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A STUDY OF THE VISUAL PIGMENTS OF THE CLAWED TOAD

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It is generally accepted that the spectral regions in which the sensory mechanisms of vision are active are determined by the light-absorbing properties of pigments. Relationships between visual sensitivity curves and the absorption spectra of photosensitive substances extracted from retinal end organs have been established in a few instances. This has led to the belief that other visual sensitivity curves will eventually be explained in similar terms, even though pigments with the necessary properties have, so far, escaped detection.

Recently a sensitivity curve of peculiar form has been reported. This was for the clawed toad, *Xenopus laevis*, and was obtained by measuring the lowest intensities of light from different spectral regions which were required to produce certain responses from the animal (Denton & Pirenne, 1951). The results, when plotted against wave-length, did not give a smooth bell-shaped curve as would be expected for a scotopic function; on the contrary they showed marked irregularities which included a pronounced maximum at 570 mμ.

These findings suggested that extracts of *Xenopus* retinae might provide interesting solutions for photochemical experiments. A study of the light-sensitive visual pigment solutions prepared from these animals is presented in this paper.

APPARATUS AND METHODS

Measurement of absorption spectra

Apparatus suitable for measuring the absorption spectra of highly photosensitive solutions has been described (Dartnall, 1952). The same apparatus and techniques were used in the present work.

The stability of the solutions during these measurements was tested in the following way. Optical densities were measured consecutively from 380 to 620 mμ, at 20 mμ intervals; then, sometimes after an interval, from 610 to 390 mμ, again at 20 mμ intervals. If the return measurements retraced the path of the outward ones this showed that the solution was stable, at least

during the period of measurement. The outward and return sweeps took about 30 min each to complete. In certain illustrations in this paper, attention is drawn to this stability test by showing the results for the outward and return measurements by different symbols (empty and filled circles, respectively). Whether so indicated or not, however, this procedure was always followed.

All density measurements were made at $20{\cdot}0 \pm 0{\cdot}2°$ C (uncorrected) and were for an optical path length of 0·5 cm.

The bleaching apparatus

This, again, was as previously described. Suitable intensities of the approximately monochromatic bands of light for bleaching the solutions were obtained by adjusting the widths of the entrance and exit slits of the monochromator. The corresponding band widths for each dominant wave-length of bleaching, and the stray light filters employed, are given in Table 1. During bleaching, the temperature was controlled at 20° C.

TABLE 1. Purity of the spectral bleaching lights

Dominant wave-length (mμ)	Approximate range (mμ)	Auxiliary stray-light filter
650	±16	Ilford 608
630	±15	Ilford 204
580	±13	Ilford 626
530	±10	Ilford 625
480	± 5	Ilford 621
480*	± 7	Ilford 621
430	± 6	Ilford 601

* For the second bleaching of extract III*b* with light of dominant wave-length 480 mμ (see Table 4) the intensity was increased by widening the slits of the monochromator.

Preparation of the visual pigment solutions

The toads (*X. laevis*) were kept in the open in a large bath filled with water. They were fed on raw liver and, occasionally, on raw meat. The eight typical animals used in preparing extract IV weighed 950 g, or an average of just under 120 g apiece.

Before the preparation of a retinal extract the toads were kept in darkness for at least 40 hr. All procedures following this period of dark adaptation were carried out in a dark room, in the general illumination of a deep red photographic safe-light. Additional light, where necessary for operations such as removal of retinae, was provided by a cycle torch lamp fitted with Ilford 'monochromatic' filter no. 609 (transmitting from 650 mμ to the infra-red). Unnecessary exposure of the retinae or of the photosensitive solutions prepared therefrom, even to these relatively ineffective radiations, was avoided. For example the tube, into which the retinae were put after extraction, stood in an aluminium cigar protector and was withdrawn only for the purpose of adding a retina or for some other essential operation.

Removal of retinae

The toads were beheaded and the heads washed free from blood in running water. A single cut was then made across the cornea with sharp scissors. The retinae were removed by a method based on that used by Lythgoe for the frog (Lythgoe, 1937). In this method the head was held in the left hand 'with the forefinger on the palate just behind the eye and the thumb in the corresponding position on top of the head'. By this means the forefinger served as a backing to the eye and prevented inward collapse when first the lens, and then the retina, were picked out with forceps. The retinae were accompanied by considerable amounts of black pigment.

Isolation of the outer limbs of the retinal end organs

Because of the difficulty in obtaining the retinae clean it was decided to separate the outer limbs of the end organs from the rest of the retina, using Saito's technique (Saito, 1938). The retinae were placed, therefore, in a tube and vigorously shaken with 2 ml. of 35% (w/v) sucrose

solution. This treatment caused the outer limbs to break away from the retinae. On spinning the mixture at 4000 rev/min for 20 min, the heavier black pigment and other retinal debris separated to the bottom, leaving the outer limbs suspended in the supernatant sucrose solution. After withdrawal of this, a further suspension of outer limbs could be obtained by adding 2 ml. more of sucrose solution to the residue and repeating the whole procedure. The quantity of outer limbs obtained was judged, in the safe-light illumination, by the cloudiness of the suspensions. Examination of small samples of first and second supernatants in daylight confirmed that the first contained most of the outer limbs; these gave to the mixture a reddish violet colour which changed to orange on exposure.

The residues were stratified in three layers: the lowest was black; the middle one, dark brown; the top layer—consisting of retinal debris—usually colourless, but sometimes showing patches of a reddish violet colour which faded on exposure to light.

Each suspension of outer limbs was diluted with 4 ml. of pH 4·6 buffer (pH 6·5 in the case of extract II). Because of the reduction in specific gravity and viscosity resulting from this dilution, the outer limbs sank to the bottom of the tube, a process hastened by centrifuging at 4000 rev/min for at least 20 min. The clear supernatants, which were either colourless or very pale yellow (faintly pink and photosensitive when the diluent was pH 6·5 buffer), were withdrawn from the compacted precipitate and discarded. The outer limbs were then washed into one tube with 2–4 ml. of pH 4·6 buffer (pH 6·5 in the case of extract II), centrifuged, and the colourless—or nearly colourless—washings removed and discarded.

