THE NATURE OF THE LAMPREY VISUAL PIGMENT*

By FREDERICK CRESCITELLI

(From the Department of Zoology, University of California, Los Angeles)

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Quite apart from their role in vision, the visual pigments are of interest in relation to problems of ecological adaptation and biochemical evolution. This aspect has been investigated by Wald (1, 2) whose views on this subject are widely known and respected. According to Wald the rhodopsins absorb maximally in the vicinity of 500 mμ and are found in the retinae of most marine fishes and of terrestrial vertebrates. In contrast, the porphyropsins, Wald believes, are characterized by absorption maxima in the neighborhood of 522 mμ and are present in the retinae of fresh water fishes. The problem as to which of these two pigment systems was primary in the evolutionary history of the vertebrates can, if we accept Wald's interpretation, be illuminated by an examination of the visual pigments of some of the most primitive living vertebrates. Of such vertebrates the lampreys are especially useful. These ancient animals are taxonomically remote from other living vertebrates. Moreover, they are numerous, easily obtained, and, as adults, the lampreys have eyes large enough for adequate study. The lamprey is a euryhaline animal migrating from the sea into fresh water streams where spawning occurs.[1] In addition, land-locked populations which live their entire lives in fresh water are available. If fresh water habitat is the primary determinant these animals should possess the porphyropsin system. From the results of an analysis of the vitamin A in the bleached retina Wald (3) concluded that porphyropsin is present in the lamprey retina and that, to quote Wald (1), "the sea lamprey possesses an enormous preponderance of the porphyropsin system, exactly like an anadromous teleost." No attempt was made to extract, and to analyze, the pigment itself. In the absence of such direct information, the nature of the lamprey pigment must still be regarded as uncertain.

This investigation was initiated in order to attempt the extraction of the

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[1] Dr. Carl L. Hubbs has called the writer's attention to the fact that there are a number of species of lampreys confined wholly to fresh water and that one of the major genera, *Ichthyomyzon*, possibly the most primitive, is so confined.

lamprey pigment and to identify it spectrophotometrically. The attempt was successful but the results, instead of confirming Wald's conclusion based on vitamin A analysis, show the pigment to be a rhodopsin. This paper will describe the results and the reasoning which led to this conclusion.

Analytical Procedure

Two species of lampreys were employed in the study: the Pacific Coast lamprey (*Entosphenus tridentatus*) and land-locked individuals of the sea lamprey, *Petromyzon marinus*. These animals were shipped alive to the laboratory, *Entosphenus* coming from one of the streams of the Columbia River basin in Oregon while *Petromyzon* was taken out of the Carp Lake River, Emmet County, Michigan.[2] The Pacific Coast lampreys were sexually mature adults while the sea lampreys were in the stage known as recently transformed downstream migrants. A comparison of the results obtained with these two lampreys is of interest for one of these populations (*E. tridentatus*) is migratory between the sea and fresh water while the animals of the second species have a fresh water environment throughout their entire life cycle. This population of *P. marinus* has been land-locked in the upper Great Lakes region for at least 34 years since the first specimen of this species was taken from Lake Erie in 1921. Entrance into the upper Great Lakes is believed to have occurred by way of the Welland Canal (4, 5).

The procedures employed in extracting and analyzing the lamprey pigment were those customarily utilized in this type of study, and for this reason unnecessary details will be omitted. The description of a typical experiment with *P. marinus* will serve to illustrate the procedure. In this experiment 18 lampreys were dark-adapted for an hour, following which the eyes were removed, utilizing illumination provided by a deep red photographic safe light. The eyes were placed in 4 per cent alum for about 15 minutes and then the retinae were removed through an opening in the cornea and immersed in 4 per cent alum overnight. The next morning the hardened tissue was washed twice with distilled water and once with borate buffer at a pH of 7.9. The retinae were then extracted in two successive steps with a total of 0.8 ml. of 2 per cent digitonin made up in borate buffer at a pH of 8.0. This extract was then stored in a refrigerator and later analyzed with a Beckman DU spectrophotometer provided with a photomultiplier attachment and with a water-cooled circulating system. Water from a constant temperature bath maintained the temperature of the Beckman cell compartment at 20°C. ± 0.5°C. The absorption spectrum obtained with an aliquot of this extract is to be seen as curve 1 of Fig. 1 A. In obtaining the data for this curve, measurements were begun at 700 mμ and were continued down to 320 mμ, readings being taken at 20 mμ intervals. Following this traverse the wave length scale was retraced starting at 330 mμ and recording the optical density at 20 mμ intervals. This procedure was employed in order to detect any instability in the solution. If evidence of instability was found the extract was discarded or else returned to the refrigerator for further examination at a later date. Following these

