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VISUAL PIGMENT 467, A PHOTOSENSITIVE PIGMENT PRESENT IN TENCH RETINAE

By H. J. A. DARTNALL
From the Group for Research in the Physiology of Vision, Institute of Ophthalmology, London

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In an investigation of the retinal densities of the scotopic pigments in various animals it was necessary to select for study a representative of the class in which the scotopic response is mediated by visual violet.

This class comprises the fresh-water fishes (Wald, 1937*a*, 1939). Of these, the tench was chosen, since its scotopic sensitivity has been measured (Granit, 1941).

Aqueous digitonin extracts of dark adapted tench retinae were prepared for the purpose of estimating the optical density of visual violet in the retina of this fish. On exposing these extracts to light it was found, however, that bleaching was maximal at approximately 520 mμ. instead of at 535–540 mμ. as reported by Köttgen & Abelsdorff (1896) and by Bayliss, Lythgoe & Tansley (1936).

As repeated determinations yielded the same unexpected result, it seemed probable that the extracts contained, in addition to visual violet, a photosensitive component absorbing maximally in the blue and not present in the extracts of previous workers. The bleaching of such a component, together with visual violet, could account for the displacement of the maximum photosensitivity from its expected position at 535 mμ.

A study of this additional photosensitive component is presented in this paper.

APPARATUS AND METHODS

Apparatus for measurement of absorption spectra

The only method known for the identification and estimation of the visual pigments is measurement of their absorption spectra.

In principle, measurement of an absorption spectrum consists of a series of determinations of the optical density at various wave-lengths throughout the desired range. Since the visual pigments are photosensitive, it is necessary in these measurements to employ light of very feeble intensity to reduce photodecomposition to insignificant proportions. Consequently, the light-detection apparatus must be of high sensitivity.

The density measurements were made in the usual way, that is, by comparing the light transmitted by the visual pigment solution with that transmitted by a 'reference' solution. Similar measurements were also made after the visual pigment solution had been exposed to light. As these procedures involved removal and replacement of the optical cells containing the solutions, a high standard of construction of apparatus and cells was required to achieve the desired precision of measurement.

Optical system. The source of light was an 18 W. car headlamp bulb run off a 6 V., 130 amp.-hr. accumulator. An image of the vertical filament of the lamp was formed on the entrance slit of a Hilger Barfit monochromator.

The light emerging from the monochromator had a band width which varied with the wave-length setting. The total width, $\Delta W = \Delta w_1 + \Delta w_2$, where Δw_1 was the band width due to dispersion and Δw_2 that due to curvature of the spectral lines (both slits being straight). The widths of the entrance and exit slits were 0·04 mm. for all wave-length settings. The length of the entrance slit was 11 mm. For these conditions the band widths, calculated from data supplied with the monochromator, were as shown in Table 1.

The light emerging from the exit slit passed through a filter (Fig. 1) to remove 'stray' light. Ilford filters nos. 601–608 in the 'monochromatic series' were used in the wave-length range 410–700 mμ. The feebleness of the light intensity in the range 380–400 mμ. precluded the use of a stray light filter, except on rare occasions.

TABLE 1

Wavelength (mμ.)	Δw_1 (mμ.)	Δw_2 (mμ.)	ΔW, total band width (mμ.)
400	0·4	0·2	0·6
450	0·7	0·3	1·0
500	1·0	0·5	1·5
550	1·4	0·6	2·0
600	1·9	0·8	2·7
650	2·5	1·1	3·6
700	3·1	1·5	4·6

Optical cells and holder. The optical cells containing the solutions for absorption spectra measurements were of fused glass construction (Fig. 1). They had a capacity of approximately 0·4 ml. The internal distance between the optical faces was 0·5 cm.

The cells were a sliding fit in the cell carrier (at 20° C.). All cells were of identical dimensions to a high standard of precision. However, no two cells transmitted exactly the same proportion of light when filled with the same liquid. The differences in transmission, which were almost independent of wave-length, were about 1%, corresponding to a density difference of 0·004.

The cell holder (Fig. 1) comprised a movable carrier supported by an outer case. The body of the carrier was an ⊔⊔-shaped piece of brass accommodating two optical cells. It was covered on both sides by thin plates, *P*, having circular apertures to correspond with the optical cells. The base, *S*, and the plates, *P*, were of plastic to give thermal insulation from the outer case. Passages were drilled in the carrier to allow the circulation of water from a thermostat. The temperature was indicated by a thermometer, *Th*, inserted in the central column of the ⊔⊔.

The base *S* fitted into a groove in the base, *B*, of the outer case, this arrangement permitting a lateral movement limited by the end-pieces, *E*. These were so positioned that full movement of the carrier in either direction brought each cell in turn into alinement with the circular apertures in the outer case. The apertures, *A* (front and back) were of slightly smaller diameter than that of the cells to ensure equalization of 'edge' effects.

The front aperture in the outer case was enclosed by a spigot, *Sp*, threaded to engage with the telescopic connecting tubes *TC*. The back aperture was similarly enclosed in an unthreaded spigot which was a sliding fit in the corresponding member of the photocell housing.

Entry of light between the carrier and the outer case was prevented by a wafer, *W*, fixed to the carrier. Caps, *C*, were placed over the cells after insertion to prevent entry of light from above.

The cell holder and photocell housing were rigidly mounted on an optical bench.

Temperature control. The temperature of the solutions was controlled during measurement of the optical densities by circulating water (at 300 ml./min.) from a thermostat through the channels (of capacity 4 ml.) in the carrier. To allow temperature equilibrium to be established, at least 5 min. elapsed between inserting the cells in the carrier and commencing the measurements.

The thermostat was adjusted to run at 20·0±0·1° C. At equilibrium, the temperature indicated by the thermometer in the carrier was 0·1° C. above or below that of the thermostat, depending on the room temperature. Thus all absorption spectra were measured at 20·0±0·2° C. (uncorrected).

The photoelectric cell. After passing through the cell holder the monochromatic light beam was received on the cathode of an R.C.A. 931 A multiplier photocell. Although the maximum response of this cell was in the near ultra-violet, its sensitivity was sufficiently high to allow measurements to be made at 700 mμ. with the present apparatus.

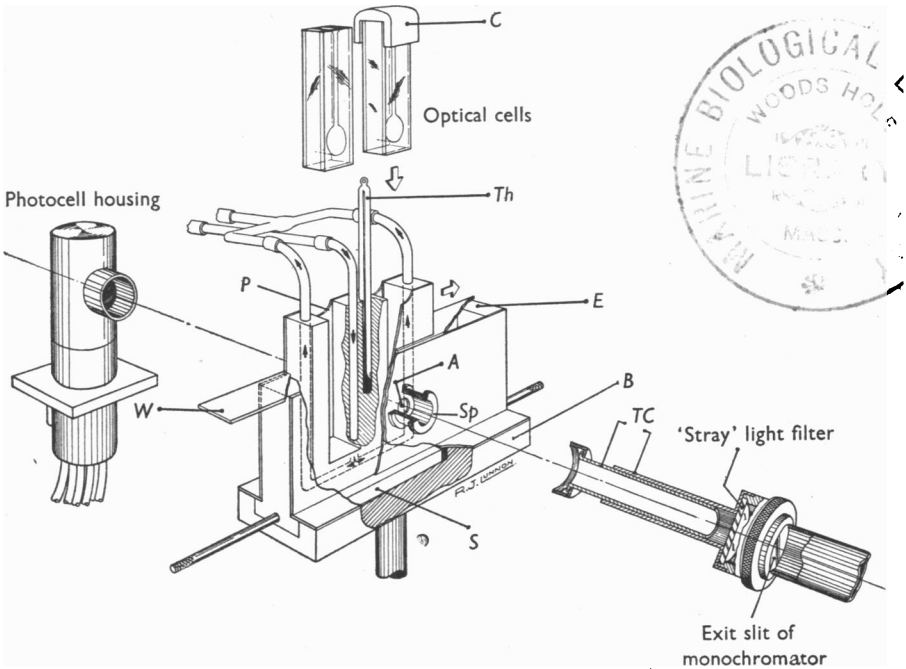

Fig. 1. The optical cell holder. For explanation, see text.

The photocell was activated by a specially constructed mains unit. The smoothed and stabilized output voltage from this unit was applied across a chain of matched resistances connected to the dynodes of the photocell. By varying a resistance in series with this chain the potential difference between dynodes could be varied from 90 to 110 V. The photocell was normally activated at 100 V. per stage.

The output leads were connected to a critically damped d'Arsonval galvanometer of period 2 sec. Readings were observed on a translucent scale, 50 cm. long, calibrated in mm. The scale was rigidly mounted at a distance of 1 m. from the reflecting mirror attached to the galvanometer coil.

Technique of density measurements

Method. Optical density is defined as $\log_{10}(I_{inc.}/I_{trans.})$, where $I_{inc.}$ is the intensity of light entering the front surface of the medium, and $I_{trans.}$ the intensity leaving the back surface. It is impracticable to measure $I_{inc.}$ and $I_{trans.}$ directly owing to reflexions which, in the case of liquids, take place at two vessel/air interfaces and at two vessel/liquid interfaces.

This difficulty was avoided in the following, standard, manner. A second ('reference') optical cell of identical construction to that containing the solution under test was filled with solvent alone. The two cells were then successively placed in the light beam issuing from the monochromator, the intensities of light falling on the photocell after passing through them being recorded. It may be shown by simple theory that D_d, the density of the solute, is given by

$$D_d = D_s - D_r = \log_{10} I_r/I_s,$$

where D_s and D_r are the optical densities of the solution and solvent respectively, and I_r and I_s are the intensities of light leaving the rear surface of the 'reference' and 'solution' cells (and hence falling on the photocell).

The method gives the density of the solute in the dissolved condition, not the density of the solution as a whole. In a retinal extract, which contains digitonin, buffer salts and impurities in addition to the visual pigments, the absorption due to the digitonin and buffer salts may be eliminated by using as the reference solution an aqueous buffered digitonin solution of the same strength. The density measured in this case is that of the visual pigment and accompanying impurities of retinal origin.

The observations were carried out as follows. Having made the required wave-length setting and inserted the appropriate stray light filter (Fig. 1), the light intensity was adjusted (by means of a neutral wedge of density range 0–2 placed between the light source and the entrance slit) so that full-scale deflexion of the galvanometer was obtained with the 'reference' cell in position.

Between the light source and the entrance slit was a slowly rotating sector disk (1 rev./min.). This alternately prevented and allowed the passage of light for 15 sec. periods and thus ensured that the photocell/galvanometer combination had a regular history during the experiment.

When the disk prevented the passage of light a 'zero' reading, a, was taken. When it allowed passage of light a further reading, b, was taken. The cell carrier was then quickly transferred to bring the 'solution' cell into the light beam and the new reading, c, noted. The deflexions $(b-a)$ and $(c-a)$ were measures of the intensity of the light falling on the photocell after passing through the 'reference' and 'solution' cells respectively. The density was calculated from the formula

$$D = \log_{10} \frac{(b-a)}{(c-a)}.$$

Readings were estimated to 0·1 mm.

Accuracy and precision. For the density measurements to be accurate, the response of the photocell-galvanometer unit must be proportional to the light intensity. Tests showed that the photocurrent was strictly proportional to the light intensity. The galvanometer deflexion, however, was not proportional to the photocurrent. The small deviations, principally due to geometrical distortion caused by the use of a straight galvanometer scale, resulted in errors in the density measurements almost exactly proportional to the magnitude of the measured density, the latter being uniformly 1·75 % too low. (Within the density range 0·0–0·7, the residual errors from assuming that the density error was 1·75 % never exceeded 0·0005 and were usually much less).

As the present investigation was concerned only with the relations between densities at different wave-lengths (absorption spectra) the need for applying such proportional corrections did not arise. The densities quoted in this paper are, therefore, uncorrected.