TABLE 2. Preparation details of the extracts

Extract	I	II	III*a*	III*b*	IV
Date of preparation	24. ix. 51	3. x. 51	29. iv. 52		15. ix. 52
Duration of dark-adaptation (hr)	66	42	89		68
Number of toads	6	11	10		8
Number of retinae	12	22	20		15
Volume (ml.) of 2% digitonin solution used in extraction of outer limbs	1·0 + 1·0	2·0	1·5 + 1·5		1·0 + 1·0
Volume (ml.) of buffer added	0·2 s.b.	0·2 s.b.	0·15 p.h.p. (to 1·5 ml.)	0·15 s.b. (to 1·5 ml.)	0·2 s.b.
Total volume (ml.) of extract (approx.)	2·2	2·2	1·65	1·65	2·2
pH of extract, at 20° C	8·60	8·61	4·55	8·62	8·65

The following solutions were used: 's.b.', a saturated solution of sodium borate; and 'p.h.p.' a molar/20 solution of potassium hydrogen phthalate.

Extraction of visual pigments from the outer limbs

The washed outer limbs were stirred with freshly prepared 2% digitonin solution (Tansley, 1931) for 2 min and then centrifuged at 4000 rev/min for 20 min. The supernatant, containing any visual pigment released by the digitonin, was withdrawn into a clean tube. The residue was usually treated with a further portion of digitonin solution and the procedure repeated, the second supernatant being added to the first. The resulting extract was then brought to the desired pH by the addition of suitable buffer solution. Details of the extracts are given in Table 2.

Keeping qualities of the extracts

The extracts were stored in darkness in a refrigerator (about 3° C) and portions were withdrawn as required for experiments. The extracts were spun at 4000 rev/min on each occasion before sampling. Since only 0·4 ml. was required to fill an optical cell and since the experiments were often of long duration, each extract provided work over a considerable period.

The alkaline extracts showed a gradual increase in density on storage, most of which was due to an increase in light-scattering power. There was, however, little change in the amount of photosensitive material. Thus the density change at 520 mμ on complete bleaching of a sample of extract I, taken on the day of preparation, was 0·1244; for one drawn on the 2nd day, it was

0·1262 and for one drawn on the 4th day, 0·1263. The slight gain in photosensitive material (also evident in other extracts—see Table 4) was probably due to regeneration of pigment from small amounts of bleached products present in the freshly prepared solution, but could also have been due to the existence of a slight density gradient in the storage tube set up through prolonged centrifuging.

The behaviour of the only acid solution prepared was entirely different. There was a rapid initial loss of density due to settling out of a light precipitate, some hours after preparation, with consequent clearing of the originally opalescent solution. Also, a steady loss of photosensitive pigment occurred, amounting to about 15% in 6 days.

RESULTS

Absorption spectra of the extracts

Four extracts were prepared between September 1951 and September 1952. Extracts I, II and IV were made alkaline, while extract III was divided into two portions, one of which was made acid (III*a*) and the other alkaline (III*b*).

The absorption spectra of samples of each of these five solutions with respect to similarly buffered solutions of digitonin are shown in Fig. 1, in which the optical density is plotted against wave-length. The curves are of different heights (except those for extracts II and IV which, by chance, are identical) because of the varying concentrations of the solutions. In other respects, however, the curves are similar. Thus each has a maximum between 509 and 516 mμ and a minimum between 429 and 435 mμ.

TABLE 3. 'Purity' of extracts and wave-lengths of maximum absorption

Extract	pH	Ratio $D_{min.}/D_{max.}$	$\lambda_{max.}$ (mμ)
I	8·60	0·47	516
II	8·61	0·52	514
III*a*	4·55	0·68	509
III*b*	8·62	0·63	511
IV	8·65	0·52	514

All the solutions contained impurities which, being yellow in colour, absorbed light with increasing strength as the short wave-length extreme of the spectrum was approached. The different proportions of impurity to visual pigment in the extracts accounts for most of the variation in the wave-length positions of the maximum and minimum.

The 'purity' of each solution can be roughly assessed (cf. Collins & Morton, 1950) by the ratio of the optical density at the minimum (429–435) to that at the maximum (509–516 mμ). In Table 3 these ratios are tabulated, together with the wave-lengths of maximum absorption.

Solutions of frog visual purple, prepared in this laboratory by a method similar to that used in the present work, have had ratios varying between 0·38 and 0·47. That solution with the ratio 0·47 had an absorption maximum at 499–500 mμ, i.e. 2–4 mμ lower than the accepted maximum for visual purple (502–503 mμ). As Table 3 shows, the *Xenopus* extract which also had

this ratio absorbed maximally at 516 mμ. This suggests that, in the absence of impurities, the maximum would have been some 2–4 mμ higher, that is at 518–520 mμ.