[2] The author is indebted to Dr. V. C. Applegate for his kindness in sending these animals.

measurements, which normally required about 35 minutes, the solution in its cell was transferred to a temperature-controlled bleaching chamber which allowed illumination of the total volume of solution with selected light from a B and L grating monochromator. The bleached solution was then transferred back to the Beckman cell compartment and its absorption spectrum was measured as before. In this manner the action of light of different wave lengths was examined. This general procedure was followed, except for minor variations in a few of the experiments. The total number of extracts, the age of the extract, the wave length employed in bleaching, and other relevant data are summarized in Table I.

RESULTS

Petromyzon marinus

The absorption spectrum of a relatively pure solution from the retinae of *P. marinus* is shown as curve 1 (Fig. 1 A). For this spectrum the ratio of the minimum density at 424 mμ to the maximum density at 496 mμ ($D_{\text{min.}}/D_{\text{max.}}$) is 0.55, a value which is remote from the best values (0.22 to 0.26) reported for visual pigment extracts (2, 6, 7) but one which is still acceptable as indicative of a reasonably pure solution. The point of maximum absorption occurred at a wave length of about 496 mμ. This cannot be said to be the true spectral peak of the lamprey pigment. Even assuming the occurrence of only one visual pigment in the extract, the presence of yellow impurities alone must have caused some shift to shorter wave lengths from the true absorption peak of the visual pigment. The essential question is whether this shift was a slight one, thus retaining the position of the visual pigment in the region of the rhodopsin maximum, or whether it was a shift great enough to account for a true peak at about 522 mμ. It will be demonstrated in the arguments which follow that the shift was only a small one, so that from a spectrometric point of view the absorption curve of Fig. 1 A may be taken to indicate the presence of a pigment of the rhodopsin group.

1. One approach to this problem is to compare the maximum of the lamprey curve at 496 mμ with the maxima of absorption curves of rhodopsin solutions of comparable purity. From a number of absorption curves of frog rhodopsin prepared in this laboratory, three were selected with $D_{\text{min.}}/D_{\text{max.}}$ ratios of 0.51, 0.52, and 0.54. The first of these figures refers to an extract of retinae from *R. pipiens* while the last two figures relate to retinal extracts from *R. catesbiana*. The maxima for these three solutions were at 501, 502, and 501 mμ, respectively. It is clear that absorption curves obtained with solutions containing rhodopsin in the same state of relative purity as the lamprey pigment have absorption maxima which differ from the peak of rhodopsin (8) by only 1 to 2 mμ. Assuming that only one component was present in the lamprey extract, this reasoning leads to a value of 497 mμ as the maximum for the lamprey pigment.

