroxylamine had been added.

by red light at 606 mμ (curve 3 of Fig. 1). This experiment suggests that only one photosensitive pigment was present in the extracts prepared.

To demonstrate the adequacy of the above method a control experiment was performed with an artificial mixture of bullfrog (*Rana catesbiana*) rho-

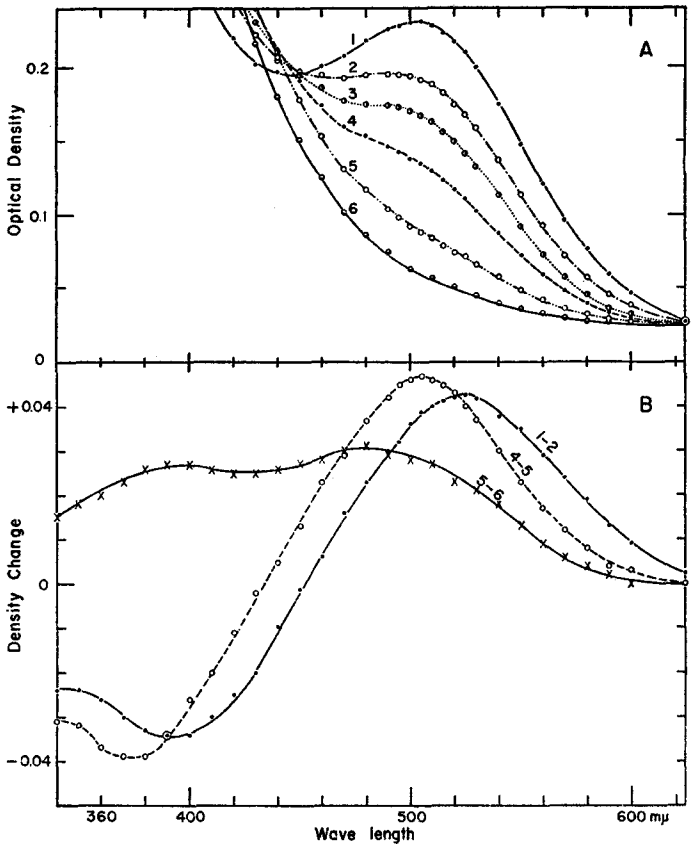

FIG. 4 A. A selective bleaching experiment with a mixture of bullfrog rhodopsin and carp porphyropsin. Curve 1, absorption spectrum of unbleached extract; curves 2 and 3, absorption spectra measured after successive 1 hour bleaches with 640 mμ light (monochromater exit 5 mm.); curves 4 and 5, absorption spectra measured after successive bleaches of 15 minutes and 1 hour, respectively, with 606 mμ light (exit 5 mm.); curve 6, absorption spectrum measured after 10 minute exposure to white light (100 watt tungsten bulb).

FIG. 4 B. Difference spectra of carp-bullfrog experiment. Curve 1–2 is the change due to the first exposure to 640 mμ light. Curve 4–5 is the result of the last 606 mμ bleach. Curve 5–6 is the final change due to exposure to white light. Omitted for the sake of clarity are curves 2–3 (second 640 mμ bleach) and 3–4 (first 606 mμ bleach); the characteristics of these difference spectra are listed in Table II. Both were intermediate between curves 1–2 and 4–5.

dopsin (λ_{max} 500 to 502 mμ) and carp (*Cyprinus carpio*) porphyropsin (λ_{max} 523 mμ). Both of these pigments are well known and have been adequately studied (Wald, 1938; Crescitelli and Dartnall, 1954). Outer segment extracts of two adult bullfrogs and of three carp were prepared separately in the man-

