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A PHOTOSENSITIVE PIGMENT OF THE CARP RETINA

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Two visual pigments, rhodopsin (or visual purple) and porphyropsin (or visual violet) appear to have a widespread distribution. Thus Wald (1949), referring to rod pigments, writes: 'To summarize our present information, true land and sea vertebrates have the rhodopsin system; true fresh-water vertebrates have porphyropsin; while those equivocal forms which can distribute their lives between fresh water and one of the other environments frequently possess both photopigments, mixed or in temporal succession'.

These visual pigments are carotenoid-protein complexes. The above conclusion was reached mainly as the result of studies of the decomposition products of the carotenoid moieties (Wald, 1935*a*, *b*, 1936*a*, *b*, 1942). These products were found to be either vitamin A_1 or vitamin A_2, the former associated with rhodopsin and the latter with porphyropsin. The absorption spectra of the retinal extracts were found to fall into one of three groups; they had absorption maxima either around 500 mμ (rhodopsin group), or 520 mμ (porphyropsin group) or between the two (mixtures of rhodopsin and porphyropsin) (Wald, 1938, 1939, 1941).

However, the presence of differing amounts of yellow impurities in the extracts complicated the inter-species comparison of absorption spectra and wide limits were tolerated by Wald (1938, 1939) in assigning a visual pigment to the rhodopsin or porphyropsin groups. Furthermore, no tests of homogeneity were applied.

It has been shown (Dartnall, 1952*a*) that difference spectra—the changes in absorption spectra caused by bleaching—are highly reproducible under certain conditions and hence provide a means of precise comparison between extracts from different species. Moreover, the homogeneity of the extracts can be put to test by comparing the difference spectra obtained by bleaching with lights from different parts of the visible spectrum.

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This paper describes some experiments carried out on the light-sensitive visual pigment solutions obtained from a freshwater fish—the carp—and presents evidence that the visual pigment of this fish is different from that of other fish so far examined by these methods.

APPARATUS AND METHODS

Measurement of absorption spectra

Apparatus and technique for measuring the absorption spectra of light-sensitive solutions have been described before (Dartnall, 1952*a*) and were used in the present work.

The stability of the solutions during measurement was tested in the following way. Optical densities were measured at 20 mμ intervals from 380 to 620 mμ; then, sometimes after a short period of time, from 610 to 390 mμ, again at 20 mμ intervals. If the returning measurements retraced the path of the outward ones, this showed that the solution was stable since the outward and return measurements each took about 30 min to complete.

All absorption spectra, whether of unexposed, partly bleached or fully bleached solutions, were determined in this way. All measurements were made at 20·0±0·2° C and were for an optical path length of 0·5 cm.

Bleaching of solutions

Complete bleaching of the visual pigment solutions was achieved by exposing them for 10 min to 'white' light from a 15 W lamp, placed about 9 in. away.

Bleaching by instalments was carried out with the bleaching apparatus previously described (Dartnall, 1952*a*). Suitable intensities of the approximately monochromatic lights used were obtained by adjusting the widths of the monochromator slits. The band widths for each dominant wave-length of bleaching light, and the stray-light filters employed, were similar to those in previous work. During bleaching (which, at these low intensities, was often of some hours duration) the temperature of the solutions was controlled at 20° C.

Preparation of the visual pigment solutions

The fish (*Cyprinus carpio*, variety King Carp, length 7–8 in.) were kept in the open in a large bath of running water.

Before the preparation of an extract, five to ten carp were placed in running water in a tank from which all light could be excluded. The fish were kept in darkness for various times (Table 1). All procedures following this period of dark adaptation were carried out in a dark room in the general illumination of a deep red safe-light, as used in photography. Additional light, when necessary for operations such as the removal of retinae, was provided by a cycle torch lamp fitted with Ilford monochromatic filter no. 609. Unnecessary exposure of the retinae, or of photosensitive solutions, even to these relatively ineffective radiations, was avoided.

Removal of retinae

The carp were guillotined and the heads washed free from blood in running water. The tapering end of a centrifuge tube was then thrust deep into the gullet of the fish until, with some tearing of tissue, the end lay behind and between the eyes. In this position the tube supported the eyes against pressure applied in the subsequent operations. Also, by using the tube as a lever, either eye could be made to protrude. The point of the blade of a small pair of scissors was inserted into an eye at the corneal-scleral junction and a cut made right across the cornea. Gentle pressure, applied with a pair of forceps held with its limbs on the eyeball at either side of the cut, caused extrusion of the lens, which was then lifted away. With additional pressure, the retina appeared. This was gently eased from its optic nerve connexions and placed in the first washing solution. This procedure was then repeated on the other eye. The retinae were quite coherent and were nearly always obtained whole, as was shown when they billowed out into their original shape on being placed in the washing solution.

Extraction of the visual pigments

By treating retinae with 2% aqueous digitonin, the visual pigments can be obtained in solution (Tansley, 1931). However, digitonin also releases other light-absorbing substances present in the retina. Little can be done to purify an extract once made. By previously washing the retinae with certain reagents, however, some of these unwanted impurities can be removed before extraction. Alternatively, the outer limbs of the retinal end organs can be segregated and separately extracted. Both methods were used.

Method 1. *Extraction of whole, washed retinae.* The retinae, removed as described above, were placed in about 3 ml. of the washing solution—McIlvaine's pH 4·6 or pH 4·9 buffer solution—and gently stirred. The mixture was then centrifuged at 4000 rev/min for 20 min and the supernatant washings removed by a micropipette. The retinae were then washed again a variable number of times (see Table 1). In one instance the retinae were washed with frog's Ringer's solution (once only) instead of the acid buffer. In other instances as many as six acid washes were used.

TABLE 1. Extract preparation details

Extract	Date	No. of carp	Time in dark (hr)	No. of washings*	Vol. of extractant (ml.)†	Vol. of buffer added (ml.)‡	Total vol. of extract (ml.)	Density change at 525 mμ on bleaching§
			Method 1. Extraction of whole, washed retina					
I	28. xi. 52	10	71	6	2·0+2·0	0·4	4·4	0·13
II	16. xii. 52	10	20	6	1·0+1·0	0·2	2·2	0·23
III	3. i. 53	8	24	5	1·0+1·0	0·2	2·2	0·19
IVR	16. ii. 53	Retinal residues left by extract IV procedure		1	2·0	0·2	2·2	0·084
V	20. iv. 53	7	2½	2	1·0+0·5	0·15	1·65	0·19
VI	20. iv. 53	6	2½	1	1·0+0·5	0·15	1·65	0·22
			Method 2. Extraction of end organ outer segments					
IV	16. ii. 53	9	3	3‖	1·2+1·0	0·3	2·5	0·18
VII	4. v. 53	5	2½	1‖	0·7	0·07	0·77	0·058

* With McIlvaine's pH 4·6 or pH 4·9 buffer solution, except for extract VI when frog's Ringer's solution was used.
† 2% digitonin solution.
‡ Saturated sodium borate solution.
§ A measure of the concentration of photosensitive pigment.
‖ The washed retinae were then shaken with 35% or 40% sucrose solution, as described in the text, to separate the outer segments.