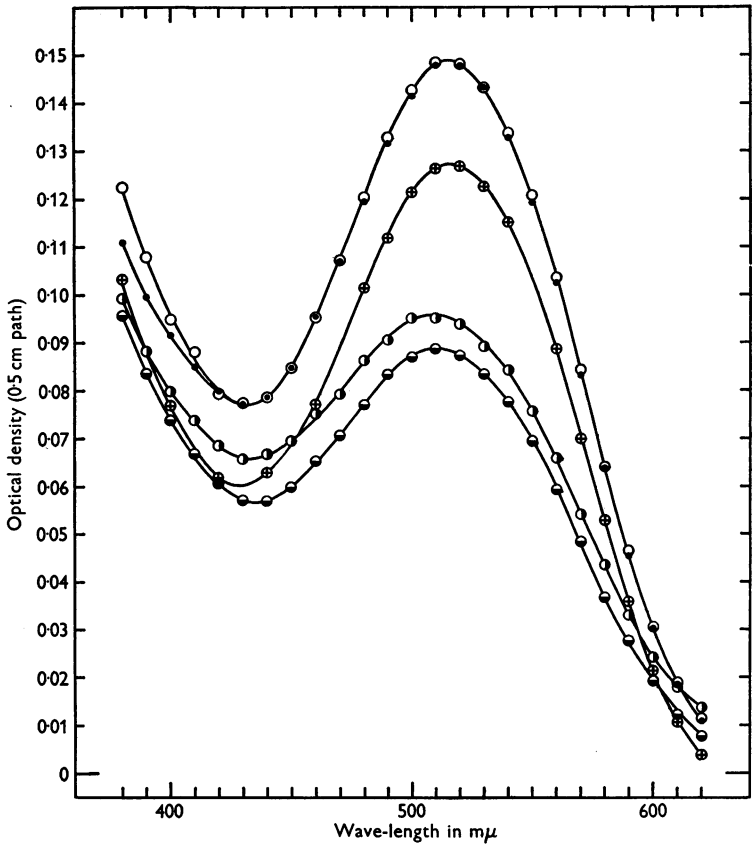

Fig. 1. Absorption spectra of the extracts at 20° C. ⊕, extract I, pH = 8·60; ●, extract II, pH = 8·61; ◑, extract III*a*, pH = 4·55; ◒, extract III*b*, pH = 8·62; ○, extract IV, pH = 8·65.

Behaviour of the extracts on exposure to light

Effect of pH

The behaviour of *Xenopus* extracts on exposure to light depends on pH. This is shown by the following experiments, for which purpose extract III was divided into two nearly equal portions; one was acidified (extract III*a*, pH 4·55) and the other made alkaline (extract III*b*, pH 8·62).

Behaviour in acid solution. About 17 hr after addition of the acid buffer, extract III*a*—which was originally opalescent—developed a precipitate and became clear. The following experiment was carried out on a sample of the extract after this clarification had occurred. The absorption spectrum before exposure to light is given by curve 1*a* of Fig. 2. Although the correspondence

between the outward and inward density measurements (empty and filled circles respectively) shows that the solution was not perceptibly changing during the course of the measurements, a slow loss of photosensitive pigment could have been detected over longer periods, and the 6-day-old sample used in the present experiment contained about 15% less pigment than originally.

When the measurements shown in curve 1*a* of Fig. 2 had been completed, the solution was exposed to a 15 W lamp for 5 min. The absorption spectrum after this exposure is given by the curves 2*a* of Fig. 2. As shown by the difference between the outward and inward measurements, the bleached solution was unstable, due to thermal fading of a substance absorbing maximally in the region of 450 mμ.

The solution was left undisturbed in darkness at 20° C overnight (17 hr) after which time measurements of its absorption spectrum (curve 3*a*, Fig. 2) showed that further major changes had taken place. To ascertain whether any regeneration of photosensitive pigment had occurred during this period, the solution was then exposed to the 15 W lamp for 5 min. However, this caused no measurable changes. Although curve 3*a* of Fig. 2 appears to indicate that after 17 hr the bleached solution had reached a stable condition, changes in absorption, too slow to show up in the 50–60 min period of measurement, were in fact still taking place. This was proved by subsequent measurements of the absorption spectrum after further prolonged periods in darkness. These measurements (not shown in Fig. 2) indicated that, even after 162 hr, changes were still occurring. During the period of 17–162 hr after bleaching there were slight losses of density at all wave-lengths greater than 391 mμ, the maximum loss (total = 0·022) being at 434 mμ. At wave-lengths less than 391 mμ (which was an isosbestic point for these later changes) there were corresponding slight increases of density. The later slow fading was thus different in nature from the original rapid fading.

This experiment shows that a succession of thermal (dark) reactions occur after exposure of an acid solution to light. These reactions are not fast enough to reach early completion, and not slow enough to be negligible. Consequently at no reasonable time is a satisfactorily stable condition reached. This, together with the fact that the visual pigment itself is not very stable under acid conditions, makes it impracticable to study the behaviour of such solutions by the present slow methods.

Behaviour in alkaline solution. The results of a similar experiment on the alkaline portion of the extract are shown by the curves 1*b*, 2*b* and 3*b* of Fig. 2. The absorption spectrum of the unexposed solution (curve 1*b*) was similar to that of the acid solution. On exposure to the 15 W lamp, however, the solution was bleached to a condition (curve 2*b*) which was photometrically similar to that of the well-faded acid solution and quite unlike that of the freshly bleached one. Moreover, the newly bleached alkaline solution was

stable, apart from slight changes which occurred in the violet region of the spectrum during the course of measurement. This stable condition, reached in alkaline solution so soon after exposure, was maintained for long periods. Thus after 17 hr in darkness at 20° C the absorption spectrum (curve 3*b*) had only slightly changed.