ner described previously. The absorption spectra of the bullfrog and carp extracts used are given in Fig. 1. The extracts were mixed so as to give the summed spectral absorption curve 1 of Fig. 4 A. This curve is similar to curve 1 of Fig. 3 A, the spectral absorption curve of mudsucker extract aliquot 4B. Again the series of two bleaches at 640 mμ and two at 606 mμ was completed; the only difference between the bleaching conditions of the two experiments was that somewhat longer bleaches at 606 mμ were given the bullfrog-carp mixture. As one would expect, rhodopsin is less sensitive than the mudsucker pigment to light of this wave length and requires longer exposure to be bleached proportionately. In Fig. 4 B the difference spectra obtained after the first exposure to 640 mμ light (curve 1-2) and after the last exposure to 606 mμ light (curve 4-5) are plotted. The shift from a porphyropsin (λ_{max} = 525 mμ) to a rhodopsin (λ_{max} = 504 mμ) difference spectrum is clear. The intermediate difference spectra would make the figure confusing and were left out for this reason. The difference spectrum 2-3 (second bleach at 640 mμ), however, was maximal at 515 mμ (see Table II). The curve 3-4 (first bleach at 606 mμ) was also intermediate (λ_{max} = 510 mμ) between the first and last bleaches. There was, therefore, a gradual shift in the difference spectra during the course of bleaching; the intermediate curves are due to partial bleaching of both components. In this way a mixture of two photosensitive pigments with absorption maxima only 20 mμ apart was shown to be distinguishable by the method of partial bleaching. This assures that the evident homogeneity of the mudsucker light-sensitive pigment is real and not the product of inadequate analytic methods. It should also serve as caution that the spectral absorption curve of a given retinal extract may represent a number of possible combinations of component pigments. An interpretation attempted without adequate demonstration of the constituent nature of such a total curve may be subject to error.

4. The Hydroxylamine Difference Spectrum of Mudsucker Retinal Extracts.—An attempt to locate more prediscely the spectral absorption maximum of the mudsucker photosensitive pigment was made with the use of hydroxylamine. Hubbard and Wald (1952) stated that hydroxylamine combines with retinene to form the oxime. The absorption maximum of this oxime is shifted to shorter wave lengths than the λ_{max} of the indicator yellow. The use of hydroxylamine, therefore, results in a difference spectrum which is freer from distortion due to the absorption of light by the products of bleaching. This difference spectrum should, in fact, very closely approximate the true absorption spectrum of the photosensitive pigment to wave lengths as short as about 460 mμ. Curve 3 of Fig. 1 is such a hydroxylamine difference spectrum and agrees with the absorption spectrum of the purest retinal extract down to about 460 mμ. The maxima of the hydroxylamine difference spectra of aliquots 2C, 4C, 4D, and 4F were all at 512 mμ.

5. Spectrophotometric Analysis of Mudsucker Retinal Carotenoids.—Extrac-

tion and analysis of the retinal carotenoids were performed with the methods described extensively by Wald (1939 *a*, 1939 *b*). On June 13, 1956, 40 adult mudsuckers were obtained from a local bait dealer, and the right and left retinae of the dark-adapted fish were collected separately. The left retinae were extracted for 20 minutes in the dark with petroleum ether (b.p. 66–