The first washings were white and cloudy (sometimes yellow because of blood pigments), the cloudiness becoming progressively less with each operation. Even after six washings the supernatants were never completely clear, but the prospect of improvement by a reasonable number of additional washings seemed slight.

After this treatment the retinae were extracted with a freshly prepared 2% aqueous solution of digitonin. After centrifuging, the supernatant, containing any visual pigment extracted by the digitonin, was withdrawn into a clean tube. The residue was then re-extracted with another portion of the digitonin solution and the procedure repeated, the second supernatant being added to the first. The combined supernatants were then brought to the desired alkalinity (pH 8·0–8·6) by the addition of one-tenth volume of saturated sodium borate solution.

Method 2. *Extraction of end organ outer segments.* In this method the retinae were first given a preliminary washing with acid buffer as before. This was removed and replaced by 2 ml. of a 35 or 40%, w/v, sucrose solution. The tube was then briskly shaken. This treatment caused the outer segments of the retinal end organs to break away from the retina. On spinning the mixture at 4000 rev/min the heavier retinal debris separated to the bottom, leaving the outer segments still

suspended in the supernatant fluid. This was pipetted off into a clean tube. A second but smaller yield was obtained by repeating the procedure with another 2 ml. portion of sucrose solution. Then, to each suspension, 4 ml. of pH 4·6 buffer solution were added. As a result of this dilution the outer segments were now heavier than the fluid medium and began to settle, a process which was speeded up by spinning at 4000 rev/min. The clear supernatants were withdrawn from each compacted sediment and discarded. The two yields of outer segments obtained were then washed with a little pH 4·6 buffer. After withdrawal of this final washing, the visual pigment was extracted with digitonin solution and made alkaline as before.

In one instance the retinal debris remaining after two such sucrose flotations was extracted with digitonin. The resulting solution (extract IVR) contained a considerable amount of photosensitive material, showing that not all the outer segments had been disengaged from the retina by two sucrose treatments.

RESULTS

Absorption spectra and storage stability of the extracts

The visual pigment solutions were prepared between November 1952 and May 1953; five of them (extracts I, II, III, V and VI) by extraction of washed whole retinae, two (extracts IV and VII) by extraction of isolated outer segments, and one (extract IVR) by extracting the denuded retinal debris of extract IV.

The extracts were stored in darkness in a refrigerator (about 3° C) and were spun at 4000 rev/min on each occasion before withdrawal of a sample for experiment.

Absorption spectra are shown in Fig. 1 (A–D) in which the optical density of typical extracts is plotted as a function of wave-length. Fig. 1A shows the absorption spectra of an extract (VI) made from whole retinae which had been washed only once before extraction. In addition to the broad maximum at 500–505 mμ, the absorption curves also possess a sharp maximum at 407 mμ. This is probably due to the presence of a haem pigment and resembles a similar peak found with extracts of inadequately washed tench retinae (Dartnall, 1950, 1952*a*). Another extract (extract V) in which the whole retinae were washed only twice before extraction also had this peak.

Typical of extracts of adequately washed whole retinae are the curves in Fig. 1B. This extract (I) was prepared from whole retinae which had been washed 6 times before extraction and has a single maximum close to 520 mμ. There is no trace of the 407 mμ peak and this was also the case for extracts II and III in which the retinae were washed 6 and 5 times respectively before extraction.

Fig. 1C shows absorption spectra of an extract (IV) made from isolated outer segments. The maximum is at 520 mμ in this case also, but the curves rise somewhat less steeply at the extreme short wave-length end of the spectrum indicating that the extract was slightly freer from yellow impurities. This small improvement is well demonstrated by comparison with Fig. 1D, which shows the curve for an extract (IVR) made from the segment-denuded debris of extract IV.

For the first few days after preparation, the extracts became clearer by precipitation of a colourless material which, in suspension, had caused them to be opalescent in varying degrees. This is illustrated in Fig. 1 by the absorption curves of samples of different age. Thus, considering Fig. 1 B there was

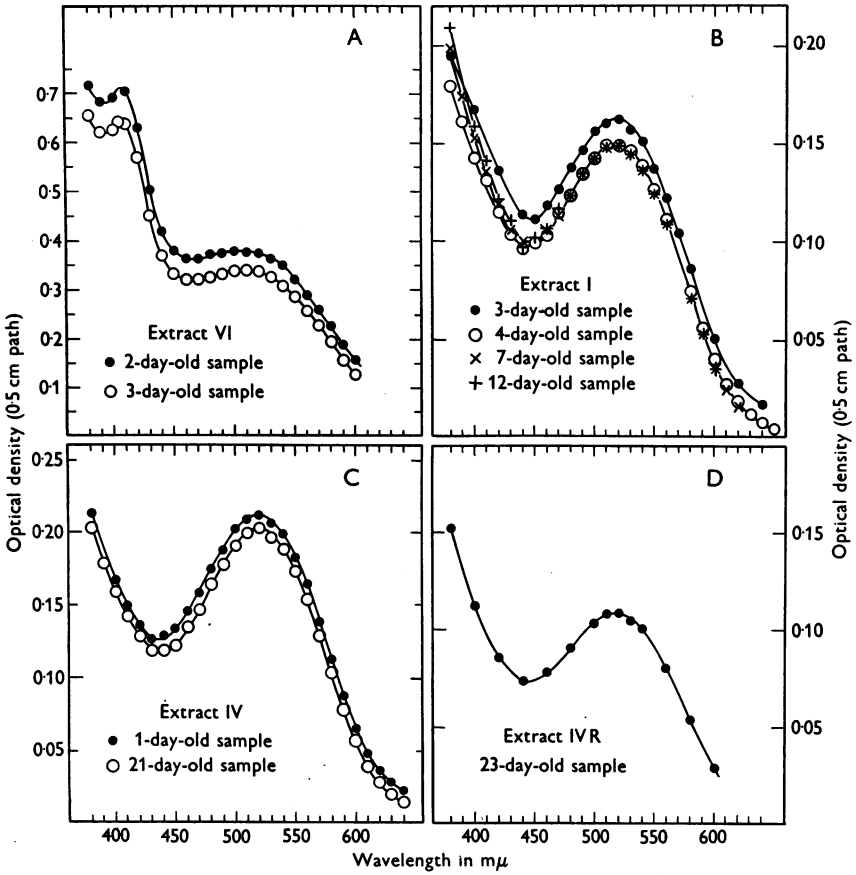

Fig. 1. Absorption spectra of representative digitonin extracts of: A, whole retinae after one washing; B, whole retinae after six washings; C, separated retinal outer limbs; D, retinal debris remaining after removal of outer limbs. pH 8–8·6, $T = 20°$ C.

a marked fall in density (approximately equal at all wave-lengths) up to the 4th day, but little change thereafter. Because of this phenomenon, which is new in our experience, the solutions were aged before use in the more critical or longer experiments. It was found that solutions aged at 3° C for as long as 35 days showed no measurable loss of photosensitive pigment and no apparent alteration in properties. These aged solutions had the essential advantage of absolute thermal stability for long periods.