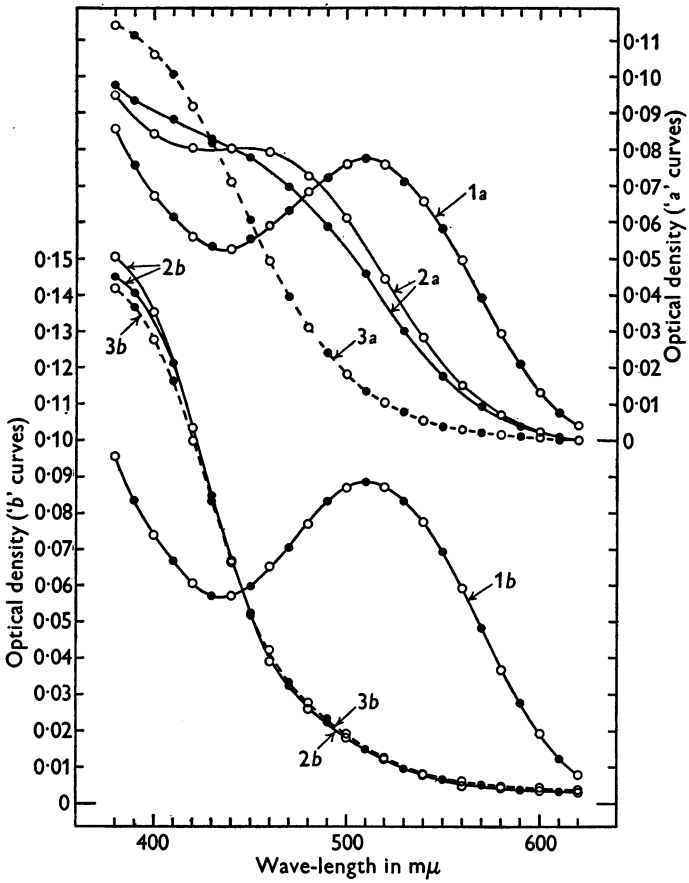

Fig. 2. The different behaviour of acid and alkaline extracts on bleaching. The '*a*' curves refer to an acid solution (extract III*a*, pH = 4·55); the '*b*' curves to an alkaline one (extract III*b*, pH = 8·62). Curves 1, initial absorption spectra; curves 2, after 10 min exposure to a 15 W lamp; curves 3, after 17 hr in darkness. ○, measurements made consecutively from 380 to 620 mμ; ●, return measurements from 610 to 390 mμ. Temperature, 20° C throughout.

In this experiment the solution had been exposed to the 15 W lamp for 10 min and a further period of 5 min or so had elapsed before observations on the bleached solution were begun. In another experiment (this time using a portion of extract II) the interval between exposure and measurement was reduced by using a 100 W bleaching lamp and cutting down the exposure time

to 1 min. Immediately afterwards a few density measurements were made as quickly as possible. These indicated that rapid thermal changes were taking place. When all changes had ceased the full absorption spectrum of the bleached solution was measured. Comparison of the data obtained at various times for the bleached solution with the original absorption spectrum showed that 86 % of the total change was achieved within 2 min and practically all within 11 min. Since, in this work, all other exposures were of at least 5 min duration (and often much longer) and a further minimum period of 5 min elapsed before observations on the bleached solution were begun, these transient changes normally passed unnoticed (cf. the transient orange stage (Lythgoe, 1937; Lythgoe & Quilliam, 1938) in the bleaching of visual purple solutions).

Partial bleaching of alkaline solutions

In addition to measurements (such as those just described) of the absorption spectra of solutions before and after bleaching to completion with white light, alkaline solutions were also investigated by the method of partial bleaching. The solutions were exposed to narrow spectral bands of light from a monochromator for times sufficient to bleach only a portion of the total photosensitive material present. After their absorption spectra were measured the partially bleached solutions could then be re-exposed to the spectral light for further bleaching. Finally, bleaching was completed by white light.

Some of the results obtained are shown in Fig. 3. Curves 1–4 (Fig. 3) give, respectively, the absorption spectra of a sample of extract IV—before bleaching, after an hour's exposure to light of dominant wave-length 580 mμ, after a further hour's exposure to the same yellow light and finally after exposure to white light (15 W lamp) for 10 min. Curves 5–8 (Fig. 3) show a similar sequence obtained with a sample of extract III*b* and give, respectively, the absorption spectra before exposure, after 1½ hr exposure to light of dominant wave-length 480 mμ, after a further 1½ hr exposure to the same blue light (but roughly twice the previous intensity) and finally after 5 min exposure to white light.

As shown by the correspondence between the outward and inward measurements (empty and filled circles) in all curves, the solutions were thermally stable, at whatever time the bleaching was interrupted. Because of this, and the great stability of the visual pigment itself, alkaline solutions are suitable for detailed study since they can be bleached by instalments with the knowledge that the bleached moiety produced by previous exposures will remain thermally stable during a further exposure.

Difference spectra of alkaline solutions

Difference spectra obtained by bleaching to completion

Since alkaline solutions were stable, both before and after bleaching to completion with white light, the changes in absorption obtained by sub-

tracting the curve for the bleached solution (e.g. curve 2*b*, Fig. 2) from that for the unbleached (curve 1*b*) are reproducible functions. Such difference spectra are independent of stable impurities since these have contributed equally to the absorption both before and after exposure to light.

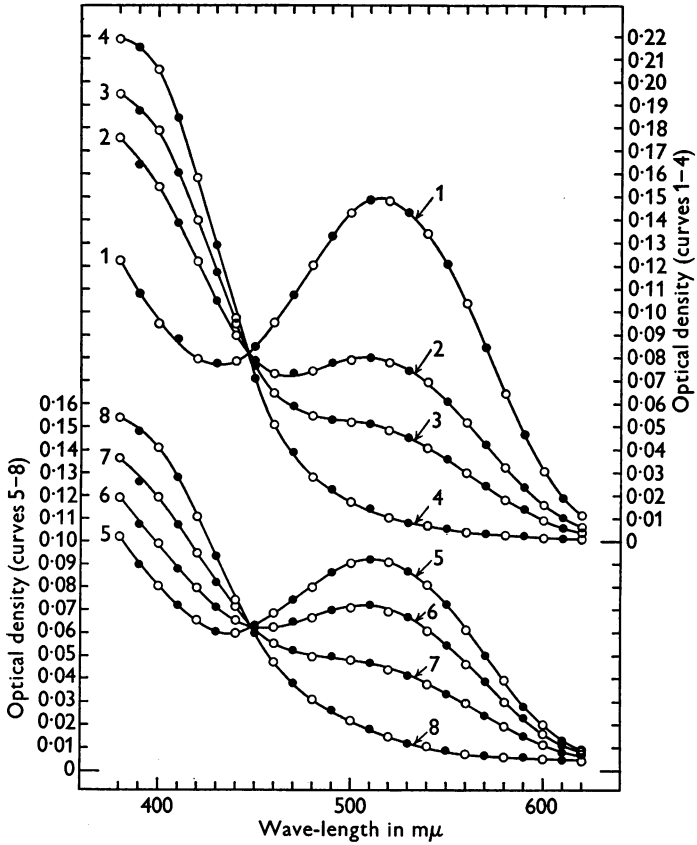

Fig. 3. The partial bleaching of alkaline solutions. Curve 1, original absorption spectrum of a sample of extract IV (pH 8·65); curve 2, after exposure to yellow light (580 mμ); curve 3, after further exposure to yellow light; curve 4, after exposure to 'white' light; curve 5, original absorption spectrum of a sample of extract III*b* (pH 8·62); curve 6, after exposure to blue light (480 mμ); curve 7, after further exposure to blue light; curve 8, after exposure to 'white' light. ○, measurements made consecutively from 380 to 620 mμ; ●, return measurements from 610 to 390 mμ. Temperature, 20° C.