To ascertain the precision of the method the standard deviations, σ_D, of the density measurements were determined for various density levels between 0·0 and 0·5. Each estimation of σ_D was calculated from ten consecutive determinations of the density. Because of variation in the 'steadiness' of the galvanometer readings, some day to day variations in the standard deviations were observed. The averages of values observed at various times were as shown in Table 2.

In the investigation proper, all density measurements were made at least twice. The standard deviations of the means of two determinations are given by $\sigma_D/\sqrt{2}$. Because of other, not easily assessable errors, however, the figures quoted in Table 2 may be used as a rough guide to the total errors.

The bleaching apparatus

For complete bleaching of the visual pigment solutions a 15 W. clear, wire filament lamp was used. The solution to be bleached was placed on white paper about 9 in. from the lamp and exposed for 15–20 min. Such bleachings are referred to in this paper as bleachings by 'white' light.

For bleachings with approximately monochromatic light an apparatus similar in principle to that used for measurement of absorption spectra was employed. The source of light was a tungsten ribbon filament lamp (6 V., 18 amp.) run off a 310 W. 'Westat' constant potential power unit. The light from this lamp was focused on the entrance slit of a Hilger Barfit monochromator.

TABLE 2

Density, D	Standard deviation, σ_D
0·0	0·0004
0·1	0·0005
0·2	0·0006
0·3	0·0007
0·4	0·0009
0·5	0·0011

A 'monochromatic' image of the telescope lens of this instrument was formed on the front face of the optical cell, containing the solution to be bleached, by means of a lens screwed into the mounting of the exit slit. The dominant wave-length and purity (band width) of the bleaching lights used are given in Tables 5 and 8.

A holder, similar to that described for the absorption spectrum apparatus, supported the 'solution' and 'reference' optical cells, the latter cell being protected from the bleaching light. The temperature was controlled at 20° C. during bleaching by the circulation of water from a thermostat through passages drilled in the holder.

Preparation of visual pigment solutions

The major part of the experimental work was carried out with retinal extracts of the dark adapted tench (*Tinca tinca*), but for certain comparative experiments a retinal extract of the pike (*Esox lucius*) was also prepared.

All operations were performed in a dark room in the general illumination of a deep red photographic safe light. Additional illumination, where necessary for operations such as removal of the retinae, was provided by a cycle torch lamp (6 V., 3 amp.) with reflector, fitted with Ilford 'monochromatic' filter no. 609 (transmitting from 650 mμ. to the infra-red). Tests showed that photochemical bleaching of visual violet (and visual purple) solutions in these illuminations was negligibly small for the periods of necessary exposure. Even so, exposure was avoided as far as possible and many procedures were carried out in total darkness.

Removal of retinae

Tench. The fish was laid on its side and the centre of the cornea of the upper eye punctured with the point of one blade of a sharp pair of scissors. The point was gently pressed into the eye and a cut made from the corneal centre. Three other cuts, mutually at right angles, were then made, the final appearance of the incisions being that of a cross with its mid-point at the corneal centre.

A pair of forceps, held with its limbs an eye-diameter apart, was carefully pressed on the eye. This caused the lens to protrude. The forceps were then partially closed and the lens eased away.

The forceps were lowered into the eye and a portion of the retina gently grasped. The retina was surprisingly coherent and, provided the forceps were gradually raised, came away in one piece. The retina so obtained, with an accompanying drop of aqueous and vitreous humours, was placed in the first washing solution, whereupon it resumed its shape to reveal a hole in its base where the optic nerve had entered. In some cases the size of this hole indicated that a considerable portion of the retina had remained behind with the optic nerve.

Pike. The fish was laid on its side and a diagonal cut made across the cornea which was then removed by a peripheral incision. After lifting out the lens, the retina was obtained piecemeal in as large portions as possible.

Washing of retinae prior to extraction of visual pigments

Method. The retinae, removed as described above, were placed directly in the washing solution (McIlvaine's pH 4·6 buffer) in a centrifuge tube. The mixture was stirred for 1 min. by introducing a small ball-bearing into the tube and moving the latter vigorously between the poles of a powerful magnet. This procedure broke up the retinae into small fragments. The well-stirred mixture was centrifuged at 4000 rev./min. for 20 min. The clear, yellow supernatant was completely withdrawn from the compacted sediment by means of a micropipette and discarded.

A further quantity of the pH 4·6 buffer was then added to the residue in the tube and the stirring, centrifuging and removal of the supernatant, now paler in colour, repeated. In all, the retinae were washed three or four times (see Table 3), the last washing being almost colourless.

Absorption spectra of the washings of tench retinae. The absorption spectra of successive washings of eleven tench retinae with 3 ml. portions of McIlvaine's buffer solution of pH 4·6 are shown in Fig. 2.

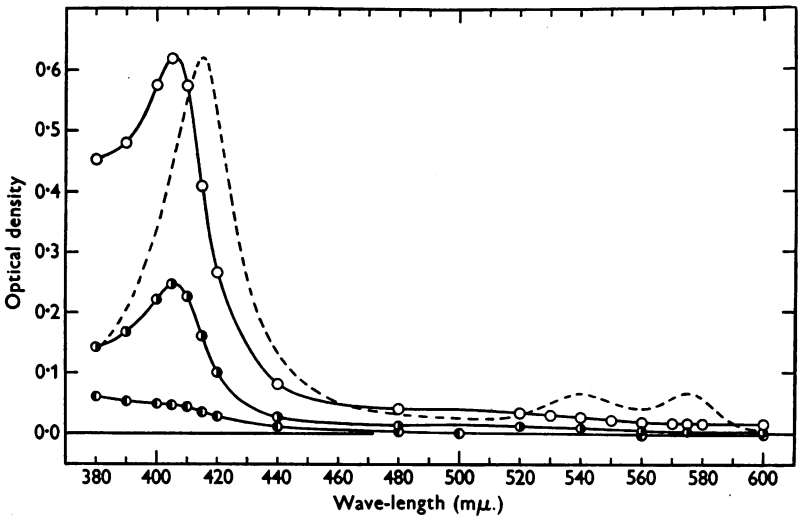

Fig. 2. Absorption spectra of successive washings of tench retinae with McIlvaine's buffer solution of pH 4·6. ○, first washing; ◑, second washing; ◐, third washing; – – –, absorption spectrum of oxyhaemoglobin (I. G. Wootton, unpublished).

The spectra of the washings, particularly the first and second, were characterized by a strong absorption band, maximal at 405 mμ. Traces of this band also appeared in the third, and in this case final, washing. The absorption spectrum of the subsequent digitonin extract of these washed retinae (extract 8) also showed a very slight inflexion in this spectral region, indicating that, in this case, the triple washing with pH 4·6 buffer had not been completely effective in removing the material responsible for this absorption. For this reason the retinae were usually washed four times before extraction with digitonin (Table 3). In such cases the digitonin extracts appeared to be quite free from the '405 chromophore' (see, for example, Fig. 3).

McIlvaine's buffer of pH 6·5, unlike that of pH 4·6 is not very effective in removing the '405 chromophore'. In a previous communication (Dartnall, 1950) an extract of tench was described in the preparation of which pH 6·5 buffer had been used for washing the retinae prior to extraction with digitonin. It is apparent from the absorption spectra given that the '405 chromophore' was present in large amount in this extract of visual pigments. This material was doubtless responsible

for the slight loss in density, maximal at 407 mμ., observed on bleaching as such a loss has not been observed on bleaching extracts free from the chromophore, i.e. extracts prepared from retinae which had been adequately washed with pH 4·6 buffer. The claim (Dartnall, 1950) that this substance was a visual pigment is now considered to have been unfounded. Collins & Morton (1951) have also criticized the original claim and have suggested that the '405 chromophore' is oxyhaemoglobin. Oxyhaemoglobin, however (Fig. 2, broken line) has three well-defined absorption bands, at 415, 540 and 575 mμ., respectively, while the '405 chromophore' has a band at 405 mμ. and, possibly, a much weaker band between 470 and 550 mμ. The '405 chromophore' resembles, in this respect, the substance extractable from the choroidal gland of certain fish (Barnet, 1951). Barnet considers that this substance may be cytochrome *c*.

Extraction of visual pigments from the washed retinae

The extraction of the visual pigments from the retinal centrifugate remaining from the last washing with pH 4·6 buffer was effected by the addition of a freshly prepared 2 % w/v solution of digitonin (Tansley, 1931) and by stirring for 5 min. The mixture was centrifuged for 20 min. at 4000 rev./min. The supernatant was pipetted into another vessel and the residue re-extracted with a further portion of the digitonin solution. After centrifuging, the second supernatant was withdrawn and added to the first. The bulked extract was then brought to the required pH by the addition of a suitable buffer solution.

TABLE 3. Preparation details of the extracts

Fish*	Extract no.	Date of preparation	Size (cm.) of fish and no. of retinae	Time of dark adaptation (hr.)	Retinae washing solutions	Volume (ml.) of extractant (2 % digitonin)	Volume (ml.) of buffer added†	Weight of buffered extract (g.)	pH at 20 ° C.
Tench (5)	2	16. v. 50	15–17 (10)	16	1 × 4 ml. 0·6 % NaCl 1 × 4 ml. pH 4·6‡	3 × 1·5	0·45 d.h.p.	4·99	7·74
Tench (6)	5	26. vi. 50	13–18 (11)	64	1 × 4 ml. 0·6 % NaCl 2 × 4 ml. pH 4·6	2 × 2·0	0·4 d.h.p.	4·43	7·82
Tench (8)	7	19. ix. 50	11–13 (16)	19	1 × 4 ml. 0·6 % NaCl 4 × 4 ml. pH 4·6	1 × 4·0	0·5 s.b.	—	8·72
Tench (6)	8	26. ix. 50	13–15 (11)	88	3 × 3 ml. pH 4·6	2 × 1·5	0·3 s.b.	3·27	8·68
Tench (5)	9	3. x. 50	14–15 (10)	88	4 × 3 ml. pH 4·6	2 × 1·7	0·3 p.h.p.	3·65	4·17
Tench (8)	10	9. x. 50	13–15 (16)	69	4 × 4 ml. pH 4·6	2 × 2·0	§	2·10 2·10	4·22 8·66
Tench (6)	11	1. xi. 50	13–15 (12)	44	4 × 4 ml. pH 4·6	2 × 1·1	0·22 s.b.	2·41	8·54
Tench (7)	12	8. i. 51	14–23 (13)	66	4 × 5 ml. pH 4·6	2 × 1·5	0·3 s.b.	3·23	8·40
Pike (1)	1	6. xi. 50	55 (2)	‖	4 × 4 ml. pH 4·6	2 × 1·0	0·2 s.b.	—	8·24

* The figures in brackets give the number of fish used.
‡ McIlvaine's buffer solution, pH 4·6.
† The following solutions were used: 'd.h.p.', a molar solution of disodium hydrogen phosphate; 's.b.', a saturated solution of sodium borate; and 'p.h.p.', a molar/4 solution of potassium hydrogen phthalate.
§ The bulked extract (3·97 g.) was divided into two portions. To one portion (1·902 g.) was added 0·195 g. of p.h.p. bringing the pH to 4·22 and to the other portion (1·900 g.), 0·197 g. of s.b. bringing the pH to 8·66.
‖ The pike was dead when received from the angler. It was kept in darkness for a short period before commencing the extraction.

Examination of the retinal residues after the final treatment with digitonin solution often showed that extraction of the visual pigments was incomplete. Some variation in extractive efficiency was noticed among the samples of digitonin used, which were from various sources.

The buffered extracts were stored in darkness in a refrigerator (3° C.), portions being withdrawn for experiments over a period of 3–4 days. At the end of this time (and, indeed, for much longer periods) the extracts retained their original clarity apparently unimpaired, though measurement showed a gradual increase in density with time in the short wave-length region of the spectrum. The extracts were centrifuged at 4000 rev./min. on each occasion before withdrawal of a portion for experiment.