Behaviour on exposure to light

Anomalous shape of the carp difference spectrum

In the upper half of Fig. 2 (redrawn from Dartnall, 1952*a*) are shown the absorption spectra, before and after total bleaching, of an extract of pike retinae. The difference between the unbleached and bleached curves, gives the

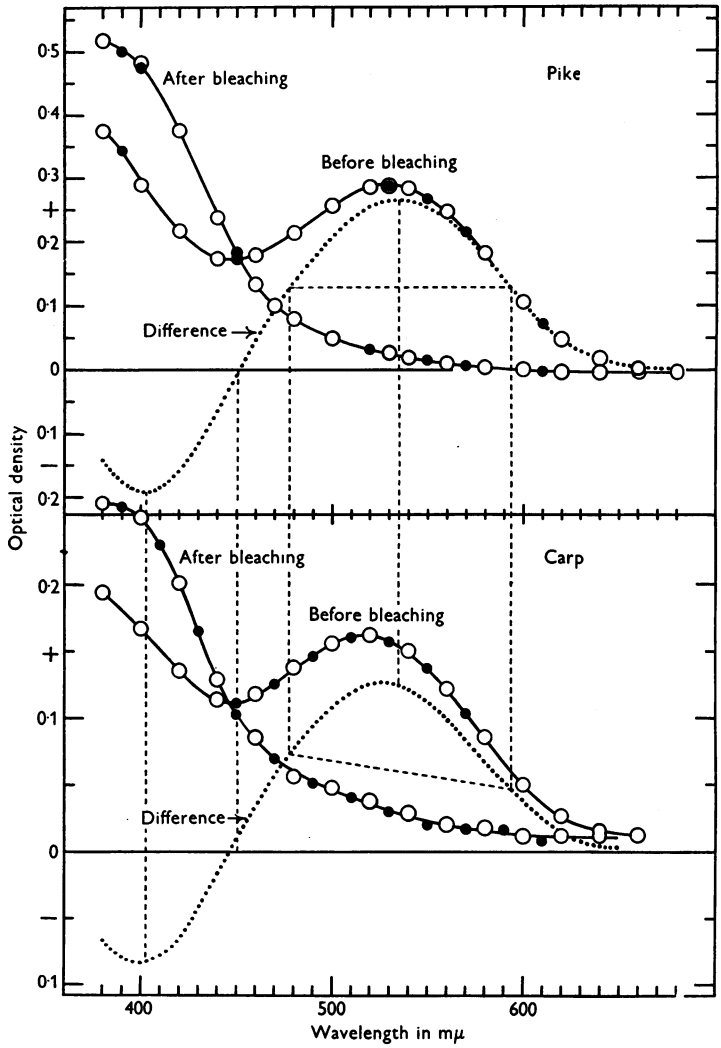

Fig. 2. Comparison of the absorption spectra of pike and carp extracts, before and after bleaching by white light. The interrupted lines are to facilitate comparison of the two difference spectra (shown by the dotted curves). ○, measurements made consecutively from 380 to 680 mμ; ●, return measurements from 610 to 390 mμ. Pike extract, pH = 8·24, T = 20° C (after Dartnall, 1952*a*); carp extract I, pH = 8·16, T = 20° C.

changes in density on bleaching and is not complicated by the presence of stable impurities since these contribute equally to the absorption both before and after bleaching. In this instance the difference spectrum shows that, on bleaching, density is lost maximally at 535 mμ and gained maximally at 405 mμ (owing to the formation of a photoproduct). Such behaviour is generally accepted as typical of porphyropsin (visual violet) (Wald, 1939). The pike retinal extract was shown by Dartnall to contain only one photosensitive pigment. The same pigment was also found in tench and bleak (Dartnall, 1952*a*, 1952*b*).

In the lower half of Fig. 2 are plotted the results of a precisely similar experiment but with an extract of carp retinae. In this case the difference spectrum has a maximum at 525 mμ and is displaced towards shorter wave-lengths by comparison with that for the pike.

Since both extracts were stable, both before and after bleaching, these differences cannot be ascribed to thermally unstable impurities. In view of the identical experimental conditions there seem only two likely reasons for the discrepancy. The carp extract might contain, in addition to visual violet, another light-sensitive component with maximum absorption at short wave-lengths. These two pigments bleaching together could then yield a displaced difference spectrum. The alternative explanation is that the carp difference spectrum is due to a single pigment with properties different from those of 'normal' visual violet.

Behaviour on partial bleaching

The homogeneity of the carp extracts was tested by the method of partial bleaching (Dartnall, 1952*a*, *b*, 1954). In this method the photosensitive solutions are bleached in instalments by exposing them to monochromatic lights for times (found by trial) to be sufficient to bleach only a fraction of the total photosensitive material present. The process can then be repeated by a second exposure and so on. Measurements of the absorption spectra are made initially and also at every stage until the solution is fully bleached. By studying the effect of lights of different spectral locations it is then possible to decide whether or not the photosensitive component is homogeneous.

One such experiment is illustrated in Fig. 3A. Curve 1 shows the original absorption spectrum of an unexposed solution (a sample of extract IV). After 3 hr exposure to violet light (430 mμ) the absorption changed to curve 2. At this stage in the experiment the solution was left undisturbed in darkness overnight (18 hr), but this resulted in no significant changes. The solution was then exposed to red light (630 mμ) for 1 hr which caused bleaching to curve 3. This was followed by a 1 hr exposure to green light (530 mμ) which caused further bleaching to curve 4. The experiment was again interrupted at this point, the solution remaining in darkness overnight (22 hr). During this period