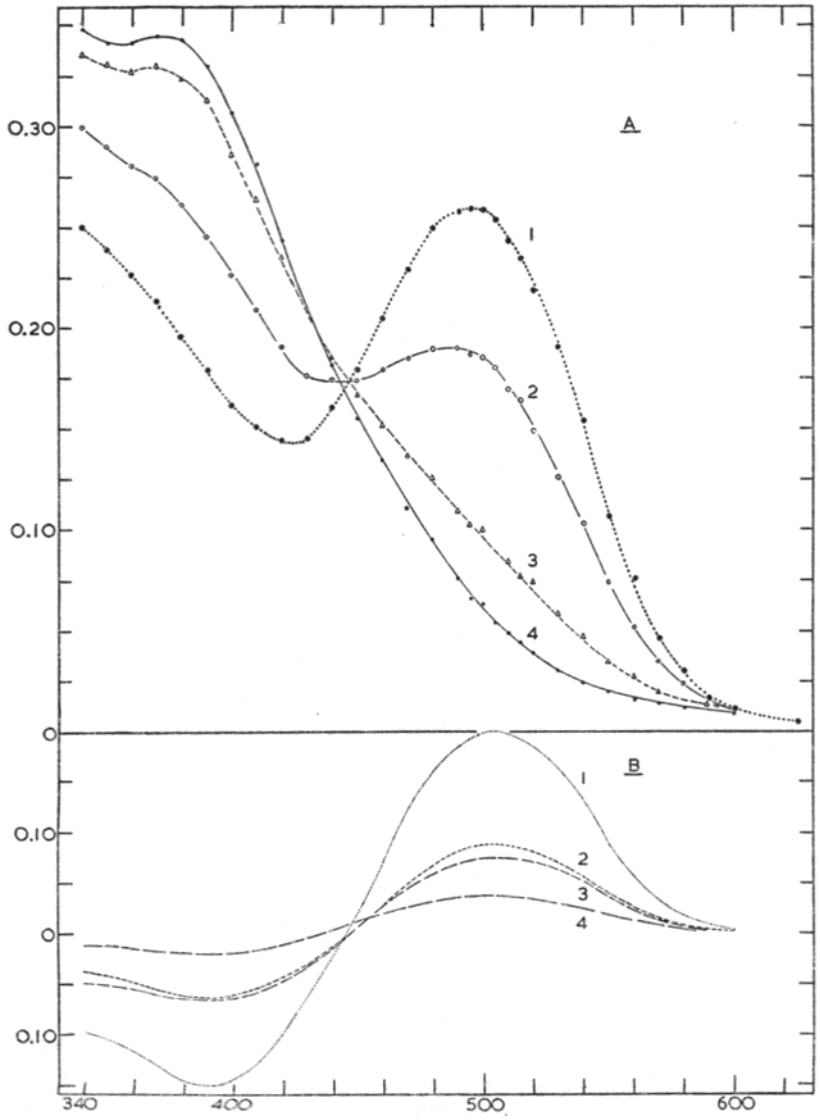

FIG. 1 A. *P. marinus*. A selective bleaching experiment (II-1). Curve 1, absorption spectrum of unbleached extract; curve 2, after 175 minutes' exposure to light of 700 mμ; curve 3, after 77 minutes' exposure to light of 630 mμ; curve 4, after 10 minutes' exposure to a 40 watt light. Optical density is plotted as a function of wave length (mμ).

FIG. 1 B. Difference spectra of experiment II-1. Change in density plotted as a function of wave length. Positive changes (upward) indicate loss in density; negative changes (downward) indicate gain in density. Curve 1, difference between curves 1 and 4 of Fig. 1 A; curve 2, difference between curves 2 and 3; curve 3, difference between curves 1 and 2; curve 4, difference between curves 3 and 4.

2. For analytical purposes the difference spectra obtained after bleaching have proved useful in identifying the approximate position of the absorption peak of a visual pigment. These difference spectra depend, of course, upon the color of the products formed as the result of bleaching, as well as upon the color of the original pigment. In alkaline solutions the absorption maxima of these

TABLE I

Extract Details and Spectrophotometric Characteristics

Species	No. of animals	No. of experiment	Extract age	Density 700 mμ	Density maximum	Density minimum	Maximum mμ	Minimum mμ	Ratio*	Bleach‡	Difference maximum mμ§	Maximum density‖
			days									
E.t.	5	I-1	23	0.020	0.198	0.175	488	442	0.88	630	503	0.025
E.t.	5	I-1	23							600	502	0.068
E.t.	5	I-1	23							W	501	0.032
E.t.	5	I-2	25	0.014	0.201	0.174	490	440	0.87	420	502	0.119
P.m.	18	II-1	7	0.001	0.259	0.142	496	424	0.55	700	503	0.074
P.m.	18	II-1	7							630	504	0.090
P.m.	18	II-1	7							W	504	0.036
P.m.	18	II-2	32	0.061	0.294	0.294	480	470	1.0	570	503	0.112
P.m.	20	III-1	7	0.006	0.262	0.130	497	415	0.50	631¶	501	0.061
P.m.	20	III-1	7							580¶	503	0.144
P.m.**	20	III-2	9	0.050	0.334	0.298	490	440	0.89	W	497	0.184
P.m.	15	IV-1	15	0.055	0.299	0.295	480	460	0.99	580	504	0.092

All extracts were at pH values between 7.9 and 8.1.