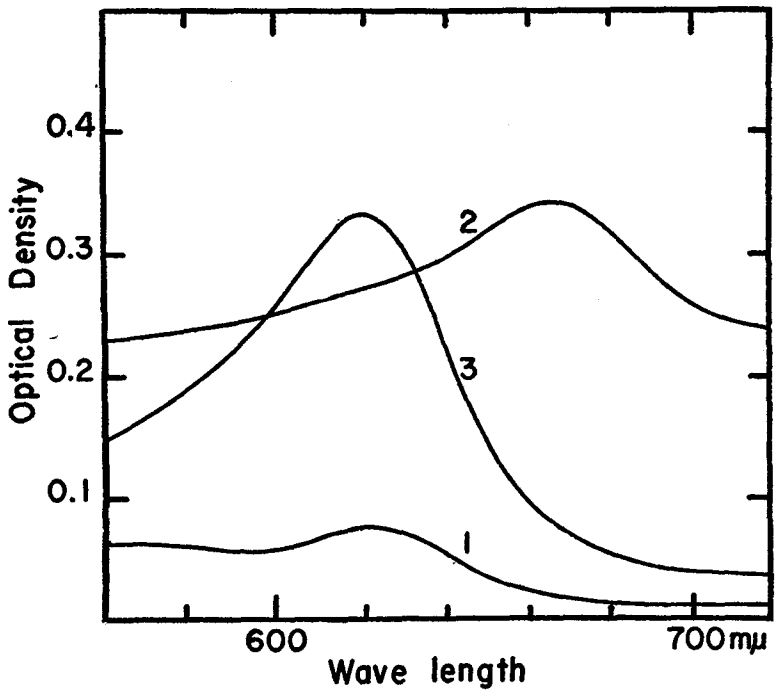

FIG. 5. Antimony trichloride reactions with petroleum ether extracts of mudsucker retinal tissue. Curves are tracings of records drawn by recording spectrophotometer. Curve 1 shows a small amount of the 620 mμ chromogen, vitamin A_1, extracted from dark-adapted retinae. In curve 2 retinene$_1$, absorbing maximally near 664 mμ, has been liberated by irradiation. Faded retinae, curve 3, show that retinene has been converted to vitamin A_1.

75°C.), then exposed to bright white light and immediately reextracted. After irradiation the right retinae were allowed to remain at room temperature for 1 hour before extraction. The antimony trichloride analyses of the three extracts (transferred to chloroform) were performed with a recording spectrophotometer (Process and Instruments Co.). The spectrophotometer was started immediately after combination of the reactants; each curve required about 2 minutes to record. Fig. 5 shows the results of these tests; a small amount of vitamin A_1 (λ_{max} 620 mμ) was obtained by preextraction in

the dark (curve 1). After exposure to white light (curve 2) a larger amount of retinene$_1$, absorbing maximally near 664 mμ, was recovered. From the other group of retinae which had been allowed to stand after exposure to light, vitamin A_1 was obtained (curve 3). After 30 minutes the absorption bands of all extracts almost entirely disappeared. No evidence of vitamin A_2 or retinene$_2$ was found. The data do suggest that the mudsucker photosensitive pigment is a member of the family of retinene$_1$ visual pigments, which include classical rhodopsins and iodopsin.

DISCUSSION

1. Absorption Maximum of the Mudsucker Photosensitive Pigment.—The location of the absorption maximum of the mudsucker photosensitive pigment may be estimated by considering the data together. The λ_{max} is certainly at a somewhat longer wave length than the absorption maximum (510 mμ) of the purest extract. As already stated, Fig. 1 combines such a curve with that of the hydroxylamine difference spectrum. The latter is a closer approach to the true pigment absorption spectrum than are the difference spectra (λ_{max} = 514 to 515 mμ) without hydroxylamine, which are distorted by the color of the violet-absorbing products of bleaching. Assuming 512 mμ to be the actual pigment maximum, a curve was constructed using Dartnall's nomogram (1953). This is curve 2 of Fig. 1. The nomogram is based upon the similarity in shape of known visual pigment absorption curves when frequency rather than wave length is plotted on the abscissa; the entire curve is merely translated according to the pigment's λ_{max}. The agreement among the three curves of Fig. 1 down to a wave length of 460 mμ is very good.

An attempt was made to correlate the spectral absorption maxima of the mudsucker extracts and their relative purity (indicated by the $D_{min.}/D_{max}$ ratio) in the manner employed by Crescitelli and Dartnall in their work (1954) on carp porphyropsin (λ_{max} = 523 mμ). In Fig. 6 is the same curve, which was the curve of best fit for the carp data, as in their Fig. 8; but the wave length scale is shifted as appropriate for a pigment maximum of 512 mμ. The open circles represent the absorption maxima of the extracts tested; they fit this line approximately (perhaps a slightly better fit would be obtained by drawing the line appropriate for a pigment at 511.5 mμ). The closed circles represent the maxima of the difference spectra without hydroxylamine and are scattered about the vertical line which is the average for the carp data also translated for a pigment with λ_{max} at 512 mμ. The half-filled circles are the λ_{max} of hydroxylamine difference spectra, and all fall at 512 mμ. Combination of all lines of evidence leads to the condlusion that the photosensitive pigment of the mudsucker has its absorption maximum at 512 $\pm$ 1 mμ and may, therefore, be named a retinene$_1$ pigment 512 of *Gillichthys mirabilis*.