Details of preparation of the extracts are shown in Table 3.

Measurement of hydrogen-ion concentration

The pH of the buffered retinal extracts were measured with the Cambridge Bench pH meter using a glass-calomel electrode assembly. All measurements were carried out at 20±0·5° C.

About 2 ml. of solution were required for the test. Accordingly, the bleached solutions from experiments were 'saved' in a closed tube stored in a refrigerator until this volume had been accumulated. Special tests indicated that after a week's storage the pH of the solutions had not changed and also that there was no significant difference in pH between bleached and unbleached buffered solutions.

RESULTS

Stability of the solutions

As a test of thermal stability the absorption spectra of all solutions, unbleached and bleached, were determined twice. The method adopted may be illustrated with reference to the curves in Fig. 3. The 'unbleached' curve for tench was obtained as follows. The densities at wave-lengths of 380–620 mμ. were measured at intervals of 20 mμ. (time taken, 25 min.). Immediately following this, densities were measured from 630 to 390 mμ., again at 20 mμ. intervals (time taken, 27 min.). The 'bleached' curve was obtained in a similar manner, the outward measurements taking 27 min. and the inward 26 min.

This is typical of a 'rapid' determination. In some instances a considerable interval elapsed between finishing the outward and starting the inward measurements. For example, in the case of the 'unbleached' curve for the pike (Fig. 3), there was a 2 hr. interval between the last outward and the first inward measurement, and similarly in the determination of the 'bleached' curve, an interval of 23 min. between the two sets of measurements.

The fact that the inward measurements in all cases retraced the paths of the outward measurements indicated that both unbleached and bleached solutions were thermally stable.

Observations were not always taken at every wave-length interval of 20 mμ. but in all experiments sufficient 'interlacing' of the results was obtained to reveal the stability of the solutions.

It would be confusing in the various graphs of this paper to indicate outward and inward measurements by different symbols, particularly when dealing with 'difference' spectra. The above procedure was invariably adopted, and all absorption spectra described in this paper refer to thermally stable solutions unless indicated to the contrary.

Experiments with alkaline retinal extracts

The anomalous shape of the tench difference spectrum

The absorption spectra before and after bleaching of an extract of pike retinae, buffered at pH 8·24, are shown in the upper half of Fig. 3. These curves show that on bleaching the solution with 'white' light, the density decreases at

wave-lengths greater and increases at wave-lengths less than 451 mμ. At 451 mμ. the density of the solution is unchanged by bleaching.

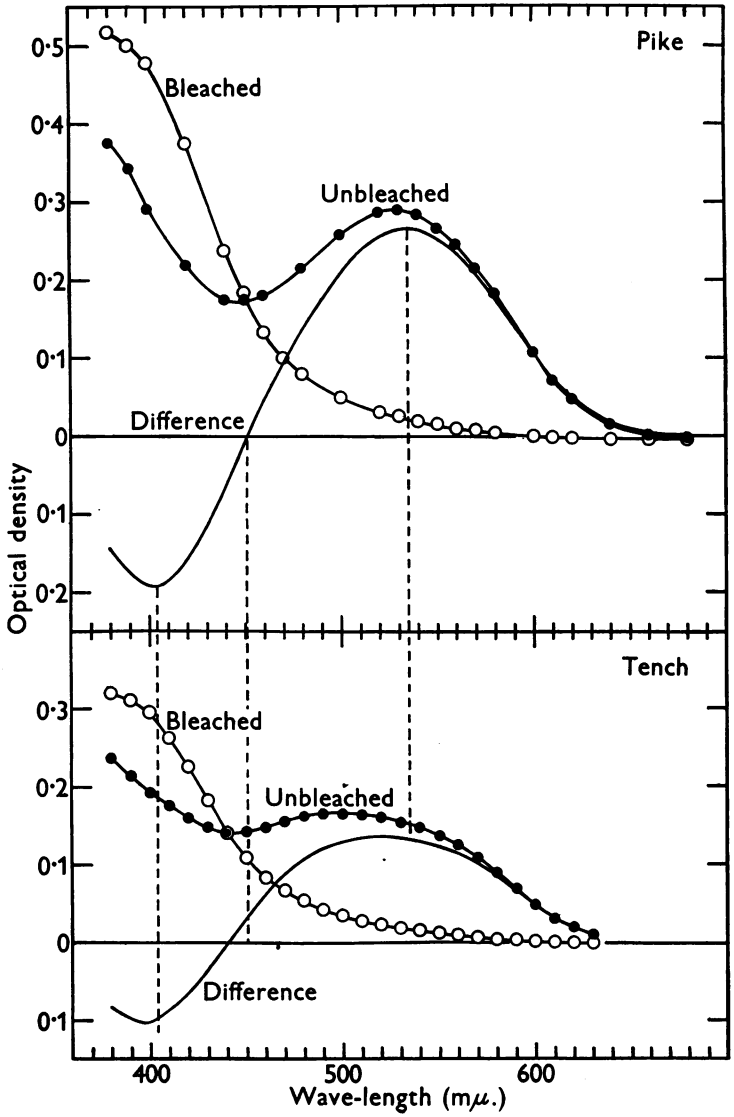

Fig. 3. Comparison of the absorption spectra of pike and tench extracts before and after bleaching with white light. The vertical interrupted lines facilitate comparison of the two difference spectra. Pike extract 1, pH 8·24; tench extract 12, pH 8·40.

The 'difference' spectrum is obtained by subtracting the bleached curve from the unbleached and gives the *change* in density on bleaching. The difference

spectrum is uncomplicated by the presence of stable impurities, as these contribute equally to the absorption in the unbleached and bleached solution. It shows (Fig. 3) that, on bleaching, density is lost maximally at 535 mμ. and gained maximally at 405 mμ., behaviour typical of a visual violet solution. The loss in density is due to the bleaching of visual violet and the gain to the consequent formation of the 'indicator' substance, that is the substance analogous to the indicator yellow formed by bleaching visual purple.

The difference spectrum is, in fact, the difference between the true absorption spectrum of visual violet and the true absorption spectrum of its indicator substance. The upper and lower portions of the difference spectrum are hence approximations to the absorption spectra of visual violet and its indicator substance respectively. The proximal limbs of these curves, however, mutually distort each other, with the result that the true absorption maximum of visual violet must be at a shorter wave-length than that indicated by the difference spectrum (535 mμ.) while the true absorption maximum of the indicator substance (at pH 8·24) must be at a longer wave-length than 405 mμ.

TABLE 4

Difference spectrum of	Wave-length (mμ.) of Maximal density loss	Maximal density gain	No change in density
Pike	535	405	451
Tench	520	395	440

The absorption spectrum of an extract of tench retinae (extract 12), prepared in exactly the same way and buffered, as nearly as possible to the same pH (8·40) is shown in the lower half of Fig. 3. The 'unbleached' curve has a maximum at a wave-length of 495 mμ. instead of at 530 mμ. as in the pike.

The dissimilarity between the pike and tench extracts is best shown, however, by their respective difference spectra. The shape of these is not the same even after allowing for their difference in height (due to the solutions being of different strengths). The differences may be summarized as shown in Table 4. Since the experiments were carried out in almost identical conditions there are only two likely reasons for this dissimilarity; either the visual violet of the tench is different from that of the pike, or the tench extract contains one or more photosensitive pigments in addition to visual violet.

The composite nature of the tench difference spectrum

If tench extracts contain, besides visual violet, another photosensitive component this would need to have an absorption maximum in the short wave-length region of the spectrum in order to account for the anomalous shape of the difference spectrum. In this event it should be possible to bleach out the visual violet preferentially with light of long wave-length.

That this is, in fact, the case is shown by the following experiments on another extract (extract 10, pH 8·66). First, the changes in density on exposure of a sample of this extract to 'white' light were determined. These changes, given by the curve marked 'total' in Fig. 4, are similar to those obtained with the previous tench extract (Fig. 3).

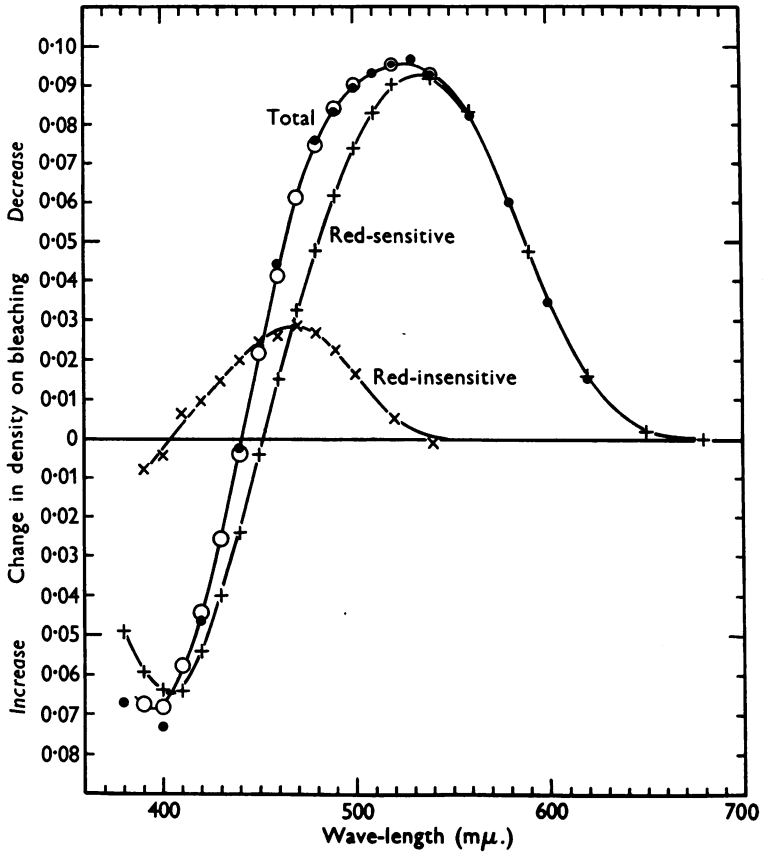

Fig. 4. The two light-sensitive components in tench extracts. ●, density changes on exposure to white light ('total'); +, density changes on exposure of another sample of the same extract to light of wave-length 610 mμ. ('red-sensitive'); ×, density changes on further exposure to white light ('red-insensitive'); ○, algebraic sum of the red-sensitive and red-insensitive components. Tench extract 10, pH 8·66.

The following, day, two absorption cells were filled with further samples of extract 10. After measuring the initial differences in density between the two cells, one of them was exposed to orange-red monochromatic light (610 mμ.) for 4¼ hr. The resulting changes in density with respect to the unexposed solution were then measured. These changes (after allowance for the small initial differences) are shown in Fig. 4 (curve marked 'red-sensitive'). This

curve shows that bleaching with orange-red light caused a maximal density loss at 535 mμ., a maximal gain at 405 mμ. and no change at 450 mμ., changes similar to those which took place on bleaching an extract of pike retinae with 'white' light (Fig. 3).

The solution which had been exposed to the orange-red light was then exposed to green light (530 mμ.) for 30 min. The object of this was to bleach any traces of the red-sensitive component remaining. In fact, measurement showed that there was no further loss of density at 535 mμ. but only a very small amount of bleaching in the short wave-length region of the spectrum (maximal density loss, at 470 mμ., 0·003). The solution was then exposed to white light for 15 min. This caused further bleaching, again maximal at 470 mμ. (density loss 0·026). The changes in density due to the exposures to green and white light are shown in Fig. 4 (curve marked 'red-insensitive').

The algebraic sum of the difference spectra of the red-sensitive and red-insensitive components of the extract is also shown in Fig. 4. This is in good agreement with the original difference spectrum for white light obtained on the previous day, showing that the two components of the solution were thermally stable.