* Density minimum/density maximum.

‡ Wave length of bleaching light; *W* signifies tungsten light.

§ Wave length for maximum loss of density following bleaching.

‖ Maximum density loss after bleaching.

¶ Interference filters used in conjunction with the monochromator.

** 0.02 ml. of 0.1 M NH_2OH (freshly neutralized to pH 7.0) added to 0.5 ml. of extract.

products are located well below 400 mμ and the absorption at wave lengths longer than 500 mμ is relatively small (Fig. 1 A). Under these conditions the difference spectrum at wave lengths longer than 500 mμ is therefore an approximation to the true absorption spectrum of the photolabile pigment. At shorter wave lengths, however, the difference spectrum is influenced more and more by the color of the products which, in turn, may depend upon a number of factors including the wave length of the light employed in bleaching (9).

The results of a bleaching experiment are graphically summarized in Fig. 1 A in which is shown the outcome of successive bleaches to light of 700 mμ (curve 2), 630 mμ (curve 3), and finally to white light (curve 4). These curves need not be discussed in detail since they are identical with descriptions of the bleaching of visual purple which appear in the literature (8, 10). The important point for our purpose is that the maxima for these difference spectra are located at about 502 to 504 mμ (Figs. 1 B, 2). Since the product of bleaching (curve 4, Fig. 1 A) absorbs increasingly as the wave length decreases, the peak of the difference spectrum is, with respect to the peak of the true absorption spectrum, shifted slightly toward longer wave lengths. In other words, it is not possible for the pigment of *P. marinus* to have an absorption maximum even as high as 502 to 504 mμ. This clearly eliminates porphyropsin as the photosensitive pigment of the lamprey extracts.

Not all the extracts obtained from the retinae of *P. marinus* were as pure as that from which the data for Fig. 1 were obtained. Nevertheless all extracts, upon bleaching, yielded difference spectra with maxima at about the same values (Table I).

3. The arguments advanced in the two preceding sections are valid only if it can be shown that only one pigment contributed to the results. The existence of several pigments can be most simply revealed by comparing the difference spectra obtained after selective bleaching with light of different wave lengths. This is the method which was employed in the demonstration of the existence of iodopsin (11, 12) and of several other pigments (13–16). Unpublished data from this laboratory have shown that, with the equipment and procedures of the present investigation, this method is able to resolve porphyropsin and rhodopsin from a prepared mixture of both these pigments. The method of selective bleaching has revealed only one component in the retinal extracts from *P. marinus*. This statement is supported by the data of Fig. 2 which shows, gathered together for comparison, the difference spectra obtained after bleaching with light of different wave lengths. There appears to be no significant variation in position of maximum, or in position or shape of the long wave length segment of the curves correlated with wave length of bleaching light. Only one photosensitive pigment was apparently present in the extracts. This statement is not intended to mean that only one visual pigment is necessarily present in the retina of *P. marinus*. There may be pigments not extractable by digitonin, or too low in concentration or too labile to withstand the laboratory treatment. Walls (17) examined the lamprey retina histologically and noted, as others had before him, two groups of visual cells: long and short types. He considered rhodopsin to be present in the short cells. The nature of the pigment, if any, in the long cells remains to be discovered.

The data of Fig. 2 suggest, however, the occurrence of a selective action of wave length on the short wave length limb of the difference spectra. This prob-

ably means that the products of bleaching are slightly different according to the wave length of the bleaching light. The effect is similar to that already reported in connection with retinal extracts of the carp (9) and may be related to the isomerizing actions of light reported by Hubbard and Wald (18). Irrespective of the exact nature of this secondary effect, it is not considered to influence the argument bearing on the nature of the lamprey pigment.