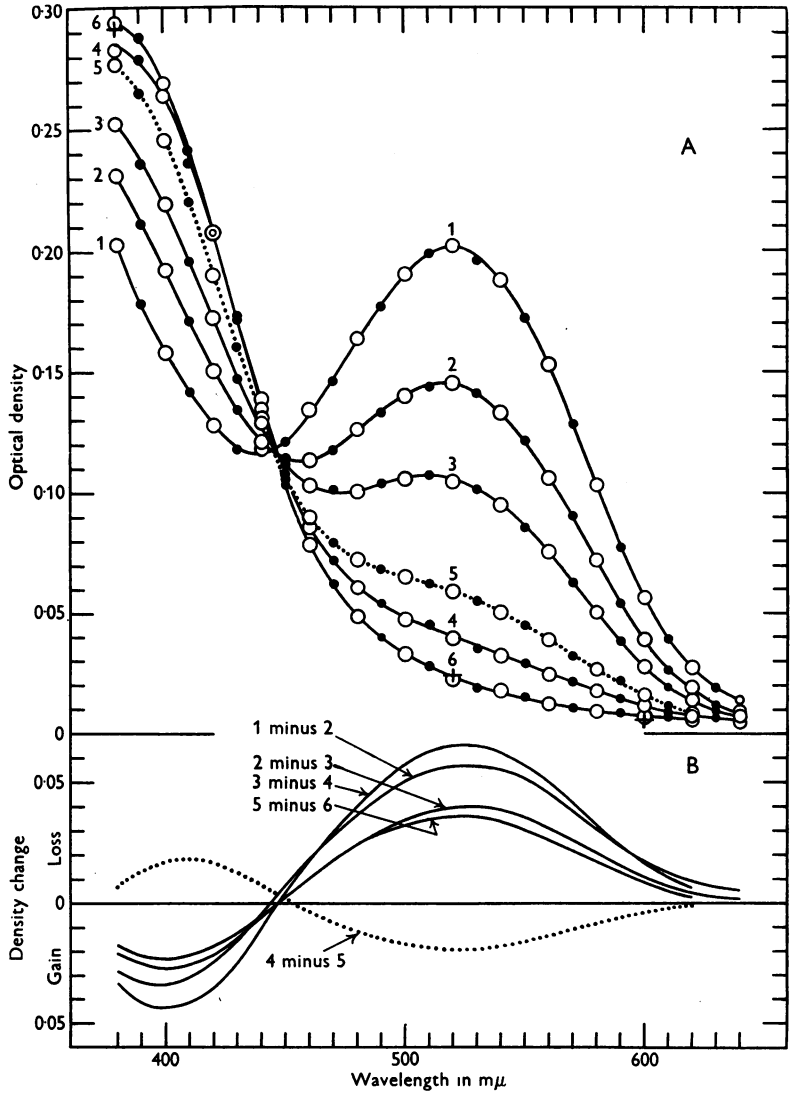

Fig. 3. A: a typical partial bleaching experiment (carp extract IV, pH = 8·24, $T = 20°$ C). Curve 1, original absorption spectrum; curve 2, after 3 hr. exposure to violet light (430 mμ)—unchanged after 18 hr in darkness; curve 3, after 1 hr further exposure to red light (630 mμ); curve 4, after 1 hr further exposure to green light (530 mμ); curve 5, after 22 hr in darkness; curve 6, after 10 min exposure to white light; +, observations made after a further 18 hr in darkness. ○, measurements made consecutively from 380 to 640 mμ; ●, return measurements from 630 to 390 mμ. B: difference spectra, formed from the data of A by subtracting the absorption spectrum at any stage from that for the preceding stage.

considerable regeneration of the photopigment took place (curve 5). Finally the solution was exposed to white light (15 W lamp) for 10 min to complete the bleaching (curve 6). The solution was then left in darkness overnight (18 hr). Optical density measurements on the following day at 380, 520 and 620 mμ (+in Fig. 3) then showed that no further changes had occurred.

Homogeneity of the carp pigment

Difference spectra can be constructed from the results of Fig. 3A by subtracting the absorption spectrum at any stage from that for the preceding stage. Such curves have an advantage over the difference spectrum obtained by complete bleaching in a single exposure. This latter (Fig. 2) merely showed that the carp extract is not the same as the pike extract. It did not enable a decision to be made whether or not this difference was due to the presence of a second pigment in the carp extract. By partial bleachings with suitably chosen monochromatic lights, however, it is possible to affect one pigment in a mixture more than another with consequent variation in the difference spectra. If there is no variation—in spite of changes in the wave-length of light used for bleaching—then this is strong evidence that the pigment is truly homogeneous.

The answer is in part provided by Fig. 3B which shows the various difference spectra constructed from the results of Fig. 3A. These difference spectra form a family of similarly shaped curves.

A large number of experiments of the type shown in Fig. 3 were carried out. A few results with the less pure extracts had to be discarded because of concurrent changes in impurities during the bleaching, but satisfactory difference spectra by *partial* bleachings were obtained in twenty-one cases; eight with red light (630 or 640 mμ), one with green (530 mμ), one with blue (480 mμ), four with violet (430 mμ) and seven with white—these last being the results of a final exposure after previous bleachings with monochromatic lights. Each of these difference spectra was plotted as a percentage of its maximum. The average percentage difference spectrum for each of the red, violet and white groups is shown in Fig. 4. The single results from the green and blue bleachings were similar. From the similarity of these difference spectra it is concluded that the carp extracts were homogeneous, that is they contained only one photosensitive substance.

Comparison of the difference spectra of the carp pigment and of visual pigment 533

Dartnall's values (mean of three determinations) for the difference spectrum of visual pigment 533 found in pike are shown by the vertical crosses in Fig. 5. The same pigment was also found by Dartnall (1952*a*) in the tench, but in this case, unlike the pike, a second photosensitive substance, visual pigment 467,

was also present. To avoid affecting this second pigment, Dartnall bleached the tench solutions with light of long wave-length. The difference spectrum obtained, shown in Fig. 5 by the oblique crosses (mean of five determinations), was in precise agreement with the pike data.

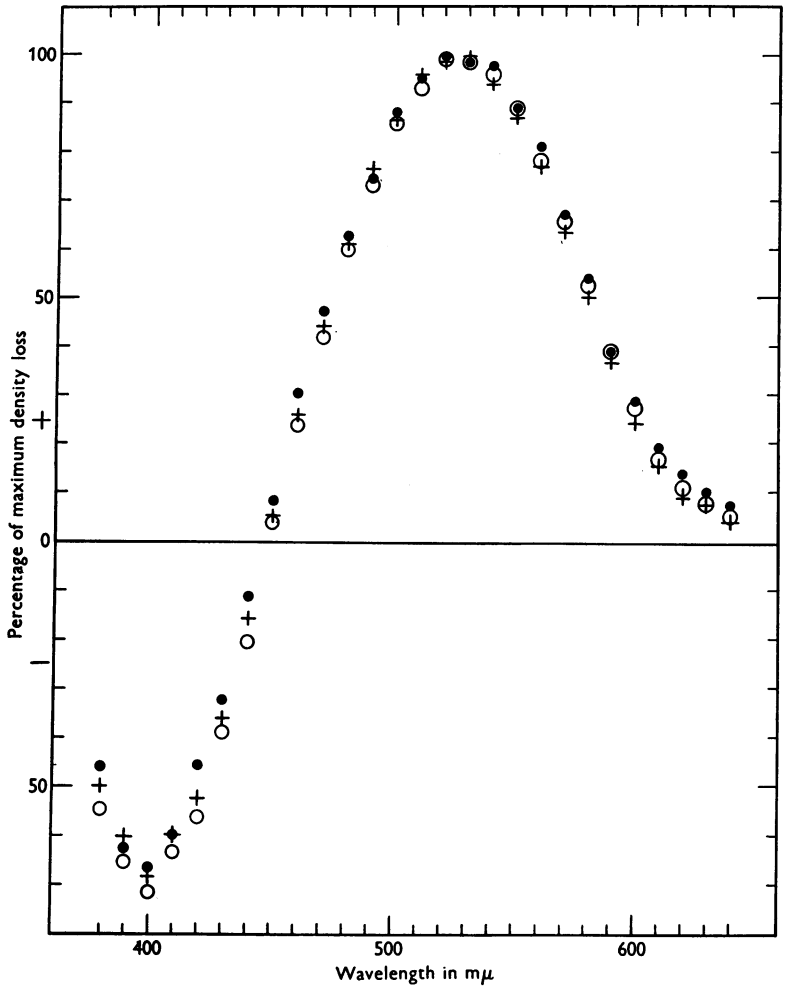

Fig. 4. Difference spectra of the carp pigment (pH 8–8·6, $T = 20°$ C). ○, mean of eight difference spectra obtained by partial bleachings with red light (630 or 640 mμ); ●, mean of four difference spectra obtained by partial bleachings with violet light (430 mμ); +, mean of seven difference spectra obtained by terminal bleachings with white light (following previous exposures to monochromatic lights of various wave-lengths).