These experiments indicated that extracts of tench retinae are not homogeneous but contain two light-sensitive components, one showing density changes on bleaching which are maximal at 535 mμ. (the 'red-sensitive' component) and the other changes which are maximal at about 470 mμ. (the 'red-insensitive' component).

The red-sensitive component of tench extracts

It has already been noted that the red-sensitive component has absorption characteristics similar to those of visual violet as reported in the literature. In order to obtain data for precise comparison, a number of determinations of the difference spectrum of the red-sensitive component were made under controlled conditions (pH 8·40–8·68) by bleaching various extracts with monochromatic lights of wave-lengths 600–650 mμ. Two methods were employed. In method A the absorption spectrum of the unbleached solution was first measured with reference to a digitonin solution buffered to the same pH. The solution was then exposed to the long wave-length monochromatic light, after which the absorption of the solution was measured again. The difference spectrum was obtained by subtracting the final from the initial observations; in method B both optical cells were filled with the unbleached solution and after first measuring the small density differences between them one cell was exposed to the monochromatic light. The density differences between the cells, after this exposure, were then measured. Apart from the correction which must be applied for the small initial density differences, the difference spectrum is measured directly by this method.

The difference spectra obtained by bleaching four separate extracts of tench retinae with red light of wave-lengths varying between 600 and 650 mμ. are shown in Fig. 5. To make comparison easy, the difference spectra obtained have

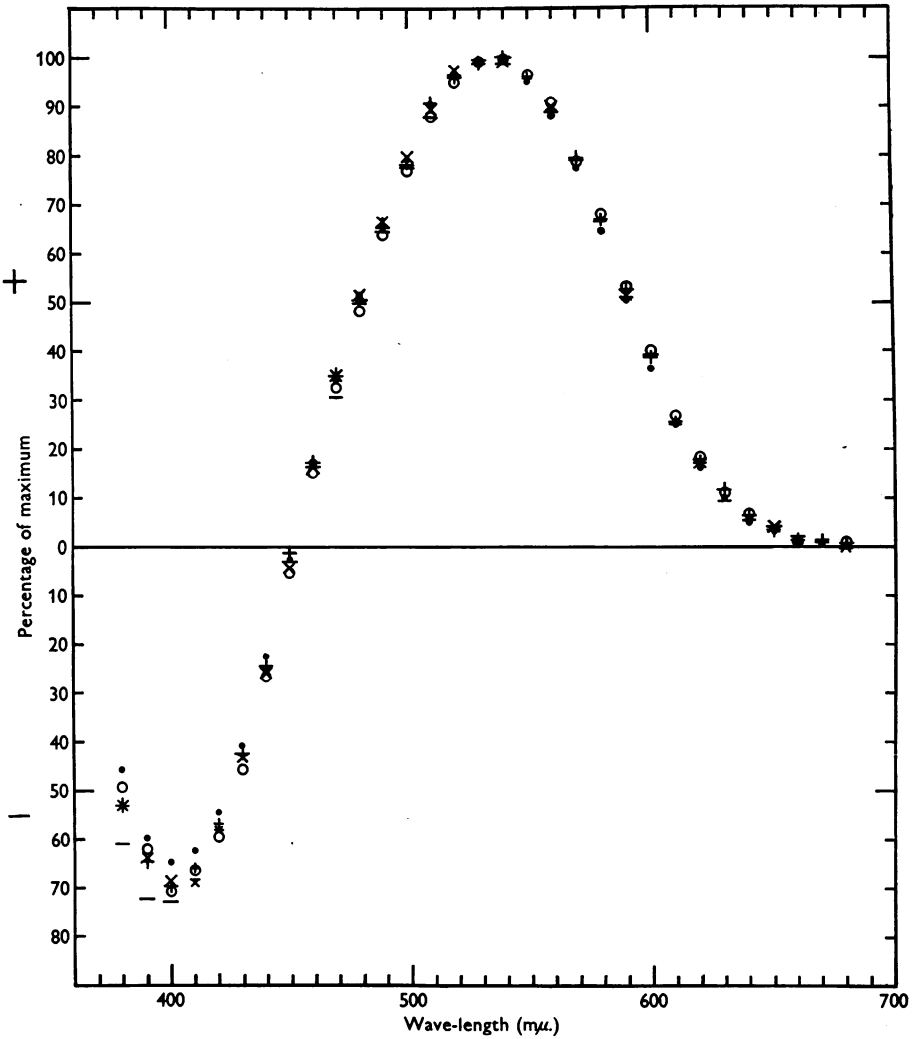

Fig. 5. Difference spectrum of the red-sensitive component in tench extracts (obtained by exposing solutions to light of wave-lengths which do not bleach the red-insensitive pigment). +, extract 8 (610 mμ.); −, extract 8 (650 mμ.); ×, extract 10 (610 mμ.); ○, extract 11 (600 mμ.); ●, extract 12 (630 mμ.). Mean pH, 8·6.

in all cases been multiplied by such factors that the values at the maximum (535 mμ.) become 100. The actual density losses at this wave-length are shown in Table 5.

The standard deviation of a density determination increases with increasing density. In the present series of experiments the lowest density was zero and the highest about 0·3. Over this range the standard deviation, σ_D, varies from 0·0004 to 0·0007. Taking 0·0006 as applicable throughout the measurements, and bearing in mind that difference spectra are the differences between two sets of measurements, the standard deviation at any point of the difference

TABLE 5. Details of bleaching experiments with extracts of tench and pike retinae.

See Figs. 5 and 6.

				Bleaching conditions				Density loss at 535 mμ.‡	
Fish	Extract no.*	Date of experiment	Method†	Duration (hr.)	Mean wave-length (mμ.)	Total wave-length Range (mμ.)	Stray light filter	Actual	Possible
Tench	8	27. ix. 50	A	2	610	594–626	None	0·120	0·134
Tench	8	28. ix. 50	B	2	650	631–669	None	0·057	(0·134)§
Tench	10	12. x. 50	B	4¼	610	597–623	None	0·093	0·093
Tench	11	3. xi. 50	B‖	2	600	587–613	Ilford 607	0·091	0·130
Tench	12	10. i. 51	B	16	630	615–645	Ilford 204	0·124	0·124
Pike	1	7. xi. 50	A	⅛	(White)	All	—	0·265	0·265
Pike	1	9/10. xi. 50	B	18	430	422–438	Ilford 601	0·142	0·251
Pike	1	10. xi. 50	B¶	2	530	517–543	Ilford 625	0·107	0·109

* Full details of the extracts, viz. dates of preparation and pH, are shown in Table 3.

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

‡ The density loss at 535 mμ. is a measure of the amount of visual violet. The actual loss is that resulting from the bleaching conditions set out in previous columns, the possible loss is that which would occur if the total amount of visual violet present at the beginning of the exposure were bleached.

§ Assumed to be the same as in the immediately preceding experiment.

‖ Solution previously partly bleached for 17 hr. with light of mean wave-length 430 mμ. (see Table 8, experiment of 2/3. xi. 50).

¶ Solution previously partly bleached for 18 hr. with light of mean wave-length 430 mμ. (this table, preceding experiment).

spectra is 0·0085 ($\sigma_D\sqrt{2}$). Since the difference spectra shown in Fig. 5 have been plotted with the values at 535 mμ. adjusted to 100, there is no variation at this point. In consequence, the standard deviation at any other point is 0·0012. Since the actual density losses at 535 mμ. range from 0·06 to 0·12 (Table 5) this standard deviation corresponds to 1–2 ordinate units. Fig. 5 shows that the difference spectra obtained under various conditions (Table 5) are in agreement within experimental error except at the short wave-length extreme of the spectrum where the scatter is greater than expected.

The difference spectrum of the pigment present in pike extracts (visual violet) was obtained under nearly identical conditions (pH 8·24) by a number of methods. The difference spectrum for white light is shown in Fig. 3. On

another sample of the same extract, the difference spectrum for blue light (430 mμ.) was determined by method B. The bleaching was interrupted after the solution had been approximately half bleached and was completed by

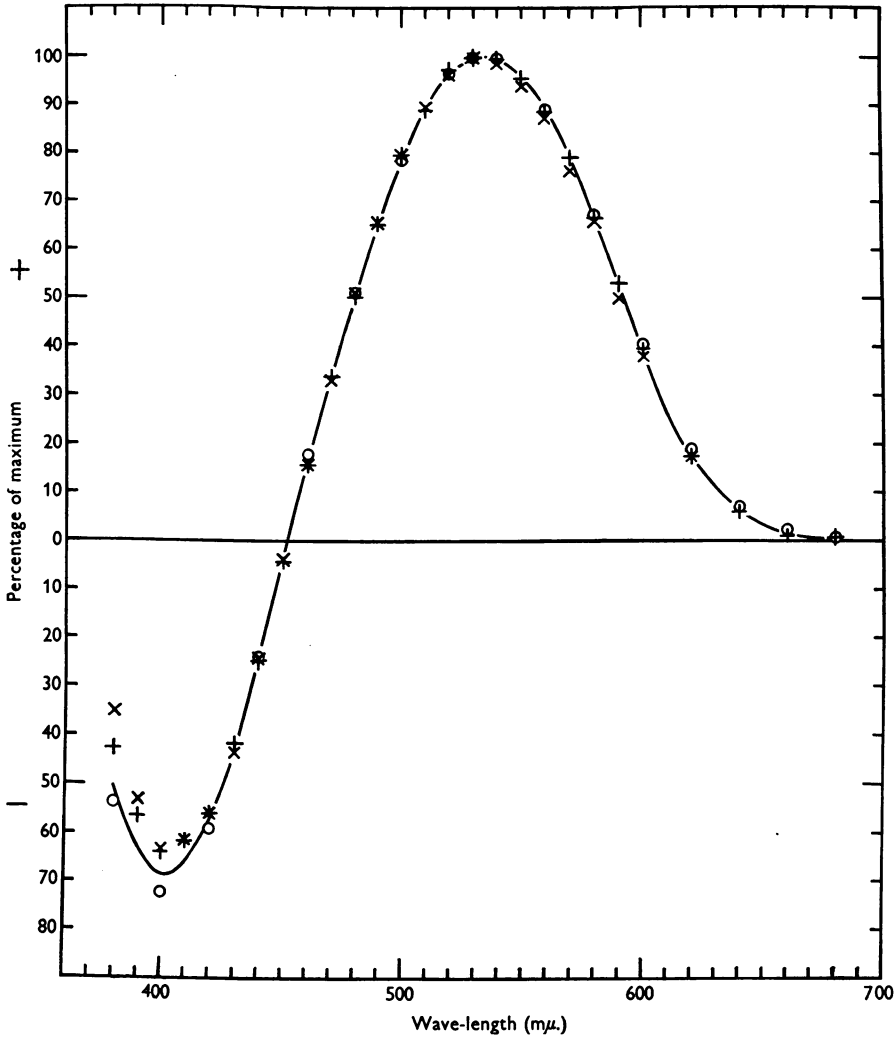

Fig. 6. Comparison of the difference spectrum of the visual pigment of pike (visual violet) with that of the red-sensitive pigment of tench. ○, + and ×, difference spectra for pike to white, 430 and 530 mμ. respectively (extract 1, pH 8·24); ———, average difference spectrum for the red-sensitive pigment of tench (Fig. 5), mean pH 8·6.

exposure to green light (530 mμ.), thus giving the difference spectrum for light of this wave-length also.

The three difference spectra obtained ('white', green and blue) are shown in Fig. 6. As in the case of the tench the results have been multiplied by suitable

factors to bring the maximum to 100 in each case. The actual density losses at 535 mμ. are given in Table 5. Apart from minor discrepancies, again in the short wave extreme of the spectrum, the difference spectra are in close agreement.

The continuous line in Fig. 6 represents the mean of the results, shown in Fig. 5, for the red-sensitive component of the tench extracts. The agreement with the results for pike is within experimental error.