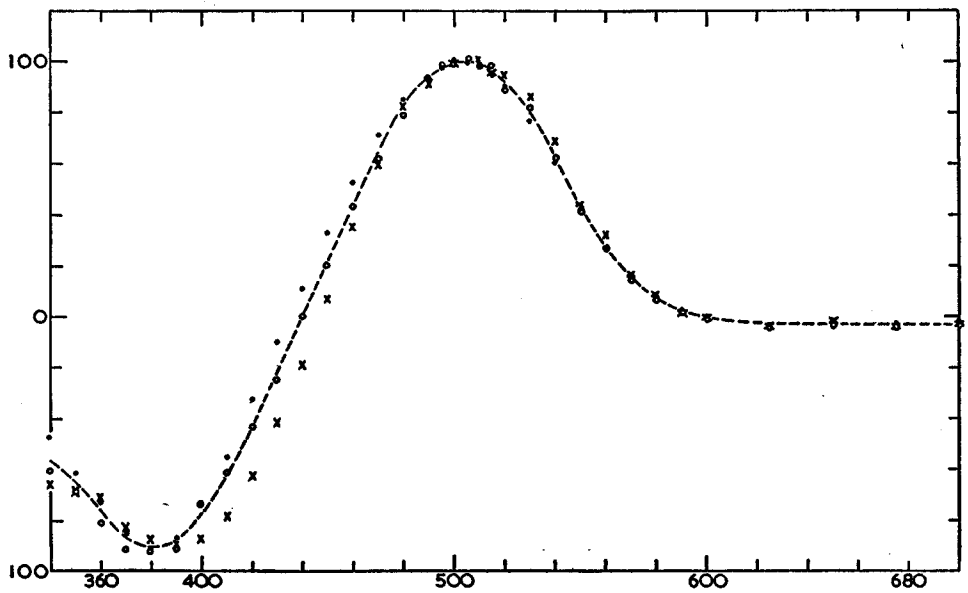

FIG. 2. *P. marinus*. Difference spectra after selective bleaching. All data scaled so that positive maximum of each curve is set at 100 per cent. Dotted line is mean of all bleachings; ×, after 700 mμ bleach; open circles, after two bleachings, one at 630 mμ, the other at 631 mμ, these data averaged; filled circles, after two bleachings, one at 570 mμ, the other at 580 mμ, these data averaged.

4. It is obvious that neither the absorption curve of the unbleached pigment (Fig. 1 A) nor the difference spectra (Fig. 2) approximate the true absorption spectrum of the visual pigment except for the long wave length portions of the curves. The absorption curve is distorted by the presence of yellow impurities in the extract, while the difference spectra are affected by the yellow products of bleaching. For several reasons it is desirable to obtain a curve which is closer to the form of the true absorption curve. One approach to this is to alter the nature of the products so as to reduce their absorption in the visible region. In such a case the difference spectrum will approach more closely the curve which is desired. The use of a carbonyl-trapping reagent, such as hydroxylamine, has proved very useful as Wald and his collaborators have repeatedly

shown (19). This compound reacts with retinene to form the corresponding oxime. The spectrum of the latter compound is, with reference to that of the normal products of bleaching, shifted toward shorter wave lengths. The difference spectrum obtained after bleaching an extract in the presence of hydroxylamine is shown in Fig. 3 (curve 3). This is compared with the measured absorption spectrum of a relatively pure solution of unbleached pigment (curve 1). The two agree reasonably well down to a wave length of about 495 mμ. Below