For comparison, the carp difference spectrum (mean of twenty-one determinations) obtained in the present investigation is shown by the filled circles. This is quite distinct from that of pike and tench in having a maximum at

525 mμ. The reality of this difference was confirmed in the present investigation by preparing a digitonin extract from the outer limbs of the retinal end

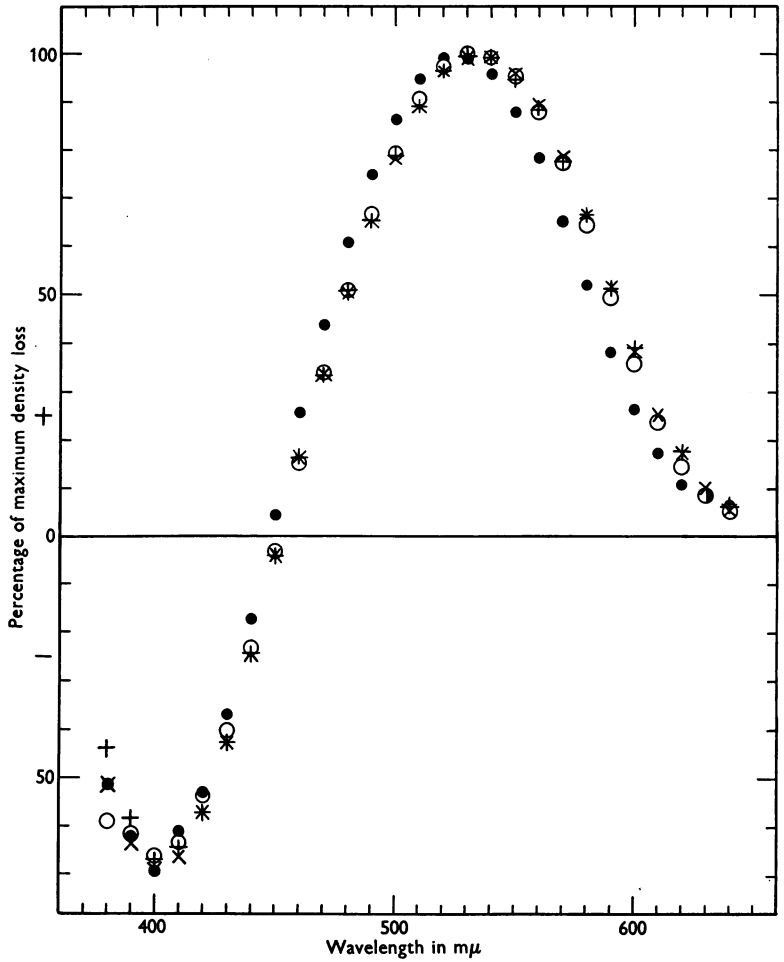

Fig. 5. Comparison of the difference spectrum of the carp pigment with that of visual pigment 533. ●, difference spectrum of carp pigment, pH 8–8·6 (present investigation, mean of twenty-one determinations). Difference spectra of visual pigment 533: ○, tench extract, pH 8·15 (single determination, present investigation); ×, tench extracts, mean pH 8·6 (mean of five determinations, Dartnall, 1952*a*); +, pike extract, pH 8·24 (mean of three determinations, Dartnall, 1952*a*). All measurements at 20° C.

organs of tench. On bleaching this extract with red light (640 mμ) the difference spectrum, shown in Fig. 5 by the empty circles, was obtained. This has a maximum at 535 mμ and is in agreement with Dartnall's measurements.

Effect of prolonged exposure to light

In all cases so far considered the times of exposure to the bleaching lights were relatively short—never more than 3 hr. The results obtained under these conditions were all substantially the same (Fig. 4), though minor differences were observed—particularly at short wave-lengths—between those curves obtained by bleaching with violet light and those by bleaching with light of longer wave-lengths. It seemed probable that these differences might be due to an action of short wave-length light on the product of bleaching of the carp pigment. Accordingly, to give this full play it was decided to investigate the effect of prolonged exposure to light.

For this purpose roughly equal parts of extracts IV and IVR were mixed together to ensure a sufficient supply of constant material. The resulting solution was thermally stable, an essential requirement for lengthy experiments.

After measuring the absorption spectrum of a sample of the solution it was exhaustively bleached (95% of possible density loss at 525 mμ) by exposure to violet light (430 mμ) for 21 hr. The changes caused are shown (max. = 100) by the circles in Fig. 6A. The solution was then exposed to red light (630 mμ) for 18 hr. Its absorption spectrum was then measured once more. The total change after these treatments (crosses in Fig. 6A) shows that the second exposure was without effect on the difference spectrum.

In the second experiment (with another sample) the order of exposures was reversed, the exhaustive (100%) bleaching with red light coming first, followed by 18 hr exposure to violet light. In this case the second exposure resulted in small density losses confined to short wave-length regions and maximal at 400–420 mμ. Because of this the difference spectra in the two cases were not quite the same, that obtained by the first exposure being shown as crosses and that by the total exposures as circles in Fig. 6B.

In the third experiment a further sample was exhaustively bleached (99%) by 2 hr exposure to green light (530 mμ). This yielded the difference spectrum shown by the crosses in Fig. 6C. The solution was then exposed to violet light for 18 hr. This caused further small changes, again maximal at 400–420 mμ, the difference spectrum pertaining to the combined exposures being shown by the circles in Fig. 6C.

The possibility that these differences could be due not to the wave-length of the bleaching light but solely to thermal changes in the solutions over long periods was eliminated by further experiments. These showed that the changes occurring on exposure to light of short wave-length were not obtained when a solution (extract VII) which had been exhaustively bleached with red light was merely kept in darkness for 20 hr. They did occur, however, as the result of only 15 min subsequent exposure to a mercury vapour lamp.