From these experiments, the following conclusions are drawn:

(*a*) The 'red-sensitive' component of the tench is identical with the photosensitive pigment of the pike (visual violet). This, however, does not preclude the possibility that the protein moiety is different in the two cases as this may not contribute to absorption in the visible.

(*b*) The pigment visual violet cannot be resolved into components by partial bleaching with lights of different spectral composition ('white', and 650, 630, 610, 600, 530 and 430 mμ.). It is therefore a homogeneous pigment.

(*c*) Variation of the wave-length of the bleaching light does not affect the nature of the end product (the indicator substance analogous to the indicator yellow of visual purple) and hence is presumably without effect on the course of the bleaching.

(*d*) Since the difference spectra of Figs. 5 and 6 are based on changes of density at the maximum (535 mμ.) varying from 0·057 (extract 8, tench) to 0·265 (extract 1, pike), i.e. a fivefold range of concentration, Beer's and Lambert's laws of light absorption (implicitly assumed in adjusting all maxima to 100) are obeyed by visual violet and also by the indicator substance ($\lambda_{max.}$ = 405 mμ. at pH 8·24 – 8·68) formed on bleaching.

The red-insensitive component of tench extracts

Having identified the red-sensitive component of tench retinal extracts with visual violet, further experiments were carried out to determine the difference spectrum of the red-*in*sensitive component and also to ascertain whether this, too, is homogeneous or, alternatively, a mixture of red-insensitive pigments.

The difference spectrum of the red-insensitive component was obtained in the following ways:

(1) By subtracting the density changes due to the bleaching of visual violet from the total density changes caused by exposure of a solution to white light.

(2) By observing the further changes in density, on exposure to white light, of a solution in which the visual violet had been either partially or completely removed by a previous exposure to light of long wave-length. In cases where the visual violet had been completely removed, the ensuing changes on exposure to white light were due solely to bleaching of the red-insensitive component; in cases where the visual violet had been only partially removed, however, the changes caused by white light were due to the bleaching of both components.

In the above methods, the red-insensitive component was completely bleached by white light. To establish the homogeneity, or otherwise, of the red-insensitive material, partial bleaching is necessary. The following additional methods were used.

(3) Partial removal of visual violet by exposure to long wave-length light as in method 3, followed by exposure to monochromatic light of shorter wave-length which caused the bleaching of some of the red sensitive material and some of the residual visual violet.

(4) Initial exposure to light of short wave-length. This caused partial bleaching, both of the red-insensitive material and of the visual violet.

In all the above methods, with the exception of that in which the visual violet was first *completely* removed, the difference spectra obtained required correction for the changes in density due to bleaching of visual violet. To apply this correction it is necessary to know the amount of visual violet bleached as well as its difference spectrum. The method of calculating the correction was the same in all cases and may be conveniently illustrated for the case of method 1.

The changes in density caused by exposure of a typical tench solution (extract 12) to white light are shown in Fig. 7 (filled circles). This curve is composite, consisting of the algebraic sum of the difference spectra of visual violet and of the red-insensitive component. The absorption of the red-insensitive material is negligible at wave-lengths of 560 mμ. and longer (Fig. 4). Consequently, that portion of the composite difference spectrum beyond 560 mμ. is due to visual violet alone. Since the difference spectrum of visual violet is accurately known (Fig. 5), an estimate of the visual violet content of the solution can be made for each observation from 560 mμ. to the last observation at 630 mμ. (Table 6). The first five estimates for the amount of visual violet are in fairly good agreement, thus confirming that absorption due to the red-insensitive component is negligible in this region. The others are less reliable, partly because the change in density (column 2) on which they are based is smaller and partly because the difference spectrum of visual violet is here (for the same reason) not so accurately known. The weighted mean density change at 535 mμ. due to visual violet is 0·129. From this mean figure and the difference spectrum for visual violet the density changes due to the bleaching of this pigment can be calculated. Since the bleaching of visual violet results in a loss of density at wave-lengths greater, and a gain at wave-lengths less than 450 mμ., the 'visual violet corrections' above and below this wave-length are of opposite sign. The magnitude of the corrections at each wave-length are represented by the lengths of the dotted lines in Fig. 7. The density changes remaining, after subtracting these, contributed by the bleaching of visual violet, are due to the red-insensitive component.

In Fig. 8A are shown the results of eight determinations of the difference spectrum of the red-insensitive component. Six of these difference spectra were

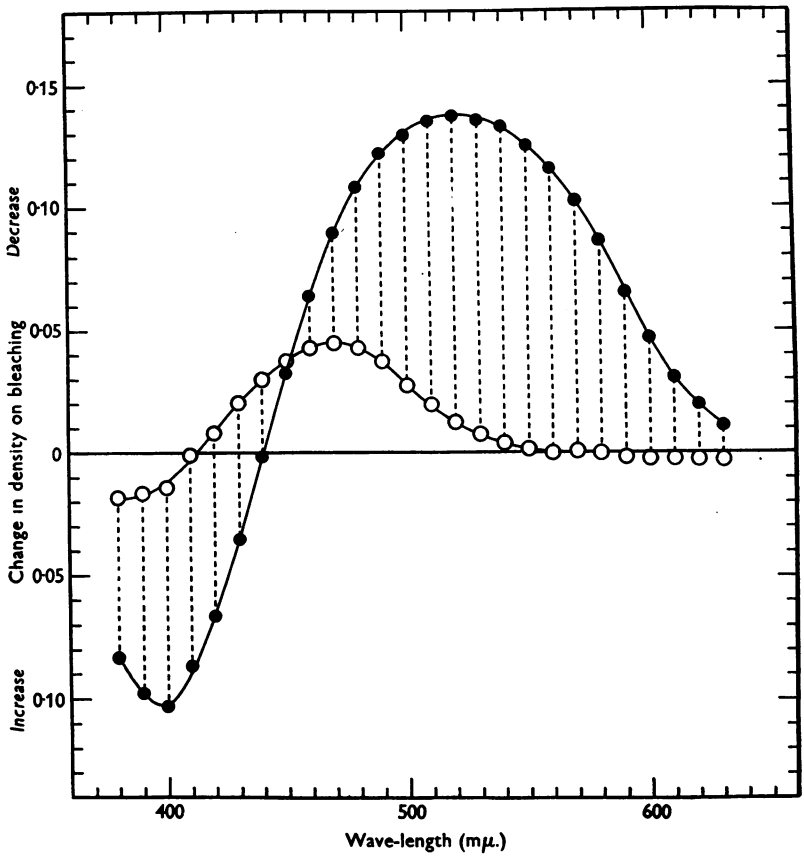

Fig. 7. Calculation of the difference spectrum of the red-insensitive pigment from the total density changes caused by exposure to white light. The density changes due to the bleaching of visual violet (lengths of the interrupted lines) are subtracted from the total changes (●) leaving a remainder (○) due to the red-insensitive pigment. Tench extract 12, pH 8·40.

TABLE 6

Wave-length (mμ.)	Change in density (Fig. 7)	Corresponding density change at 535 mμ. (calculated)
560	0·1161	0·1296
570	0·1024	0·1303
580	0·0862	0·1295
590	0·0656	0·1264
600	0·0473	0·1233
610	0·0309	0·1201
620	0·0198	0·1138
630	0·0114	0·1056

calculated from the composite changes in density taking place on bleaching solutions with white light (method 1) and two from the changes caused by exposure to white light following, in one case partial and in the other total, removal of visual violet by a prior exposure to light of long wave-length (method 2). In Fig. 8B are a further five determinations. Two were obtained from the changes in density caused by bleaching solutions with light of short wave-length (method 4) and the remaining three from the changes following exposure to white or monochromatic light after an initial partial bleaching (method 3). In all cases the difference spectra were multiplied by suitable factors to bring the maxima to 100. Details of the experiments illustrated in Fig. 8A, B are given in Tables 7 and 8 respectively.

Thus the results shown in Fig. 8A were obtained by bleaching of the whole, and those in Fig. 8B by bleaching of only a part, of the total quantity of the red-insensitive component present in the solutions.

The density changes from which the difference spectra of Fig. 8 were derived were subject to approximately the same absolute errors (standard deviation 0·0012) as were those of the visual violet determinations (Figs. 5 and 6). The errors were, however, proportionately much greater, especially in those cases where only a fraction of the total quantity of red-insensitive component was bleached (Fig. 8B). The actual density changes at the maximum (Tables 7 and 8) range from 0·056 to 0·026 in Fig. 8A and from 0·036 to 0·012 in Fig. 8B. A standard deviation of 0·0012 thus corresponds to $2\frac{1}{2}$–5 ordinate units in the former case and to $3\frac{1}{2}$–10 in the latter. In addition, the difference spectra are subject to errors of uncertain magnitude arising from any error in the visual violet corrections. Within experimental error, therefore, the results form individually homogeneous groups except at the short wave-length extreme of the spectrum where the scatter is larger than expected.

The mean curves of Fig. 8 are in precise agreement on the long wave-length side of the maximum, but there are minor differences both in the positions of the maxima and in the short wave-length limbs. Thus the curve of Fig. 8A has a maximum at 469 mμ. and crosses the axis at 408 mμ. while that of Fig. 8B has a maximum at 465 mμ. and crosses at 399 mμ.

Examination of the experimental conditions revealed that when the experiments were of short duration a 'narrow' difference spectrum was obtained and conversely, when they were of long duration, a 'broad' one. The results shown in Fig. 8A were mainly of short duration (bleachings with white light) and those in Fig. 8B mainly of long duration (bleachings with monochromatic light). The spread of results within each group could in general be similarly connected with the time factor.

Other observations, not reported here, indicated that these discrepancies were due to changes on ageing of the impurities (absorbing principally in the short wave extreme of the spectrum) and not to any lack of homogeneity of the red-insensitive component.

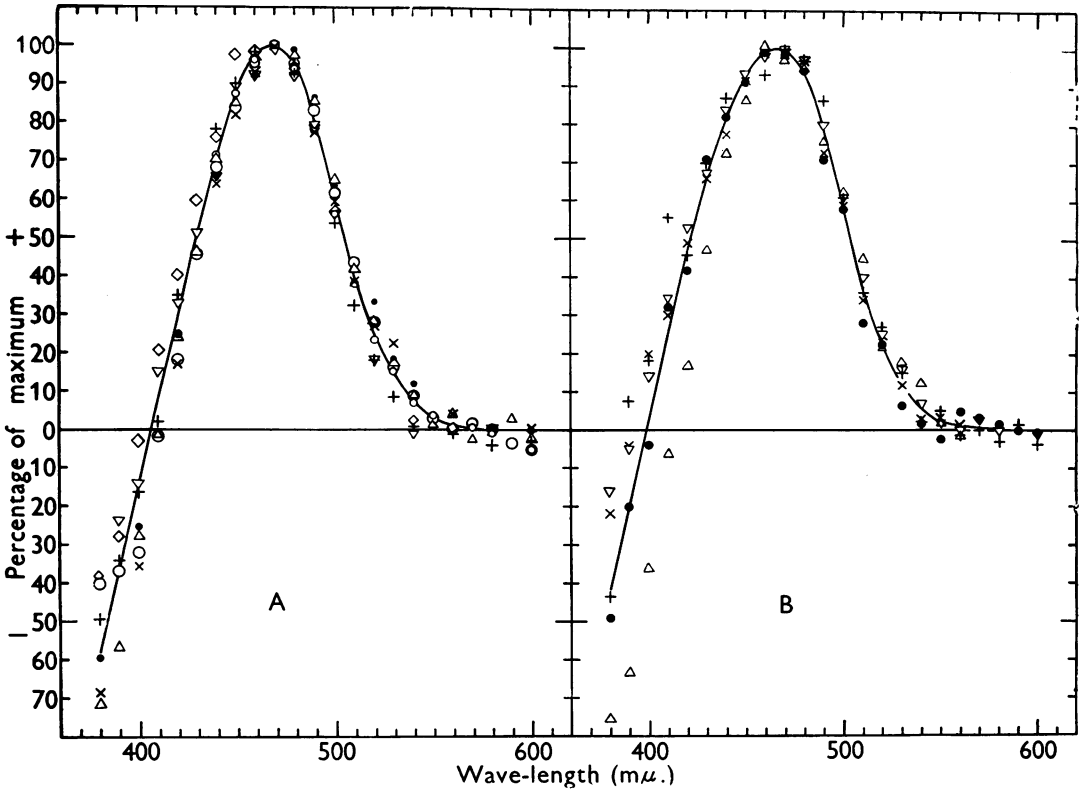

Fig. 8. Difference spectrum of the red-insensitive pigment. A, difference spectra obtained by complete bleaching of the red-insensitive component with white light; mean pH 8·3. ●, extract 2 (*a*); ○, extract 2 (*b*); ◇, extract 5; △, extract 7; ×, extract 10 (*a*); ▽, extract 10 (*b*); +, extract 11; ○, extract 12. B, difference spectra obtained by partial bleaching of the red-insensitive pigment with white or monochromatic light; mean pH 8·6. △, extract 7; +, extract 8 (*a*); ×, extract 8 (*b*); ●, extract 11 (*a*); ▽, extract 11 (*b*).