When plotted as a percentage of its maximum, the alkaline difference spectrum thus gives us a means of precise comparison between different extracts. Three difference spectra, obtained in this way by total bleaching of samples of extracts I–III, are plotted in Fig. 4*A*. Within experimental error these difference spectra are identical. This shows that, over the whole period

of the investigation, the photosensitive components of the extracts were strictly reproducible. This conclusion could not have been reached from a study of absorption spectra alone (Fig. 1), in view of the varying 'purities' and degrees of opalescence of the extracts.

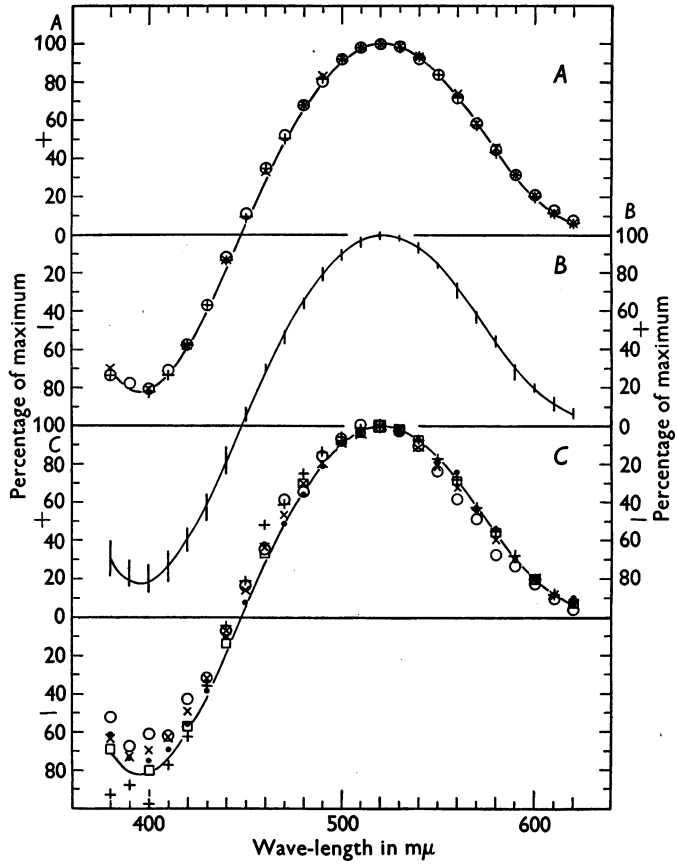

Fig. 4. The 'alkaline' difference spectra (max. = 100). *A*, difference spectra obtained on complete bleaching by white light (×, extract I; ○, extract II; +, extract III*b*). *B*, difference spectra obtained on partial bleaching by lights of dominant wave-lengths 650, 630, 580, 530, 480 and 430 mμ. The vertical lines show the range of results. *C*, difference spectra obtained on exposing, to white light, solutions which had been partly bleached by previous exposures, as follows: □, to 650 mμ; ○, to 630 mμ; ×, to 580 mμ; ●, to 480 mμ; +, to 430 mμ. The continuous line through each group of results is the mean of the *B* results. Temperature, 20° C, pH 8·60–8·65.

Difference spectra obtained by partial bleaching

In the case of solutions bleached in instalments by a series of exposures to light (Fig. 3), difference spectra can be similarly constructed by subtracting the absorption spectrum for one stage from that for the preceding stage. The

value of this procedure is that it enables us to decide whether a pigment is homogeneous or whether it is a mixture of two or more others. For instance the *Xenopus* pigment, as indicated by the difference spectra of Fig. 4*A*, is certainly neither pure visual purple nor pure visual violet, for its difference spectrum lies about halfway between those found for these two well-known pigments. But the results of Fig. 4*A* might perhaps arise from a mixture of visual purple and visual violet in about equal proportions. If this were the case *partial* bleaching with yellow light would affect chiefly the visual violet, whereas partial bleaching with blue would affect chiefly the visual purple. Clearly the difference spectra in the two cases would not be at all alike: one would be mainly that of visual violet; the other of visual purple. If, on the contrary, we have to deal not with a mixture but with a homogeneous pigment, then the difference spectra in the two cases will be identical when scaled to the same maximum.

The answer has already been provided in Fig. 3, where curves 2 and 3 show the effect of bleaching by yellow light (580 mμ), curves 6 and 7, the effect—on another solution—of blue light (480 mμ), and curves 4 and 8 the results of the final exposures to white light in the two cases. The changes from stage to stage appear to be the same whether yellow, blue or white bleaching lights are used, and the constant isosbestic point throughout confirms this. These, and all the other partial bleaching results, are displayed more clearly in Fig. 4*B*, *C* as difference spectra scaled to the same maximum.

The eight difference spectra obtained by monochromatic-light bleaching (dominant wave-lengths 650, 630, 580, 530, 480 and 430 mμ) lay within the narrow ranges indicated by the vertical lines in Fig. 4*B*: the five difference spectra obtained by the final exposures to white light are separately shown in Fig. 4*C*. All results are substantially the same, from which it can be concluded that the *Xenopus* pigment is not a mixture of visual purple and visual violet, nor, indeed (in a gross sense) a mixture at all, but a single, new pigment with absorption maximum at about 520 mμ.

Variations in the difference spectra

The evidence for the homogeneity of the *Xenopus* pigment, as shown by the small scatter of the results in Fig. 4*B*, *C*, is so good that it might be thought out of the question to extract further information by analysing such slight differences as are found. However, these differences, though small, are outside the limits of experimental error; they bear a consistent and expected relationship to the bleaching light employed and they lead to some unification with other work.