The further note may be added here that a retinal extract prepared from

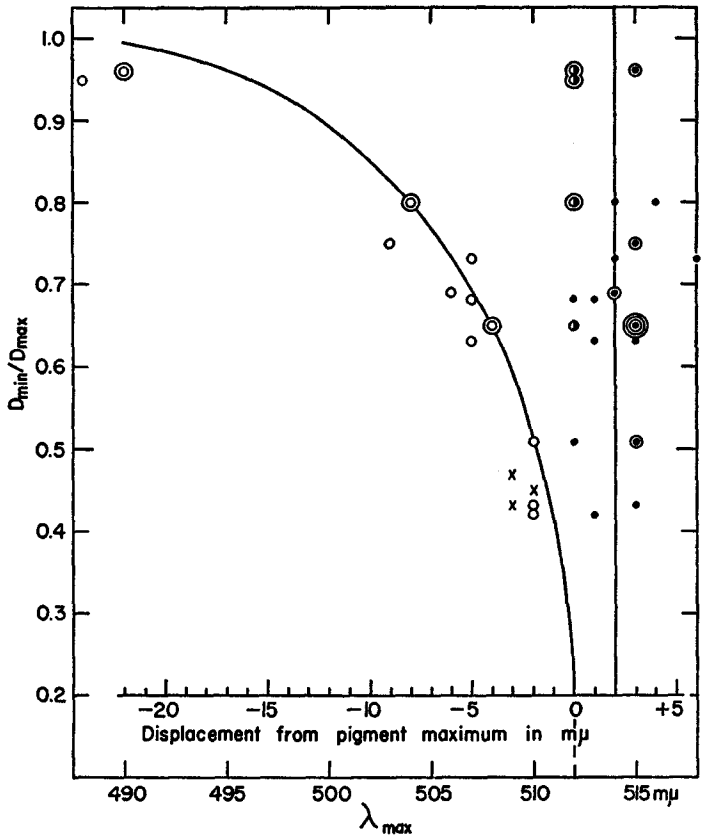

FIG. 6. Relation between the "purity" (D_{min}/D_{max} ratio) of retinal extracts and their λ_{max}. Solid line is curve of best fit for carp porphyropsin (Crescitelli and Dartnall, 1954), translated so λ_{max} = 512 mμ. Open circles are measured spectral absorption maxima. Vertical line is the average value of λ_{max} of the carp difference spectra, also translated as appropriate for a photosensitive pigment with its λ_{max} at 512 mμ. Filled circles are difference spectrum maxima obtained with each aliquot. Half-filled circles are hydroxylamine difference spectrum maxima. ×'s are three extract absorption maxima taken from Kampa's work (1953) on the mudsucker and plotted here for comparison.

members of another population of mudsuckers appeared to contain the same photosensitive pigment as those from San Francisco Bay. The mudsuckers used for this preliminary extract were collected[1] on May 14 and 15, 1955, at Fish Springs, Salton Sea, Riverside County, California, and were members

[1] Collected and kindly donated for this work by Mr. George W. Barlow, Department of Zoology, University of California, Los Angeles.

of a population introduced there. This first attempt at extraction unfortunately gave a very impure preparation, which could be studied only by the difference spectra obtained after exposure to colored light (λ = 424 to 660 mμ). The data are not tabulated, but the difference spectra were all maximal between 512 and 517 mμ and were consistent with the other results.