The mean curve shown in Fig. 8A is thus a more reliable estimate of the difference spectrum of the red-insensitive component than that of Fig. 8B though, as already mentioned, they are in precise agreement at the longer wave-lengths.

From these experiments, therefore, the following conclusions are drawn:

(*a*) The red-insensitive component cannot be resolved by partial bleaching with light of various spectral compositions ('white', 430, 450 and 530 mμ.). It is therefore a homogeneous pigment.

(*b*) The spectral composition of the bleaching light is without effect on the course of the bleaching.

(*c*) Since the difference spectra of Fig. 8 are based on changes of density at the maximum varying from 0·012 to 0·056 (Tables 7 and 8), Beer's and Lambert's

TABLE 7. Details of bleaching experiments with tench extracts. In these experiments the total amount of red-insensitive pigment originally present in solution was bleached. See Fig. 8A

Extract no.*	Date of experiment	Exposure previous to experiment	Method†	Time of exposure to 'white' light (min.)	Amount bleached‡	
					Visual violet	Red-insensitive pigment
2	16. v. 50(*a*)	None	A	10	0·061	0·031
2	17. v. 50(*b*)	None	A	20	0·065	0·037
5	28. vi. 50	2 hr.λ=610 mμ.	A	20	0·011	0·029
7	21. ix. 50	None	A	20	0·037	0·028
10	11. x. 50(*a*)	None	A	15	0·090	0·032
10	12. x. 50(*b*)	4½ hr.λ=610 mμ.§	B	15‖	Nil	0·026
11	1. xi. 50	None	A	20	0·175	0·056
12	9. i. 51	None	A	20	0·129	0·045

* Full details of the extracts, viz. dates of preparation and pH are given in Table 3.

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

‡ The amount of visual violet bleached as a result of the bleaching conditions set out in the preceding column is indicated by that change in density at 535 mμ. due to loss of visual violet; similarly, the amount of red-insensitive pigment bleached is indicated by that change in density at 469 mμ. due to loss of red-insensitive pigment.

§ See Table 5 for details of preceding experiment on this solution.

‖ Just prior to exposure to 'white' light for 15 min. the solution had been exposed to monochromatic green light (530 mμ., wave-length range 517–543 mμ.) for 30 min. This caused very little change in density (see text). The last two columns give the total change due to green and white light.

TABLE 8. Details of bleaching experiments with tench extracts. In these experiments only a proportion of the total amount of red-insensitive pigment originally present was bleached in any one experiment. See Fig. 8B

Extract no.*	Date of experiment	Exposure previous to experiment	Method†	Bleaching conditions				Amount bleached‡	
				Duration	Mean-wave-length (mμ.)	Total wave-length range (mμ.)	Stray light filter	Visual violet	Red-insensitive pigment
7	22. ix. 50	None	A	2 hr.	450	441–459	Ilford 621	0·024	0·019
8	27. ix. 50(*a*)	2 hr.λ=610 mμ.§	A	1 hr.	530	515·5–544·5	None	0·012	0·013
8	27. ix. 50(*b*)	2 hr.λ=610 mμ. and 1½ hr.λ=530 mμ.‖	A	15 min.	'White'	All	—	0·003	0·019
11	2/3. xi. 50(*a*)	None	B	17 hr.	430	422·5–437·5	Ilford 601	0·036	0·012
11	3. xi. 50(*b*)	17 hr.λ=430 μm. and 2 hr.λ=600 mμ.¶	B	30 min.	'White'	All	—	0·039	0·036

* Full details of the extracts, viz. dates of preparation and pH are given in Table 3.

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

‡ The amount of visual violet bleached as a result of the bleaching conditions set out in the preceding columns is indicated by that change in density at 535 mμ. due to loss of visual violet; similarly, the amount of red-insensitive pigment bleached is indicated by that change in density at 469 mμ. due to loss of red-insensitive pigment.

§ See Table 5 for details of preceding experiment on this solution.

‖ See preceding experiment, this table.

¶ For details of bleach with 430 mμ., see preceding experiment, this table; for details of bleach with 600 mμ. see Table 5.

laws hold for the red-insensitive pigment over this fivefold concentration range.

(*d*) The difference spectrum of the red-insensitive pigment has a maximum between 469 and 465 mμ. and crosses the axis at between 408 and 399 mμ. in alkaline solution. The former figures are more reliable.

(*e*) The increase in absorption at wave-lengths shorter than 408–399 mμ. which takes place on bleaching, indicates the formation of a photoproduct analogous to the 'indicator yellows' of visual purple and visual violet.

Experiments with acidified extracts of tench retinae

The foregoing experiments were made on alkaline solutions. To ascertain whether the spectral absorption of the red-insensitive pigment was dependent on the hydrogen-ion concentration or, like visual purple, substantially independent of it (Lythgoe, 1937), experiments were carried out on tench extracts which had been acidified to a pH of 4·2. Some preliminary experiments (extract 9, pH 4·17) showed that at this pH the unexposed solutions were thermally unstable, a gradual loss of density taking place in darkness. The products of bleaching formed on exposure of the solutions to light were even more unstable, a solution bleached by monochromatic light of wave-length 610 mμ. showing a fairly rapid loss of density in darkness maximal at 480–490 mμ. (due to thermal fading of the acid form of the indicator substance).

Owing to this thermal instability, particularly of bleached solutions, and to the proximity of the wave-length of maximum absorption (480–490 mμ.) of the acid indicator of visual violet to that of the red-insensitive pigment, the technique of preferential bleaching with monochromatic light, developed for stable alkaline solutions, was not suitable. It was accordingly decided to make a direct and timed comparison between the spectral absorption of unbleached acid and alkaline solutions.

An extract of tench retinae (extract 10) was therefore divided into two equal portions. One portion was made alkaline (pH 8·66) by the addition of sodium borate solution and the other acid (pH 4·22) by the addition of exactly the same volume of potassium hydrogen phthalate solution (see Table 3). The accuracy of the dilution was checked by weighing. The times quoted in brackets in the following description were measured from the time of addition of the acid buffer.

The two solutions were first centrifuged. Optical cell 14 was filled with alkaline solution and cell 11 with acid solution. The density of cell 11, using cell 14 as reference, was then measured (started at 30 min.; finished at 90 min.). The density differences are shown as curve 1 of Fig. 9. After an interval the measurements were repeated and are shown as curve 2 (started at 168 min.; finished at 185 min.).

Both cells were then exposed, under identical conditions, to 'white' light

for 10 min., after which the density differences between the bleached solutions were measured (curve 3, started 200 min., concluded 228 min. and curve 4, started 228 min., concluded 250 min.).

The directions in which the observations were taken are indicated by the arrows. Curves 1–4 in Fig. 9 have all been corrected for the slight differences due to the cells themselves (ascertained by measuring the density of cell 11 filled with digitonin solution plus acid buffer relative to that of cell 14 filled with digitonin solution plus alkaline buffer.)

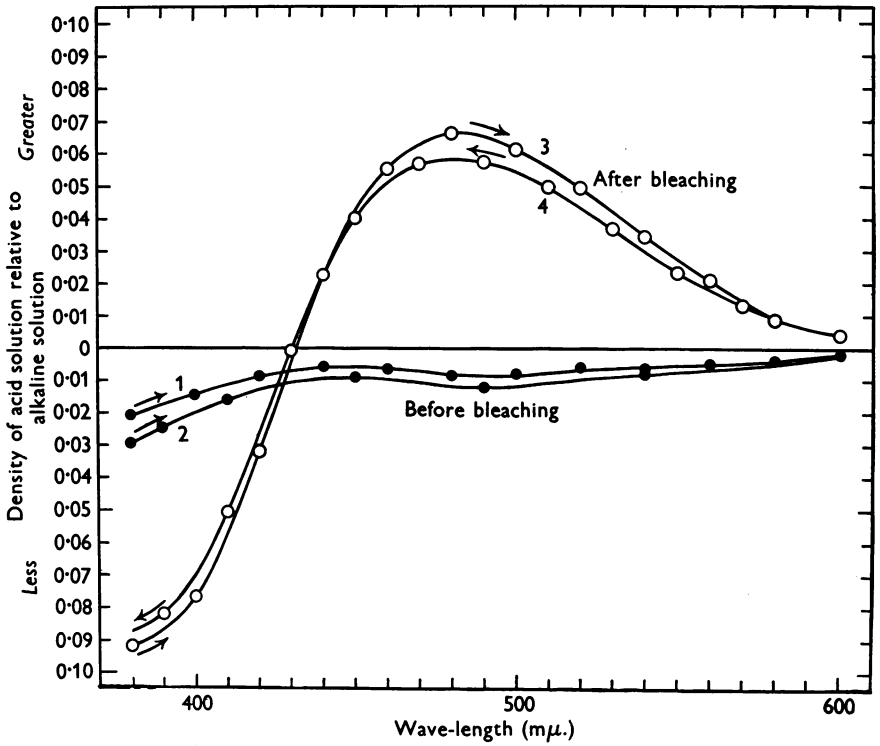

Fig. 9. The difference in absorption of acid and alkaline tench extracts before and after bleaching with white light. Curve 1, before bleaching; curve 2, ditto (later measurements); curve 3 after bleaching; curve 4, ditto (later measurements). The arrows indicate the order of taking observations. Extract 10: acid portion, pH 4·22; alkaline portion, pH 8·66.

The density differences between the unbleached solutions are thus shown by curve 1 and, at a later period, by curve 2 of Fig. 9. As the alkaline solution was known to be thermally stable the difference between these two curves must be due to thermal instability of the acid solution. Since, therefore, the determination of curve 1 was not commenced until 30 min. after the addition of the buffer solutions, the *initial* differences between the acid and alkaline solutions must have been even less than those indicated by curve 1. The differences

shown by curve 1 can thus be ascribed, at least in part, to the loss of visual pigments in acid solution by thermal decomposition during this interval. This interpretation is supported by the form of curve 1 (maximum at 490 mμ.) which is similar to the absorption spectrum of the unbleached solution itself.

In spite of these considerations, however, there still appears to be a residual difference, most marked in the violet, between the solutions, the alkaline solution being denser than the acid. A similar effect was noted by Lythgoe in his study of the effects of pH on the absorption spectrum of extracts of visual purple (Lythgoe, 1937). He considered that the greater density of the alkaline solutions at short wave-lengths was due to a dependence on pH of the absorption by impurities.