Errors of measurement. The standard error of a density determination was about 0·0005 log unit (Dartnall, 1952). Thus when one set of density measurements (constituting an absorption spectrum at one stage of the bleaching)

is subtracted from another, each member of the resulting set of differences has a standard error $\sqrt{2}$ times as great, namely 0·0007. For the sake of comparison all the difference spectra in Fig. 4 were plotted with their maxima = 100, although their actual maxima ranged from 0·022 to 0·138 (Table 4). Consequently, the standard errors of these curves vary from about 3 to 0·5%.

TABLE 4. Details of the experiments

Extract*	Age of sample (days)	Bleaching conditions: Dominant wave-length†	Duration	Method‡	Optical density at 520 mμ: Before exposure	After exposure	Density loss at 520 mμ	% bleached
I	0	15 W lamp	10 min	A	0·1268	0·0024	0·1244	100
I	1	630 mμ	2 hr	B	—	—	0·0975 } 0·1262	77
		15 W lamp	20 min		—	—	0·0287 }	23
I	3	650 mμ	2 hr	A	0·1381	0·1115	0·0266 } 0·1263	21
		15 W lamp	20 min		0·1115	0·0118	0·0997 }	79
II	2	15 W lamp	20 min	A	0·1477	0·0093	0·1384	100
II	5	100 W lamp	1 min	A	0·1496	0·0167	0·1329	96§
II	6	530 mμ	1 hr	A	0·1503	0·0190	0·1313	95§
III*a*	0	15 W lamp	10 min	A	0·0938	—‖	—	100
III*a*	6	15 W lamp	10 min	A	0·0757	—‖	—	100
III*b*	0	15 W lamp	10 min	A	0·0871	0·0123	0·0748	100
III*b*	14	480 mμ	1½ hr	A	0·0906	0·0689	0·0217 }	28·5
		480 mμ	1½ hr		0·0689	0·0435	0·0254 } 0·0758	33·5
		15 W lamp	5 min		0·0435	0·0148	0·0287 }	38
IV	1	580 mμ	1 hr	A	0·1481	0·0777	0·0704 }	51
		580 mμ	1 hr		0·0777	0·0481	0·0296 } 0·1380	21
		15 W lamp	10 min		0·0481	0·0101	0·0380 }	28
IV	2	430 mμ	17 hr	B	—	—	0·1055 } 0·1453	73
	3	15 W lamp	10 min		—	—	0·0398 }	27

* Details of the extracts, viz. dates of preparation and pH, are shown in Table 2.

† The band widths of the spectral lights, and the stray-light filters used are given in Table 1.

‡ In method A the absorption spectra of the sample before and after bleaching were measured with respect to a similarly buffered digitonin solution; in method B, the changes in absorption of the sample were measured directly by using an identical, but unexposed, sample as the reference solution.

§ Calculated on the assumption that the total possible density loss at 520 mμ was 0·1384.

‖ Bleached acid solutions were unstable (cf. Fig. 2, '*a*' curves).

Results. When the individual difference spectra in Fig. 4*B* were examined with these differing errors in mind, significant deviations were found in two instances. These were for the bleachings with lights of wave-lengths 630 and 580 mμ, and showed very slightly higher values on the long wave-length side of the maximum than those of the mean curve.

These deviations are more clearly seen—though in a reverse sense—in the corresponding curves '○' and '×' of Fig. 4*C*. Thus, considering values to the right only of the maximum, the points '○' and, to a lesser extent, '×' fall consistently *below* the others. This would appear to confirm that a pigment with maximum in the yellow (*c.* 570 mμ) had been preferentially removed by

the preliminary bleachings (with 630 mμ in the one case and with 580 mμ in the other). Since the various results in Fig. 4 are differently scaled (see Table 4 for actual density changes at the maximum) quantitative aspects cannot here be urged and will not be developed. Plotting the curves with a common maximum of 100 (necessary for the basic comparison) also entails that all differences between curves—differences which might arise through the presence or absence of minor amounts of other pigments—vanish at or near 520 mμ. All that can be claimed is that variations in certain of the difference spectra provide some evidence that the solutions contained a small amount of a yellow-absorbing photo-labile pigment in addition to the main pigment.

With regard to the points to the left of the maximum in Fig. 4*C* there are even greater divergences, again of a consistent kind. But the interpretation is decidedly more difficult. Hubbard & Wald (1952) have shown that when a solution of frog's visual purple, which had been bleached to completion with orange light, was exposed to white light, density losses occurred, maximally at 410 mμ. This additional change was found to be due to the isomerization, in white light, of the photo-products from the orange bleach. An apparently similar phenomenon has been observed with visual pigment 533 (visual violet from the pike) for if a retinal extract from this fish is first bleached to completion with long wave-length light and then exposed to white light, small additional density losses occur, maximally at 420 mμ (Dartnall, unpublished).

If the photo-product of the main *Xenopus* pigment isomerized in this way, the difference spectra in Fig. 4*C*, obtained by bleaching with white light, might be expected to show some density change around 415 mμ from this cause. It seems unlikely, however, that this could account for all the variations exhibited by the results to the left of the maximum. But without further analysis it would be unsafe to attempt to separate the isomeric changes from those due to possible residual pigments.

Absorption maximum of the main pigment

The means of the results obtained by monochromatic-light bleaching are described by the continuous curve in Fig. 4*B*. The positive maximum of this curve is clearly at about 520 mμ, but it can be more precisely located (to ± 1 mμ) in the following way. The mean results in the range 490–550 mμ can be accurately described by the function

$$y = Ax^2 + Bx + C,$$

where y and x are ordinate and abscissa values, $A = -0{\cdot}01985$, $B = 20{\cdot}69593$ and $C = -5294{\cdot}5$. This function has a maximum ($dy/dx = 0$), where $x = -B/2A$, i.e., at 521 mμ.