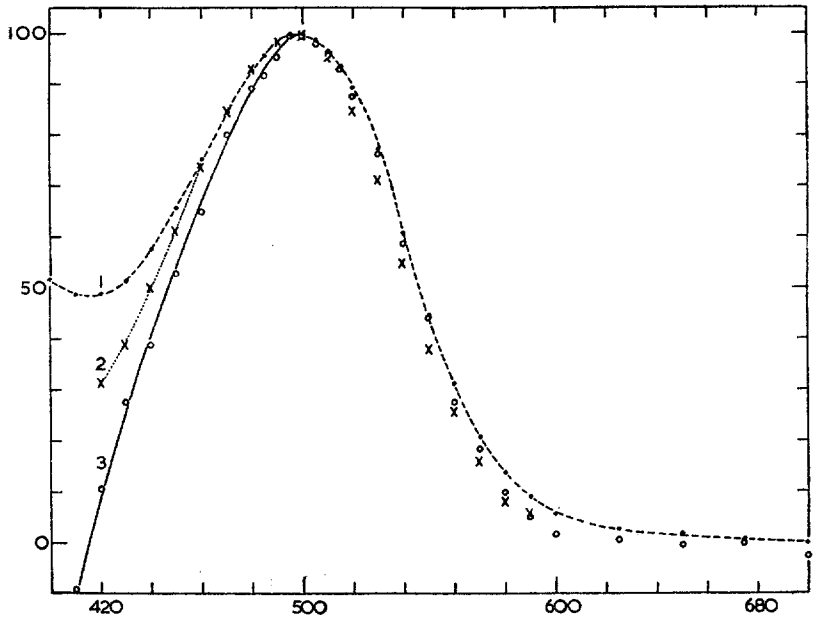

FIG. 3. *P. marinus*. Curve 1, absorption spectrum of a relatively pure solution ($D_{min.}/D_{max.}$ = 0.50); curve 2, reconstructed from Dartnall's nomogram assuming a maximum at 497 mμ; curve 3, hydroxylamine experiment, difference spectrum after exposure to a 40 watt tungsten light for 10 minutes.

this value the curves diverge, the disparity increasing with decreasing wave length. It is reasonable to suppose, because of the nature of the distortions, that the true absorption curve below 495 mμ follows a course in between these two curves. It is of interest, therefore, to compare the curve constructed from Dartnall's nomogram (20) with the two experimentally determined curves (Fig. 3). This nomogram is based on the finding that the alkaline difference spectra of a number of visual pigments, including rhodopsin and porphyropsin, are of the same form when frequency rather than wave length is used for the abscissa. This suggested to Dartnall that the absorption spectra of all these pigments were probably the same, the only difference being in position along the fre-

quency coordinate. The data for the constructed curve were obtained from the nomogram assuming the position of the lamprey pigment peak to be at 497 mμ. The reconstructed curve (curve 2) and the absorption curve of the unbleached extract agree reasonably well down to about 460 mμ. As far as the author knows the retinal sensitivity function of *P. marinus* is not available. It can be predicted, however, that such a curve would not seriously depart from the form given by curve 2 of Fig. 3. Moreover, the lamprey retinal sensitivity curve would be almost identical with the human retinal scotopic sensi-