The differences in spectral absorption between acid and alkaline solutions of tench retinal pigments are thus of a minor character and, moreover, are ascribable to the effects of pH on the absorption of impurities and to thermal instability of the acid solution. The absorption spectra both of visual violet and of the red-insensitive pigment therefore are not markedly affected by pH.

The differences between the bleached solutions (curves 3 and 4), on the other hand, are of a major character. The portions of these curves below the axis approximate to the absorption of the alkaline forms of the mixed indicator substances of visual violet and red-insensitive pigment and those above the axis to the acid forms of these substances. This dependence on pH of the absorption of the bleached solution recalls the similar behaviour of visual purple solutions (Lythgoe, 1937).

The relative amounts of visual violet and red-insensitive pigment in the tench extracts

The relation between the amounts of visual violet and of the red-insensitive pigment in the tench extracts may be expressed as the ratio of the pigment densities at their respective absorption maxima. In alkaline solution, this is closely approximated by the ratio of the respective maximum density losses occurring on bleaching. The data are shown in Table 9.

TABLE 9

Extract	Change in density at $\lambda_{max.}$ on bleaching: Visual violet	Red-insensitive pigment	Ratio
2	0·063	0·034	1·9
7	0·037	0·028	1·3
8	0·134	0·032	4·2
10	0·090	0·032	2·8
11	0·175	0·056	3·1
12	0·129	0·045	2·9
	Mean (excluding extract 7)		3·0

The results for extract 7 are excluded, for in this case the retinae were extracted only once with digitonin.

The variable proportions of the two pigments may be due to a number of causes. Since, for example, the whole retina was not always removed, any regional variations would affect the ratio. Again, the ease of extraction of the two pigments may not be equal, in which case the quantity of digitonin per retina used (see Table 3), would affect the relative yields. The extraction of pigments, moreover, was not always complete, some variation of efficiency in this respect being noted with the different samples of digitonin used.

The mean result indicates that the approximate ratio of maximal densities of the two pigments is 3. Thus the extracts contained $3n$ times as much visual violet as of red-insensitive pigment, where n is the ratio of the extinction coefficient of the red-insensitive pigment to that of visual violet at their respective absorption maxima.

DISCUSSION

'Difference' spectra

When retinae are treated with stromalytic agents, pigments in addition to the desired visual pigments are extracted. Prior washing of the retinae will remove most, but not all, of these unwanted pigments.

The absorption due to these residual 'impurities' may be eliminated by measuring the densities of the retinal extract with reference to the bleached extract. In earlier work (Köttgen & Abelsdorff, 1896) and even as late as 1936 (Bayliss *et al.* 1936) it seems to have been believed that this procedure yielded the true absorption spectrum of the visual pigment. This would be true, only if the products of bleaching were colourless. This, indeed, was believed by Köttgen & Abelsdorff (1896) and Trendelenburg (1904), though the researches of Kühne, confirmed by Garten (1906), indicated that the products of bleaching were yellow in colour.

Lythgoe's discovery that 'indicator yellow', the final bleaching product of visual purple, has an absorption spectrum dependent on pH (Lythgoe, 1937) explained the previous lack of agreement. The very pale yellow colour of alkaline bleached visual purple solutions had been overlooked or ascribed to impurities, while the deep yellow colour of acid bleached solutions was in conformity with the observations of Kühne and Garten.

Since workers prior to Lythgoe did not control the pH of their solutions, the interpretation of some of the earlier work is difficult and, to this extent, Wald's strictures on 'difference spectra' are justified (Wald, 1938, 1949). Wald has presented a large proportion of his results in the form of absorption spectra of whole retinal extracts, i.e. spectra measured with reference to a solution of digitonin. Such spectra, while approximating more or less to the true absorption spectra of the visual pigments, include absorption due to variable amounts and kinds of impurities. A strict comparison of results between different species and even between different extracts from the same species is thus impossible.

The present investigation has shown that, under suitably standardized conditions, 'difference' spectra are accurately reproducible and, through elimination of the effects of absorption by impurities, afford a means of comparing results from different species with a high degree of precision.

In the work described in this paper conditions were standardized for the majority of the experiments at a pH of 8·5 and a temperature of 20·0° C. Since the times of exposure for bleaching the retinal extracts varied between 15 min. and 17 hr., the transient stage of the bleaching process (Lythgoe, 1937; Lythgoe & Quilliam, 1938; Wald, 1939) had passed to the stable 'indicator' stage before the measurements on the bleached solutions were begun. This was confirmed by the stability of absorption of bleached solutions during measurement over prolonged periods.

Thus the measurements of absorption spectra in this investigation were made either on stable unbleached solutions or on bleached solutions at the stable 'indicator' stage or, of course, on partly bleached solutions in which the bleached moiety had reached the stable 'indicator' stage. In consequence all 'difference' spectra are differences between the absorption spectrum of the visual pigment and that of the indicator substance (alkaline form). Under the conditions chosen the absorption spectrum of the indicator substance is furthest removed from that of the parent pigment. Consequently, in the 'alkaline' difference spectrum mutual distortion of the two spectra is reduced to a minimum; the 'positive' portion approximating to the true spectrum of the visual pigment and the 'negative' portion to that of the indicator substance.

The wave-length of maximum absorption for visual violet

Köttgen & Abelsdorff (1896) found that the maximum of absorption of visual violet was at 535–540 mμ. Wald (1949) has criticized this figure as an artifact due to their measurement of difference spectra, viz. the difference in absorption between unbleached and bleached solutions. The $\lambda_{max.}$ of a difference spectrum is displaced towards longer wave-lengths compared with that of the visual pigment but, except in strongly acid solutions, the amount of displacement is small. Thus Lythgoe (1937) has shown that even at pH 6·1 the $\lambda_{max.}$ of the difference spectrum for visual purple is 506 mμ., only 4 mμ. from the true position (502 mμ.) while at pH's of 8 or more the displacement is negligible.

Wald states (1949) that the true absorption maximum of visual violet is at 522 ± 2 mμ., this being the absorption maximum of his extracts with reference to a digitonin solution. The presence of yellow impurities, which absorb more strongly as the short wave-length end of the spectrum is approached, has the effect of displacing the $\lambda_{max.}$ of an extract to shorter wave-lengths. Except in highly purified solutions the amount of displacement is likely to be serious. Unpurified extracts of visual purple, for example, may have an absorption

maximum at 490 mμ., 12 mμ. below the absorption maximum of pure visual purple. Wald's estimate of 522 mμ. for visual violet could, likewise, be influenced by the presence in his extracts of yellow impurities.

Lower and upper limits to the true absorption maximum of a visual pigment are thus given by the absorption maxima of the extract itself and of its difference spectrum respectively. From the results for pike (Figs. 3 and 6) these limits for visual violet are 530 and 535 mμ. The true absorption maximum for visual violet is probably nearer the latter figure, say at 533 ± 2 mμ.

The red-insensitive pigment of the tench

Wave-length of maximum absorption. Difference spectra of the red-insensitive pigment found, in addition to visual violet, in the tench retina are shown in Fig. 8. The mean position of the maximum for the A-group of results is 469 mμ., while that for the B-group is 466 mμ. Of these, the former is more reliable.

The displacement of the $\lambda_{max.}$ of a difference spectrum, by comparison with that of the parent pigment, is about 2 mμ. for visual purple in alkaline solution. Assuming a similar displacement in the present instance, the true absorption maximum of the red-insensitive pigment is at 467 mμ., the error probably being within ± 3 mμ. Thus the maximum, 475 ± 5 mμ., previously reported (Dartnall, 1950), was slightly high.

Independence. The claim, first advanced in a previous note (Dartnall, 1950), that the red-insensitive pigment of the tench retina is a visual pigment ('visual red') has been criticized by Collins & Morton (1951). These authors describe an experiment with a solution of rhodopsin (i.e. visual purple) prepared from isolated rods of cattle retinae. This solution was 80–90 % bleached with white light (60 W. lamp) and its absorption spectrum then measured. Completion of the bleaching with white light then resulted in a further loss of density, maximal at 480 mμ. This latter they regard as due to the fading of mixture of transient orange, acid indicator yellow and iso-rhodopsin (i.e. regenerated visual purple). They state that this experiment shows that the red-insensitive pigment of the tench 'has a doubtful and inferential existence'.

Since transient orange, acid indicator yellow and regenerated visual purple are derivatives of visual purple, a pigment not present in the tench extracts, it is assumed that Collins & Morton intend their experiment to imply the possibility of a parallel phenomenon in visual violet solutions.

The red-insensitive pigment cannot, however, be the acid indicator of visual violet since the experiments were carried out in alkaline solution; neither can it be the 'transient' of visual violet since this is thermally unstable (Wald, 1939), nor can it be regenerated visual violet because regeneration was not observed, the bleached solutions being thermally stable.

The larger issue, that is the independence or otherwise of the red-insensitive pigment appears to be settled in favour of independence by the following. The

'positive' and 'negative' portions of a difference spectrum are approximations to the true absorption spectra of the parent pigment and of its product of bleaching respectively. If the red-insensitive pigment were merely the product of bleaching of visual violet, then the negative portion of the difference spectrum should have a maximum in the region of 467 mμ., not at 405 mμ. as is the case (Figs. 5 and 6). If the mutual distortion, due to proximity of the maxima 533 mμ. for visual violet and 467 mμ. for the red-insensitive pigment, were, however, sufficiently great to give a maximum for the 'negative' portion of the difference spectrum at 405 mμ. then this same distortion would result in a maximum for the 'positive' portion at about 590 mμ., instead of at 535 mμ. (Figs. 5 and 6). Additional evidence for the independence of the red-insensitive pigment is afforded by the shapes of the absorption spectra of unbleached extracts of tench retinae (example, Fig. 3) which indicated the presence of this pigment in the unexposed solutions and also by the absence of the red-insensitive pigment from the pike extract which contained only visual violet, identical with that of tench.

Comparison with known visual pigments. The extraction of the red-insensitive pigment from tench retinae requires the use of a stromalytic agent, viz. digitonin; for example it does not pass into solution on treating the retinae with acid buffer solutions, a process which removes much other absorbing material. It is stable in the dark but bleaches on absorption of light, its photosensitivity being comparable with that of visual violet. Its absorption spectrum is not materially affected by pH (between pH 4·2 and 8·7). Its products of bleaching have a greater absorption at the short wave-length extreme of the spectrum than the parent pigment. In these respects the red-insensitive pigment resembles the known visual pigments.

Fig. 10 shows the difference spectra of iodopsin (Wald, 1937*b*), visual violet, visual purple and the red-insensitive pigment of the tench. The iodopsin curve is from Bliss (1946); the visual violet curve represents the mean of the results shown in Fig. 5; the visual purple curve is from Lythgoe (1937), and the red-insensitive pigment curve from Fig. 8A.

The main feature of the comparison is the broadening of the spectra as their location advances towards longer wave-lengths. In Fig. 10 the difference spectra have been plotted with wave-length abscissae. With frequency abscissae, for which there is a theoretical basis (Stiles, 1948), the four difference spectra are nearly identical in shape. This is shown in Fig. 11 in which the difference spectra, plotted with a frequency scale, have been shifted along the axis of abscissae so that their maxima correspond. The figure shows that the portions of the difference spectra above the 'base-line' are effectively superimposable. The agreement in shape cannot, however, be regarded as proof of the homogeneity of iodopsin, since certain combinations of the difference spectra of contiguous visual pigments yield curves which are of similar shape.

The agreement in shape between the upper portions of the difference spectra indicates that the absorption spectra of the pigments themselves are identical in shape on a frequency basis. In consequence, a knowledge of the true absorption spectrum of one pigment would make it possible to calculate the absorption spectra of the others.