Interpretation. A possible interpretation of the additional density losses

caused by exposure to violet light is that the solutions contained a small amount of another photosensitive pigment having an absorption maximum in the violet and thus insensitive to all but violet light. A more likely explanation,

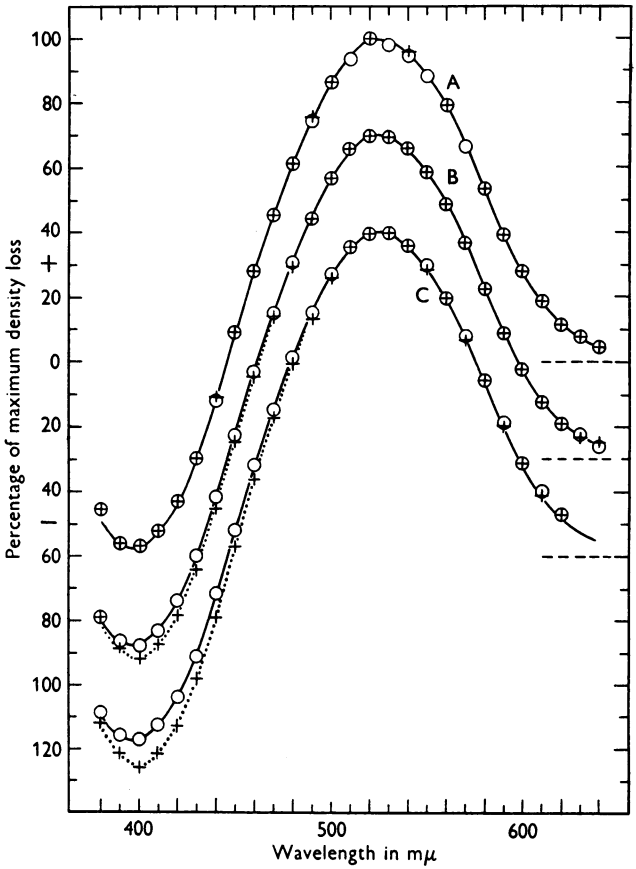

Fig. 6. Dependence of the negative portion of the carp difference spectrum on the wave-length of the bleaching light. Extracts IV and IVR mixed, pH 8·2, $T = 20°$ C. A: ○, changes caused by 21 hr exposure to violet light (430 mμ); +, total changes caused by this and by an additional 18 hr exposure to red light (630 mμ). B: +, changes caused by 19 hr exposure to red light (630 mμ); ○, total changes caused by this and by an additional 18 hr exposure to violet light (430 mμ), data displaced 30 ordinate units from A. C: +, changes caused by 2 hr exposure to green light (530 mμ); ○, total changes caused by this and by an additional 18 hr exposure to violet light (430 mμ). Data displaced 60 ordinate units from A. The full lines which pass through the ○ measurements are the same curve in all three cases.

however, is that violet light affects the product of bleaching of the carp pigment. Hubbard & Wald (1952) observed a similar phenomenon with frog visual purple (rhodopsin). When a visual purple solution, which had been bleached to completion with orange light, was exposed to white light, density

losses occurred, maximally at 410 mμ. Hubbard & Wald consider that these losses were due to an isomerizing effect of short wave-length light on the photo-product of visual purple.

Regeneration

A notable feature of the present experiments was the very marked regeneration which sometimes occurred. Regeneration after bleaching—when it happened—took place spontaneously without the addition of supplementary materials. Although a detailed study of this phenomenon was not made, some characteristics were incidentally noted.

Regeneration was more marked in some extracts than others, but was observed both in extracts made from rod outer segments and in extracts made from whole retinae. In some extracts regeneration was so active during the measurement period following a bleaching as to vitiate the experiment. This was especially noticeable after bleachings with red light. In other instances a long period in darkness was necessary to reveal evidence of regeneration.

Regeneration difference spectra can, of course, be constructed in the same way as bleaching difference spectra. An example (extract IV) was given in Fig. 3B (curves 4 and 5). Regeneration of similar magnitude was obtained in another instance (extract II) when a solution which had been partly bleached, first with red light and then with blue was allowed to stand in darkness overnight. The two regeneration difference spectra were very similar and have been combined to give the values shown in Fig. 7. The empty circles in this figure represent observations made on outward runs (380–620 mμ), the filled circles on inward runs (590–390 mμ). They do not quite correspond because of the slight regeneration occurring during the measurement periods.

For comparison the mean difference spectrum for all partial bleachings (twenty-one cases) is also shown in Fig. 7 (continuous line). The regeneration spectrum agrees reasonably well with it, bearing in mind the 'instability' of a regenerating solution and the fact that the regeneration refers to an overnight (18–22 hr) interval and hence includes any changes in this period due to general instability. The agreement is better in the 'positive' portion (which approximates to the absorption spectrum of the carp pigment) than in the 'negative' portion (which approximates to the absorption spectrum of its photo-product). The discrepancies in the negative portions are similar, though more pronounced, to those found between the negative portions of the difference spectra obtained by bleachings with short and long wave-length light (Fig. 6). This suggests that regeneration occurs only from the product of bleaching as first formed and not after it has isomerized, and this is supported by the fact that regeneration on the whole was more marked after bleachings with long than with short wave-length light.

Some of these properties are opposite to those of frog visual purple (rhodopsin). Thus Chase (1937) and Chase & Smith (1939) obtained good

regeneration after bleaching with blue light but practically none at all after bleaching with yellow light. This was confirmed by Hubbard & Wald (1952). According to their interpretation a bleaching with yellow light leaves the retinene$_1$ in a form unsuited for regeneration since it does not fit the architecture of the protein molecule (opsin). On the other hand, bleaching with blue

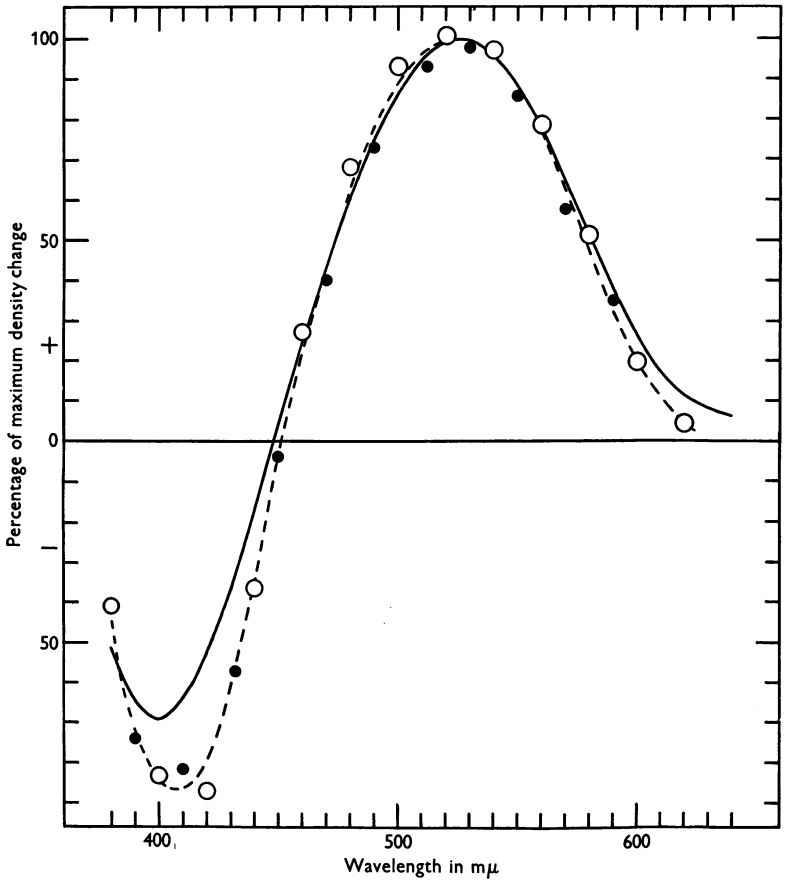

Fig. 7. Comparison of the regeneration difference spectrum with the bleaching difference spectrum. Full-line curve, mean bleaching difference spectrum; dashed-line curve, regeneration difference spectrum (mean of 2): ○, measurements made from 380 to 620 mμ; ●, return measurements from 590 to 390 mμ, $T = 20°$ C.