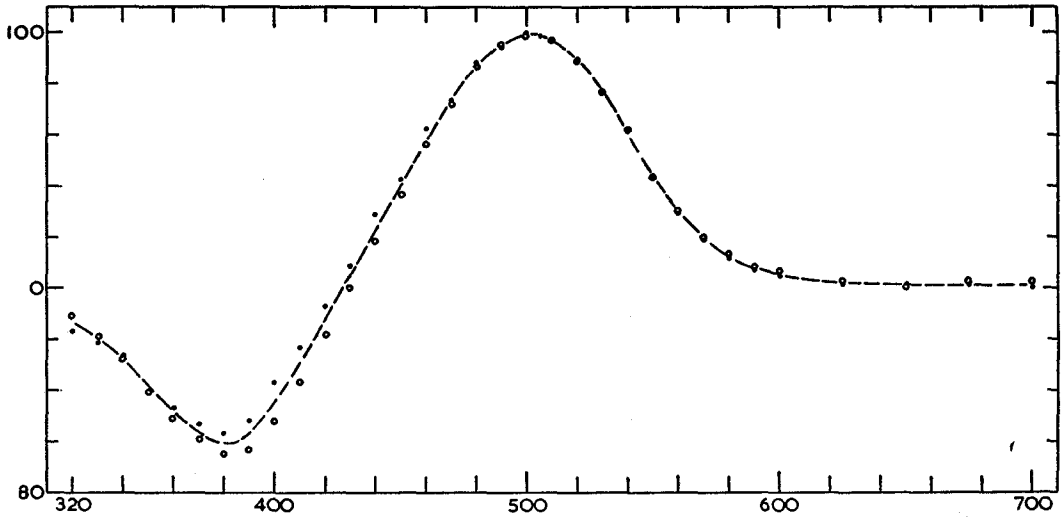

FIG. 4. *E. tridentatus*. Difference spectra scaled with maxima set to 100 per cent. Dotted line is mean curve; open circles, after two bleachings, one at 630 mμ for an hour followed by one at 600 mμ for another hour; filled circles, after 420 mμ bleach for 305 minutes.

tivity curve (21), the only conceivable factor which might cause some divergence being the *in vitro* density of the rhodopsin and this factor is probably not too important with the densities that are involved.

Entosphenus tridentatus

A singe extract of 5 sexually mature Pacific Coast lampreys was also prepared. The technique was identical with that described for *Petromyzon* except for the fact that the heads were cut off in room light and placed in a dark chamber for dark adaptation prior to removal of the retinae. The heads, rather than the entire animals, were dark-adapted in this case because the living spinal cords were required for another experiment and this part of the procedure was beyond the control of the author.

This solution was not especially pure (Table I), but the difference spectra (Fig. 4) obtained as the result of bleaching with light of different wave lengths are clear in showing that the *Entosphenus* pigment is not porphyropsin. The maxima for these difference spectra (Table I) are so similar to those of the *Petromyzon* pigment that in all likelihood the *Entospenus* pigment is identical, spectrophotometrically, with the pigment of *Petromyzon*. The *Entosphenus* experiment, though incomplete, is important for it shows that the presence of rhodopsin is a fixed characteristic of the lamprey and that it is unaffected by such factors as exposure to a marine environment and the chronological and sexual ages of the lamprey.

DISCUSSION

Kühne (22) was probably the first to note the presence of a photosensitive pigment in the retina of the lamprey. Walls (17) also observed this pigment and called attention to its purple appearance. He considered the pigment to be rhodopsin but no evidence for this appeared in his paper. Wald (3), in a communication already referred to, came to the conclusion, on the basis of the antimony trichloride reaction, that vitamin A_2 is present in the lamprey retina and that, therefore, porphyropsin is the visual pigment. He considered the porphyropsin to be present in low concentration and was therefore discouraged from attempts at extracting it.

The present results show that a photosensitive pigment can be extracted, utilizing traditional procedures, from the retinae of *P. marinus* and *E. tridentatus*. For both these species the pigment appears to be a member of the rhodopsin group and no evidence was obtained to suggest the presence of porphyropsin. The *Petromyzon* pigment, for which more data were recorded, appears to have an absorption maximum at about 497 mμ. Judging from the difference spectra, the *Entosphenus* pigment is probably located in the same position. It is to be noted, incidentally, that the liver of the lamprey contains vitamin A_1. Wald (3) also found this and commented on the disparity in vitamin content of the liver and retina, a condition which Wald (1) noted, occurs also in such fish as carp, Chinook salmon, and brook trout. The livers of the landlocked *P. marinus* also appear to contain vitamin A_1 and no significant quantity of vitamin A_2. This statement is supported by the evidence of Fig. 5 which illustrates the results of the antimony trichloride reaction on chloroform extracts of the livers. A labile peak at 618 mμ indicates the presence of vitamin A_1. No evidence of the vitamin A_2 peak of 693 mμ was recorded. In so far as the vitamin content of the liver is a factor, the present results on the nature of the visual pigment lead to complete biochemical accord between the two tissues.