2. Comparison with Previous Studies of Photosensitive Pigments of Gillichthys and of Other Species.—The retina and certain visual functions of *Gillichthys mirabilis* have been studied before by Kampa (1953). Her histological examination showed that the mudsucker retina is duplex; in addition she obtained curves of critical fusion frequency typical of animals with a duplex retina. She also determined the effect of varied dietary amounts of vitamin A upon the weight of the retina and concentration of retinal pigment. She did not recognize that this pigment was different, however, but believed it to be rhodopsin. Dr. Kampa (Mrs. Brian Boden) graciously allowed her original results to be compared with those of the present experiments. In Fig. 6 the absorption maxima of three of her retinal extracts are related to their D_{min}/D_{max} ratios. They suggest a pigment maximum at a wave length of 510 or 511 mμ, not greatly different from the conclusion drawn in the present work.

Another light-sensitive pigment at about the same position (510 mμ) as the mudsucker pigment has been described in a short paper by Dartnall (1952 *b*) from the fresh water bleak (*Alburnus lucidus*); retinal extracts of this fish contained a small amount of a pigment absorbing maximally at 510 mμ in addition to larger quantities of another photosensitive pigment (λ_{max} = 533 mμ). It seems desirable at the present time to distinguish between the trace pigment of the bleak at 510 mμ and the photosensitive pigment of the mudsucker.

The mudsucker photosensitive pigment has not been directly shown to be a visual pigment. Spectral sensitivity of this species has not been determined. The concentrations of pigment obtained in the extracts, shape of the spectral absorption curve, and analysis of the retinal carotenoids, however, all indirectly support the possibility that the pigment is a visual pigment of the rods, quite probably functioning in scotopic vision.

The mudsucker photosensitive pigment is related to rhodopsins only to the extent that it is one of a family of pigments from which retinene$_1$ may be recovered. Whether the shift of the absorption maximum from 500 to 512 mμ is due entirely to difference in the protein part of the pigment molecule or partly to some isomeric rearrangement of the carotenoid groups might be difficult at present to determine. This work on the mudsucker in no way prejudices the possible correctness for other euryhaline fishes of Wald's inference (1941) that a mixture of rhodopsin and porphyropsin combines to give an intermediate visual pigment absorption maximum. The biological

significance of a visual pigment occurring in a euryhaline fish, and absorbing maximally between the peaks of rhodopsin and porphyropsin, is not understood.

SUMMARY

Retinal extracts have been prepared from dark-adapted mudsuckers by treatment of retinal tissue or of isolated outer segments of the visual cells with digitonin solution. The extracts were examined spectrophotometrically and found to absorb light maximally between the wave lengths of 488 and 510 mμ, depending on the proportion of yellow impurities and light-sensitive pigment present. This photosensitive pigment was shown to be homogeneous by partial bleaching of the extracts with monochromatic light of various wave lengths from 390 to 660 mμ. The mudsucker pigment was specifically demonstrated not to be a mixture of rhodopsin and porphyropsin; the adequacy of the method used to analyze such mixtures was shown by performing a control experiment with an artificial mixture of bullfrog rhodopsin and carp porphyropsin.

Comparison of the hydroxylamine difference spectrum and of the absorption maximum of the purest retinal extract located the mudsucker photosensitive pigment maximum at 512 ± 1 mμ. Extraction of retinal tissue with a fat solvent after exposure to white light gave a preparation which after the addition of antimony chloride reagent developed the absorption band maximal near 664 mμ, which is characteristic of retinene$_1$. If an hour intervened between exposure of the retinal tissue to light and extraction of the carotenoid, the antimony trichloride test gave a color band maximal at 620 mμ, characteristic of vitamin A_1. No evidence of retinene$_2$ or vitamin A_2 was obtained. The euryhaline mudsucker has, therefore, a photosensitive retinal pigment with an absorption maximum halfway between the peaks of rhodopsins and of porphyropsins and belonging to the retinene$_1$ system characteristic of rhodopsins. The pigment is therefore named a retinene$_1$ pigment 512 of the mudsucker, *Gillichthys mirabilis*. It is uncertain whether this type of photosensitive pigment will be found in other euryhaline fishes.

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