Under similar alkaline conditions the difference spectrum of visual pigment 502 (frog visual purple or rhodopsin) has a maximum at 504 mμ, that is, at

2 mμ higher than for the visual pigment itself. If we may assume a like displacement in the present instance, then the indicated maximum of the *Xenopus* pigment is at 519 mμ.

The contributions of the yellow-sensitive pigment to the difference spectra would tend to make this figure somewhat on the high side. It is unlikely, however, that the error, so introduced, exceeds 2 mμ.

DISCUSSION

This investigation has shown that the visual pigment solutions, prepared from the retina of *Xenopus*, consist mainly of one photosensitive pigment having its absorption maximum at about 519 mμ. In addition, there appears to be present, though in much smaller amount, a second photosensitive pigment with maximum at about 570 mμ. Additional evidence for this second pigment is provided by quite a different consideration.

It has been observed (Dartnall, 1952, 1953) that the absorption curves of a number of visual pigments have practically the same shape when plotted upon an abscissa scale of light frequency (instead of the usual wave-length). If the curves are scaled to the same maximum they may be made to coincide with each other simply by sliding them horizontally along the frequency axis. It is interesting to see how well the *Xenopus* data fit this standard curve.

In Fig. 5 the absorption spectrum of the best *Xenopus* extract—best in the sense that it contained least of the blue-absorbing photostable impurities—is plotted as the filled circles. The continuous curve is the standard curve with its maximum at 519 mμ but now plotted against wave-length. To the left of the maximum, the whole-extract data lie above the standard curve, becoming increasingly discrepant (because of impurities) towards the short-wave extreme of the spectrum. To the right of the maximum, however, the extract data lie close to the standard curve but, nevertheless, slightly above it, the difference between them being maximal in the yellow.

This discrepancy to the right of the maximum is precisely of the sort we were led to expect by the partial bleaching experiments. The absorption due to the 570 pigment adds to that of the 519 pigment in this region. One would, therefore, expect when the 570 pigment had been largely removed from an extract by bleaching with light of 630 mμ, that subsequent white light bleaching would give the difference spectrum of pigment 519 relatively uncontaminated (circles, Fig. 4*C*). And since, to the right of the maximum, the difference spectrum is almost identical with the absorption spectrum (photoproducts absorbing very slightly here), we may obtain a better estimate of the absorption spectrum of the pure 519 pigment by simply replotting the circles of Fig. 4*C* to the right of the maximum. This has been done in Fig. 5 and the circles are seen to confirm the idea that the pigment 519, like other visual pigments, fits the standard curve.

The difference between the filled and empty circles in Fig. 5 is plotted as the dotted curve (double scaling for clearness) and gives a rough estimate of the absorption spectrum of the 570 pigment (subject to the remarks already made regarding subtraction of curves plotted with their maxima = 100). This estimate suggests that the maximum density of the 570 pigment is some 10% of that of the main pigment. It is not possible, however, to argue from this figure to the relative densities or distributions of the two pigments in the intact retina.

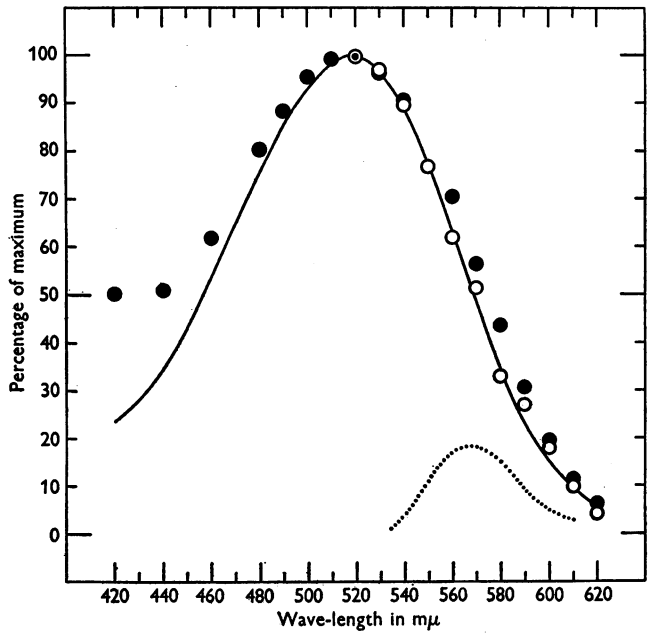

Fig. 5. Comparison of the absorption spectrum for the best extract with the calculated absorption spectrum for visual pigment 519. ●, the absorption spectrum (max. = 100) of extract I; ——, the calculated curve for visual pigment 519; ○, replotted from Fig. 4(*C*); ....., smooth curve through the differences between ● and ○ (×2).

Interpretation of the spectral sensitivity curve of Xenopus

The spectral sensitivity of *Xenopus* has been measured by two methods (Denton & Pirenne, 1951). In the first, the minimum intensities producing expansion of the skin melanophores were recorded: in the second, the intensities were measured below which the animal failed to go into the darker part of a tank, it being a characteristic of *Xenopus* to seek the shade.

The results, which were reported in energy units, have been recalculated in terms of quantum intensities, and are plotted in Fig. 6 as $\log_{10}$ relative sensitivity $\left(\text{sensitivity} \propto \frac{1}{\text{threshold intensity}}\right)$ against a frequency scale. This

method of presentation facilitates comparison with the absorption spectra of the visual pigments concerned.

The sensitivities obtained by the two methods, shown in Fig. 6 by the crosses and circles respectively, are in agreement. They show that the spectral sensitivity curve has an irregular shape, a prominent feature being the sharp maximum at 560–570 mμ.