light results in the formation of an equilibrium mixture of the various stereochemical forms of retinene$_1$. Amongst these are two forms which will combine with opsin to give 'regenerated' pigments, one of which is indistinguishable from the original visual purple ($\lambda_{max} = 502$–503) while the other has an absorption maximum at 487 mμ.

In the present instance the carp photoproduct is already suitable for regeneration and isomerization results in less active forms. The regenerated pigment is apparently identical with the original one.

DISCUSSION

Absorption maximum of the carp pigment

This investigation has shown that the visual pigment solutions—prepared in deep-red light from dark-adapted carp by treating either whole retinae, or the outer limbs of the retinal end organs, with 2% digitonin solution—contain one photosensitive pigment.

However, the absorption spectra of the solutions obtained varied considerably, according to the proportion of impurities to the visual pigment. The impurities were yellow; that is, they absorbed with increasing strength towards the short wave-length end of the spectrum. Consequently, the absorption maxima of the extracts were always at shorter wave-lengths than that of the contained visual pigment. This is illustrated in Fig. 1 which shows $\lambda_{max.}$ ranging from 500 mμ for a grossly impure extract to 520 mμ for the best one.

In contrast to this variation, the difference spectra were all substantially the same and had maxima at 525 mμ within limits of ± 2 mμ. The positive portion of a difference spectrum (see, for example, Fig. 4) would be the same as the true absorption spectrum of the visual pigment only if the product of bleaching absorbed in a spectral region so remote from that of the parent pigment that there was no overlapping. It is in alkaline solution that the product (which behaves as an acid-base indicator) has its absorption band furthest removed from that of the parent pigment, but even so the separation is not complete and there is some mutual distortion. Consequently, the positive $\lambda_{max.}$ is displaced to a slightly longer wave-length than that for the visual pigment while the negative $\lambda_{max.}$ is correspondingly displaced to a shorter wave-length than that for the product of bleaching.

Lower and upper limits to the absorption maximum of the carp pigment are thus given by the $\lambda_{max.}$ of the best extract (520 mμ) and of the 'alkaline' difference spectrum (525 mμ) respectively. The true value is probably nearer the latter figure, say at 523 mμ.

Previous work on carp

It is not always possible to compare closely the results of different workers in the visual pigment field since the solutions obtained often vary greatly in purity. In the present investigation, however, solutions of a wide range of purity were prepared and consequently a basis for comparison has been provided.

The purity of an extract can be roughly expressed by the ratio of its density at the minimum (465–435 mμ, Fig. 1) to that at the maximum (500–520 mμ). The purer the extract, the lower is this ratio. In the present experiments it ranged from 0·98 to 0·57. In Fig. 8, this $D_{\text{min.}}/D_{\text{max.}}$ ratio is plotted against the absorption maximum, each extract providing a series of data because of changes with age. It is evident that these parameters are fairly closely correlated in spite of the different types of impurities present.

The presence of yellow impurities in a visual pigment solution is not the only cause of displacement of $\lambda_{\text{max.}}$ to shorter wave-lengths. Rayleigh scattering in a slightly opalescent solution has a similar effect. According to Collins & Morton (1950) the displacement is 1 mμ when the scattering is such as to raise the $D_{\text{min.}}/D_{\text{max.}}$ ratio to between 0·33 and 0·45, 2 mμ if the ratio is raised to 0·45–55, and 3 mμ if it is raised to 0·56–0·63. These limits are shown in Fig. 8 by the vertical bars and they accord with the present data.

In 1896 Köttgen & Abelsdorff, using bile salts, prepared visual pigment solutions from eight species of fresh-water fish. In their investigation they determined difference spectra directly, that is by measuring the densities of unbleached solutions with reference to those of bleached ones. All fish examined, among which was the carp, yielded similar difference spectra with maxima at 540 mμ. Unfortunately, the *p*H of Köttgen & Abelsdorff's solutions is not known and hence no comparison with the present difference spectra is possible.

Forty-two years later the carp was again examined, by Saito (1938). Visual pigment solutions (pH not stated) were prepared with sodium glycocholate by two methods, the first by preliminary isolation of the outer limbs of the retinal end organs by flotation in sucrose solution (this being the original demonstration of the method), the second by direct extraction of the retinae. The first method yielded a solution with $\lambda_{\text{max.}} = 515$ mμ and the second, one with $\lambda_{\text{max.}} = 520$ mμ. Both solutions had $D_{\text{min.}}/D_{\text{max.}}$ ratios of 0·70. Saito's results are shown in Fig. 8 by the vertical crosses.

The most recent previous investigation was that of Wald (1939) who prepared a neutral solution by treating carp retinae, which had been hardened in alum, with 1% digitonin solution. His extract had $\lambda_{\text{max.}} = 520$ mμ and a $D_{\text{min.}}/D_{\text{max.}}$ ratio of 0·65. Wald's result is shown in Fig. 8 by the diagonal cross.

Thus, as shown in those cases (Saito, 1938; Wald, 1939) where a direct comparison is possible, the carp solutions prepared by previous workers seem to have been similar to those of the present investigation.

Porphyropsin (visual violet)

It is reasonable to suppose that the relationship shown in Fig. 8 between the $D_{\text{min.}}/D_{\text{max.}}$ ratios and the absorption maxima of carp extracts will hold good for visual pigment extracts made from other fish, provided the same visual

pigment is involved or, at least, if its absorption characteristics are not very different from those of visual pigment 523, the carp pigment. It may not hold, of course, if the extract contains more than one visual pigment.