How, then, can Wald's results be fitted into the picture? Speculations on this point are likely to be risky but one suggestion appears to be worth mentioning. Wald (3) did not deny the existence of vitamin A_1 in the lamprey ret-

ina; he merely noted the great preponderance of vitamin A_2 over vitamin A_1. Moreover, there is the observation of Lovern *et al.* (23) that in the eyes of *Lampetra fluviatilis* vitamin A_1 occurs and, apparently, in great preponderance over vitamin A_2. These observations could be reconciled on the basis of the occurrence of an intervitamin conversion in the lamprey retina. In any case, considering lampreys as a whole, the vitamin A analyses do not exclude the possibility of the occurrence of rhodopsin. The presence of vitamin A_2 in

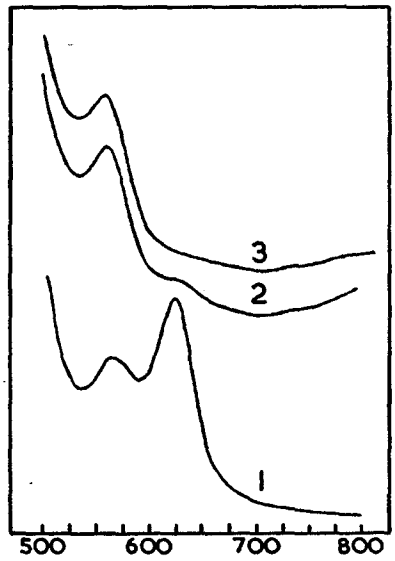

FIG. 5. *P. marinus*. Antimony trichloride reaction with liver extract. Curves are tracings of records made with a recording spectrophotometer. Each curve required about 1 minute to record with an interval of about 1 minute between the three successive recordings.

lampreys may have some functional significance in connection with the tail photoreceptors of the lamprey (24). Steven (25) determined the spectral sensitivity curve for the response of these receptors and came to the conclusion that porphyropsin might be the basis for the reaction. The writer has attempted, without success, to extract a photosensitive pigment from the tail skin of the ammocete.

From a biological point of view the finding, that the lamprey pigment is rhodopsin, rather than porphyropsin, has several interesting implications. In the first place here is a clear case of an animal which spawns in fresh water and yet which has developed the retinal pigment thought to be characteristic of marine forms. In addition, the taxonomic position of the lamprey leads to the suspicion that rhodopsin, rather than porphyropsin, was the pigment of

the original vertebrates. This notion is in better accord with the report (26) that vitamin A_1 is the constituent of invertebrates, that the visual pigment of the cephalopod retina is a retinene$_1$-compound (27), and that some invertebrates actually have vitamin A_1 itself in the visual pigment molecule (28). A direct line between invertebrates and vertebrates is thus established, rendering unnecessary, complicated arguments to explain a biochemical break in evolution. All this would place the porphyropsin system out of the main line of evolution, suggesting that the vitamin A_2 pigments arose secondarily.

There is one point further that needs emphasizing. Though the lamprey pigment is a rhodopsin, it is not necessary to assume that it is exactly the same chemical entity as other rhodopsins. The lamprey pigment, for example, is spectrophotometrically characterized by a maximum at about 497 mμ. This is significantly different from frog rhodopsin which is located at about 502 mμ (8). Even though both pigments probably have retinene$_1$, there are subtle differences in the two molecules which confer upon them slightly different spectral properties. Nature seems to have developed methods of making both coarse and fine adjustments of the visual pigments along the spectral scale without essentially changing the mode of response to light.

SUMMARY

From the retina of the land-locked population of the sea lamprey, *Petromyzon marinus*, a photolabile pigment was extracted which was identified spectrophotometrically as a member of the rhodopsin group of pigments. Using the absorption spectrum of a relatively pure solution and analysis by means of difference spectra, the peak of this pigment was placed at about 497 mμ. The method of selective bleaching by light of different wave lengths revealed no significant amounts of any other pigment in the extracts. A similar pigment was also detected in retinal extracts of the Pacific Coast lamprey, *Entospenus tridentatus*.

These results are significant for two reasons: (*a*) the lamprey is shown to be an example of an animal which spawns in fresh water but which is characterized by the presence of rhodopsin, rather than porphyropsin, in the retina; (*b*) the primitive phylogenetic position of the lamprey suggests that rhodopsin was the visual pigment of the original vertebrates.

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