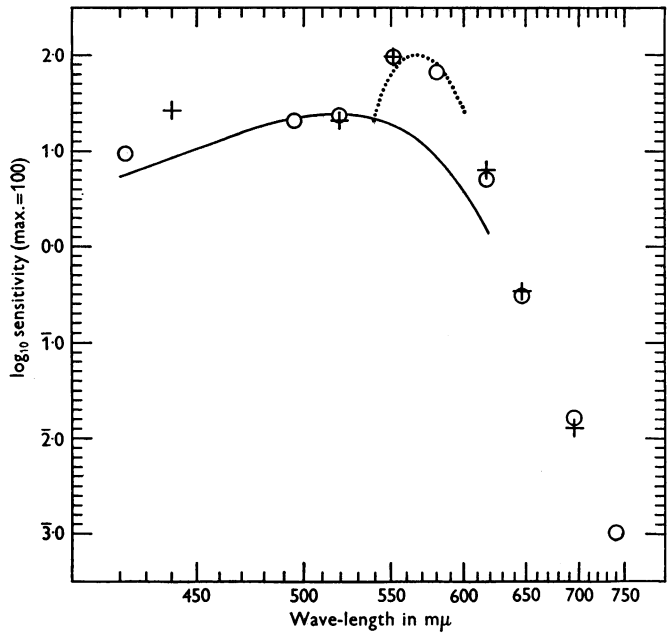

Fig. 6. Interpretation of the spectral sensitivity of *X. laevis*. +, ○, $\log_{10}$ relative sensitivity (max. = 100); +, from thresholds for expansion of skin melanophores; ○, from thresholds for the seeking of shade; ——, calculated absorption spectrum for visual pigment 519 (log. basis); ....., 'absorption spectrum' of the 570 pigment (log. basis) from Fig. 5. (Equal distances along the axis of abscissae are equal frequency intervals.)

When attempting to account for sensitivity curves in terms of absorption spectra it is, at present, impossible to make more than a shape comparison: although both sensitivity and absorption data are fixed with respect to the horizontal (wave-length) axis, there is no guide to their relative vertical placing. Normally—when a sensitivity curve is obviously mediated by a single pigment—this does not present any difficulty; the equating of maxima (for example) should result in complete coincidence of the two sets of data compared. In the present instance, however, the sensitivity data form a curve of unusual shape, not bearing any obvious relationship to the normally shaped absorption spectrum of visual pigment 519. However, visual pigment 519 is 'there' in the retinal extracts and it seems reasonable, by analogy with

extracts from other species, to suppose that the main pigment is the scotopic pigment.

If a curve to represent the absorption spectrum of the main pigment is moved upwards in Fig. 6, parallel to the vertical axis, it touches the sensitivity data first at 520 mμ. Drawn in this position (continuous line) it agrees with the sensitivity results only over the very short wave-length range of 490–520, though a reasonable extrapolation to long wave-lengths would again agree with the sensitivity data. In no other position could even this measure of agreement be obtained.

In the blue the sensitivity data lie above the 519 pigment curve. The real difference is probably greater than indicated in Fig. 6, for the sensitivity should be corrected for the absorption of light by the pre-retinal media. This difference suggests that the sensitivity is mediated in this region by a blue-absorbing pigment. No clear-cut evidence for such a pigment was obtained in the present experiments. Variations in the difference spectra were certainly observed at short wave-lengths (Fig. 4*B*, *C*), but it was not possible to interpret them with certainty because of other disturbing factors.

Even more marked than the 'blue hump' is the peak of sensitivity in the yellow. This peak is reasonably well fitted by the 'absorption spectrum' for pigment 570 (dotted curve of Fig. 5 drawn so that its maximum coincides with the peak of sensitivity in Fig. 6), suggesting that it may be, after all, a fair approximation to the true absorption spectrum for the yellow-sensitive pigment.

The fact that the *Xenopus* sensitivity curve appears to be mediated by more than one pigment raises interesting questions, one of which may be briefly stated. The *Xenopus* retina contains cones, as well as rods (K. Tansley, private communication). Presumably, therefore, *Xenopus*-vision can be photopic as well as scotopic. Do Denton & Pirenne's results (1951) refer to an exclusively scotopic condition? Although the intensities measured were thresholds, they were thresholds causing expansion of the skin melanophores, or causing the animal to seek the shade. Because of inertia, or for other reasons, such thresholds are bound to be higher than the purely visual ones. If the measured thresholds were very much higher than the absolute visual thresholds, it is possible—particularly since the responses required time to develop under the continuously applied stimuli—that the adaptation of the toads was raised to levels where photopic mechanisms came into play. By this argument visual pigment 519 could be a rod pigment, and pigment 570 a cone pigment. If, on the other hand, the animals were in a wholly scotopic condition then both pigments are presumably rod pigments.

Further work is required, both on the visual pigments and on the visual characteristics of *Xenopus*, before this and other questions can be answered.

SUMMARY

1. Photosensitive solutions have been obtained by treating the outer segments of the photoreceptors of the clawed toad, *Xenopus laevis*, with 2% aqueous digitonin. In alkaline conditions (pH = 8·6) the solutions were thermally stable—both before and after exposure to light. Consequently, the changes in optical density on bleaching (difference spectra) were reproducible.

2. Difference spectra were obtained in three ways: by complete bleaching in a single exposure to white light; by partial bleaching with spectral lights of dominant wave-lengths 650, 630, 580, 530, 480 and 430 mμ, and by exposing—to white light—solutions which had already been partly bleached by spectral lights of the wave-lengths mentioned.

3. In all cases the difference spectra obtained (when plotted with their maxima = 100), though not identical, were very similar. From this it is concluded that the photosensitivity of the solutions was due, almost entirely, to one pigment, having $\lambda_{max.} = 519$ mμ (visual pigment 519). This pigment has properties similar to those of other broad-band visual pigments.

4. Slight variations of the difference spectra in the 540–600 mμ region suggest that the solutions contained, in addition, a small amount of a yellow-sensitive, photo-labile pigment with an absorption maximum in the neighbourhood of 570 mμ. Slight variations were also observed in the short wave-length region but, because of other disturbing factors, could not be analysed.

5. An interpretation of Denton & Pirenne's (1951) spectral sensitivity curve of *X. laevis* has been attempted in the light of these results.

I wish to record my thanks to Dr E. J. Denton for the gift of the clawed toads used in this work, and to Dr W. A. H. Rushton for critically reading the original typescript and suggesting improvements to the presentation.

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