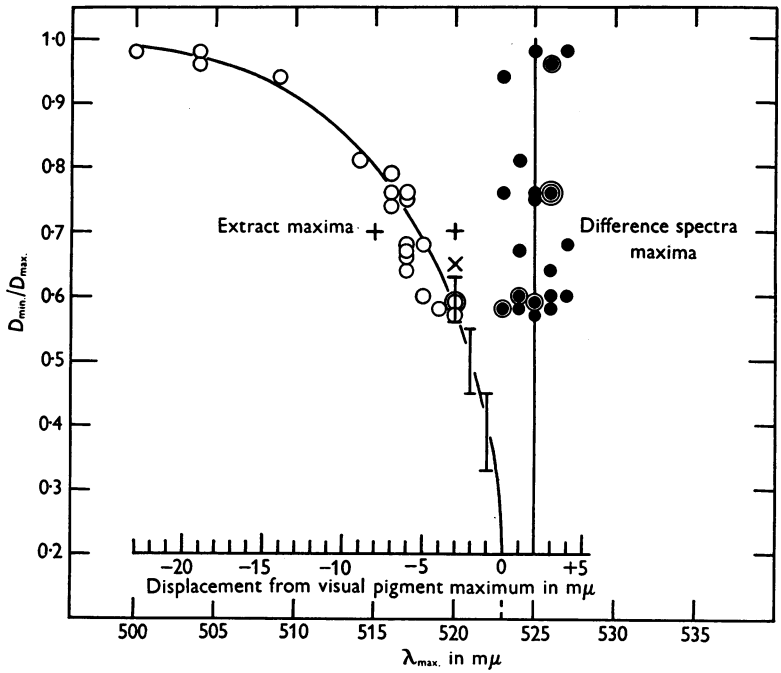

Fig. 8. Relation between the 'purity' of retinal extracts ($D_{min.}/D_{max.}$ ratios) and the $\lambda_{max.}$. ○, carp extracts of the present investigation; +, Saito's carp extracts (Saito, 1938); ×, Wald's carp extract (Wald, 1939); I, Collins & Morton's (1950) limits; ●, difference spectra maxima (present investigation).

TABLE 2. Calculation of the $\lambda_{max.}$ of visual pigments from the data of impure extracts

Fish	Reference	$D_{min.}/D_{max.}$	$\lambda_{max.}$ (mμ)	$\lambda_{max.}$ displacement† (mμ)	$\lambda_{max.}$ of visual pigment (mμ)
Carp (*Cyprinus carpio*)	Saito, 1938	0·70	515	5	520
		0·70	520	5	525
Carp (*C. carpio*)	Wald, 1939	0·65	520	4	524
Carp* (*C. carpio*)	This work	0·98–0·57	500–520	23–3	523
Perch (*Perca fluviatilis*)	Collins & Morton, 1950	0·82	524	9	533
		0·74	528	6	534
		0·83	520	9	529
Pickerel (*Esox reticulatus*)	Wald, 1939	0·77	525	7	532
Pike (*E. lucius*)	Collins & Morton, 1950	0·86	525	10	535
Pike* (*E. lucius*)	Dartnall, 1952*a*	0·58	530	3	533

* These extracts have been shown to be homogeneous, i.e. to contain only one photosensitive pigment.

† See Fig. 8.

To facilitate the use of Fig. 8 in this connexion an inset wave-length scale has been provided. This is scaled in mμ displacements from the pigment maximum.

An example of the use of Fig. 8 is as follows. Dartnall (1952*a*) found that a visual pigment solution prepared from pike retinae contained a single, homogeneous pigment. The alkaline difference spectrum of this pigment had $\lambda_{max.} = 535$ from which Dartnall deduced that the $\lambda_{max.}$ of the visual pigment was 533 mμ. Now Dartnall's extract had $\lambda_{max.} = 530$ and $D_{min.}/D_{max.} = 0{\cdot}58$. From Fig. 8, an extract with this ratio has its $\lambda_{max.}$ displaced by 3 mμ confirming 533 mμ as the $\lambda_{max.}$ for the visual pigment. Again, Collins & Morton's (1950) extract from pike retinae had $\lambda_{max.} = 525$ mμ and $D_{min.}/D_{max.} = 0{\cdot}86$. From Fig. 8 the displacement in this case is 10 mμ, giving the closely agreeing value of 535 mμ for the pike pigment. These and some further examples are tabulated in Table 2.

According to Wald (1939), porphyropsin, the visual pigment of fresh water fish, has an absorption maximum at 522 ± 2 mμ. This estimate is based on the absorption spectra of extracts from five species—the white perch, the calico bass, the blue gill, the carp and the pickerel. In all cases the absorption spectra were roughly the same.

At first sight it might appear that these five species all contained the same pigment as carp (visual pigment 523) and that by an accident no species was examined which had visual pigment 533. However, the $D_{min.}/D_{max.}$ ratios of Wald's extracts ranged from 0·51 to 0·77 and allowance must be made for this variation. The pickerel solution, for example, with $\lambda_{max.} = 525$ mμ had $D_{min.}/D_{max.} = 0{\cdot}77$. Adding 7 mμ, the displacement correction appropriate for this ratio (Fig. 8), we obtain 532 mμ as the $\lambda_{max.}$ for the pickerel pigment. Wald's carp data, on the other hand, agree with the present work (visual pigment 523). These calculations suggest that Wald's porphyropsin solutions may have included examples of visual pigment 533 as well as of visual pigment 523. A further reason for suspecting this is the statement (Wald, 1939) that the difference spectrum obtained by bleaching neutral porphyropsin had a maximum at 535–540 mμ, a property suggestive of visual pigment 533 rather than of visual pigment 523.

Conclusion

This and previous investigations (Dartnall, 1952*a*, *b*) show that the main visual pigment of the fresh-water fish so far examined occurs in at least two forms; visual pigment 533, present in tench, pike and bleak, and visual pigment 523 in the carp. In some cases (pike and carp) the relevant pigment occurs alone; in others (tench and bleak) in admixture with relatively small amounts of other pigments.

This conclusion does not necessarily conflict with Wald's conception that all

the visual pigments are related either to vitamin A_1 or to vitamin A_2. It is possible to envisage a variety of combinations of proteins with those carotenoids which are structurally related to one or other of the vitamins A. The characterization of the visual pigments by their absorption spectra (rather than by the decomposition products of their carotenoid fractions) is of interest, if only from the viewpoint of interpreting visual sensitivity curves. Much further work with many species—particularly in those cases where the retinal extracts contain more than one pigment—will be necessary before this field is adequately explored.

SUMMARY

1. Visual pigment solutions have been prepared from dark-adapted carp by treating either whole retinae or the isolated outer limbs of the retinal end organs with 2% digitonin solution. The resulting solutions had $\lambda_{max.}$ ranging from 500 to 520 mμ, depending on the proportion of yellow impurities to visual pigment.

2. The solutions (pH 8–8·6) were investigated by observing the changes in their absorption spectra which occurred when they were bleached; either to completion by a single exposure or, in a number of stages, by a succession of exposures to light. Whether white light or approximately monochromatic lights of dominant wave-lengths 640, 630, 530, 480, or 430 mμ were used, these changes (difference spectra)—when scaled to the same maximum (100)—were all practically the same. From this it has been concluded that the solutions contained a single photosensitive pigment.

3. The carp pigment—visual pigment 523—is distinct from that (visual pigment 533) found in certain other fresh-water fish.

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