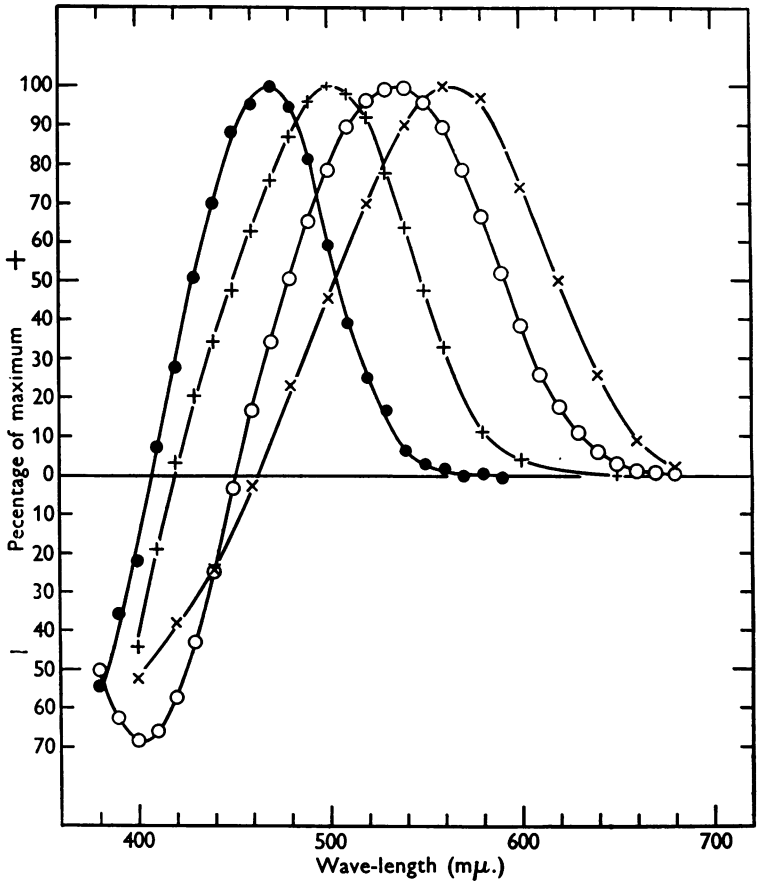

Fig. 10. Comparison of the difference spectra (wave-length abscissae) of the known visual pigments. ●, the red-insensitive pigment at pH 8·3 (mean of results in Fig. 8A); +, visual purple at pH 8·5 (Lythgoe, 1937); ○, visual violet at pH 8·6 (mean of results in Fig. 5); ×, iodopsin, pH not recorded (Bliss, 1946).

These comparisons indicate that the red-insensitive pigment and probably iodopsin also, form, with visual purple and visual violet, a series of chemically related compounds. It is possible that the chromophore parts of the molecules are successive members of a homologous series. It has, in fact, already been suggested that the visual purple and visual violet chromophores are based respectively on vitamin A_1 and vitamin A_2 (Wald, 1939, 1949).

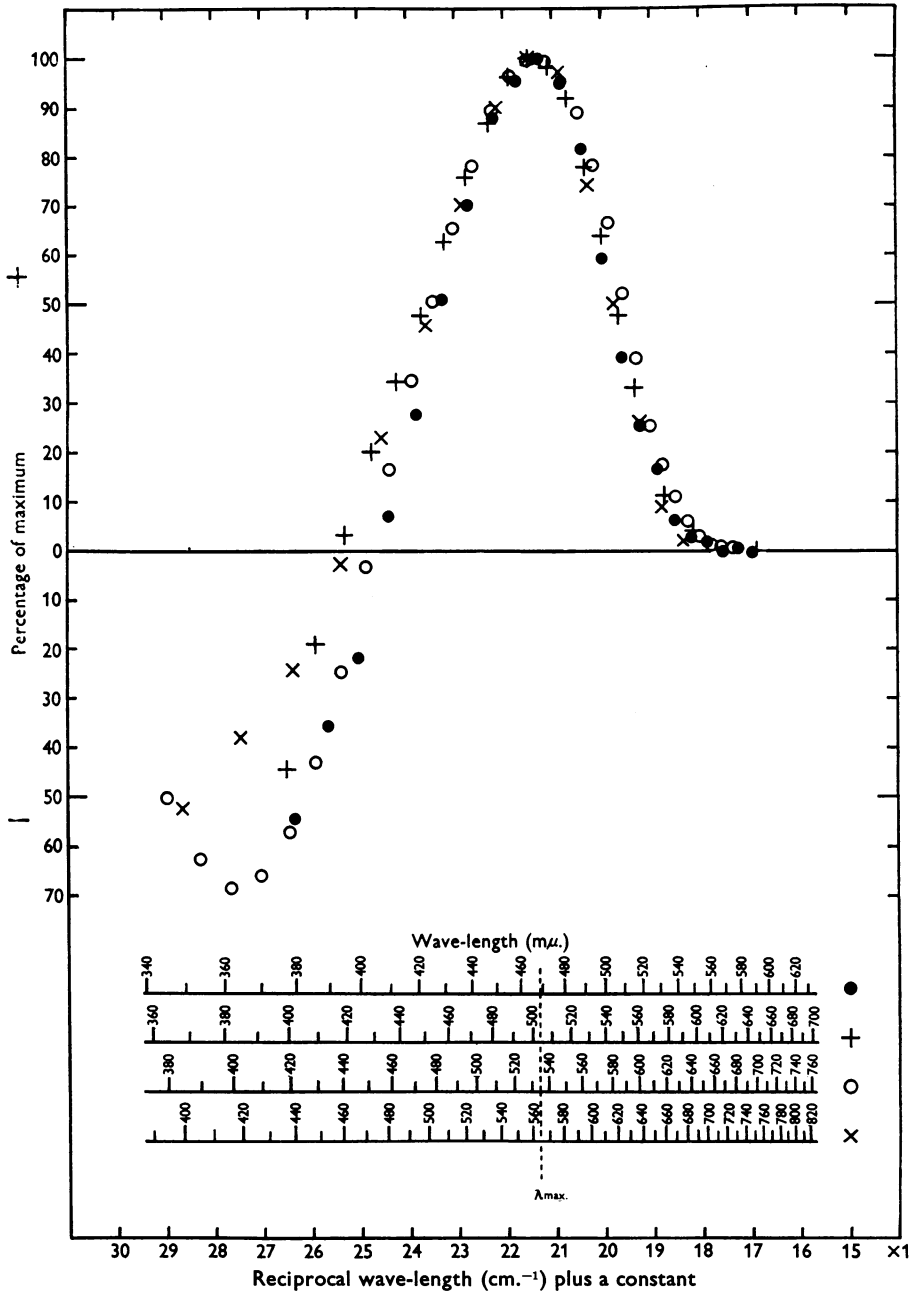

Fig. 11. Comparison of the difference spectra (frequency abscissae) of the known visual pigments. The spectra have been shifted along the axis of abscissae so that their maxima coincide with that of the red-insensitive pigment. Wave-lengths appropriate to each pigment are also shown. ●, the red-insensitive pigment; +, visual purple; ○, visual violet; ×, iodopsin.

For the same reasons the red-insensitive pigment is considered to be a visual pigment. Proof of this must, however, await the demonstration of retinal or behavioural activity in the tench in conformity with the presence of this substance in its retina. Unfortunately, Granit's micro-electrode studies of this animal in the light-adapted state were not carried below 500 mμ. (Granit, 1941). He has, however, recorded activity of the frog's retina in this spectral region and the sensitivity/wave-length curve found (Granit, 1943) is in fair agreement, both as regards shape and position of maximum, with that for the red-insensitive pigment.

Nomenclature

The term 'visual purple' is an equivocal translation of 'Sehpurpur' (Wald, 1949). Solutions of visual purple as ordinarily prepared are red. This, however, may be due to the presence of yellow impurities which would reduce the intensity of blue light otherwise transmitted. Lythgoe's solutions, which were nearly free from absorbing impurities, had a 'purple tint' (Lythgoe, 1937).

The term 'Sehpurpur' was also used to describe the pigment with $\lambda_{max.}$ = 535–540 mμ. (visual violet) (Köttgen & Abelsdorff, 1896). This lead was continued by Bayliss *et al.* (1936) who designated as varieties of visual purple the pigments with $\lambda_{max.}$ ranging from 500 to 540 mμ. they found in various fish. Wald's work indicates that intermediate absorption spectra are due to mixtures of visual purple and visual violet, but this has not been proved, and it is possible that visual pigments exist between visual purple and visual violet.

There are thus two English nomenclatures. In one the terms 'visual purple' and 'visual violet' are used to describe the pigments with $\lambda_{max.} = 502$ mμ. and $\lambda_{max.} = 533$ mμ. respectively, leaving no provision for the naming of possible intermediate pigments; in the other 'visual purple' is used as an unspecific name for all these pigments, the term 'visual violet' remaining for the description of the pigment with $\lambda_{max.} = 560 - 570$ mμ. ('iodopsin').

The American nomenclature is based on Kühne's other name for visual purple, 'rhodopsin'. Visual violet is called 'porphyrhopsin' and the pigment from chicken retinae 'iodopsin'. As in the English nomenclature, little scope is left for the naming of new pigments.

The difficulty in choosing a name for the red-insensitive pigment of tench emphasizes the shortcomings of existing systems of nomenclature. When proposing the name 'visual red' for this pigment (Dartnall, 1950) the author had been influenced by the naming of the contiguous pigment, visual purple, and by the necessity for reserving the colours, orange and yellow, to describe hypothetical pigments with absorption maxima located at even shorter wavelengths. The new pigment is orange or possibly even yellow. In any case 'visual red' is not an acceptable name, 'sehrot' having once been used for visual purple and 'visual red' for a carotenoid derivative, not visually significant obtained from cattle retinae (Krause, 1946).

The English and American systems of nomenclature are both based on a description of the colour of the pigments. However, the absorption spectra of these are not accurately known on the short wave-length sides and hence the pigment colours are not known with certainty. Moreover, the visual pigments are probably dichroic, in which case their colour would depend on concentration. Another difficulty is that pigments with intermediate spectra, which the future may reveal, would require the use of hybrid colour names. Finally, the visual pigments have colour names roughly complementary to those of the spectral regions in which they absorb most strongly, i.e. in which their physiological activity is greatest. This will lead to anomalies when the visual pigments are related with visual functions. For these reasons the colour basis is unsatisfactory.

The close agreement between the absorption spectra of visual purple and visual violet and the relevant scotopic sensitivity curves indicates that the absorption spectra of the visual pigments in aqueous solution are close to, if not identical with, their absorption spectra *in vivo*. It is therefore proposed that the wave-length of maximum absorption should be the basis for naming the visual pigments. On this basis, iodopsin is 'visual pigment 565'; visual violet (porphyrhopsin), 'visual pigment 533'; visual purple (rhodopsin), 'visual pigment 502' and the red insensitive pigment of the tench, 'visual pigment 467'.

SUMMARY

1. Aqueous digitonin extracts of dark-adapted tench retinae contain two light-sensitive components; one of these may be bleached with light of long wave-length without affecting the other.

2. The red-sensitive component ($\lambda_{max.} = 533$ mμ.) is visual violet. This pigment is homogeneous and obeys Beer's and Lambert's laws of light absorption.

3. The red-insensitive component ($\lambda_{max.} = 467$ mμ.) is likewise a homogeneous pigment, also obeying Beer's and Lambert's laws.

4. The difference spectrum of the red-insensitive pigment and the difference spectra of visual purple, visual violet and iodopsin are nearly identical in shape when plotted with frequency abscissae. In a number of other respects the properties of the red-insensitive pigment resemble those of the known visual pigments.

5. The shortcomings of existing nomenclatures for the visual pigments are discussed, and a new nomenclature system based on the wave-length of maximum absorption proposed. In this system iodopsin is visual pigment 565; visual violet (porphyrhopsin), visual pigment 533; visual purple (rhodopsin), visual pigment 502, and the new, red insensitive pigment of the tench, visual pigment 467.

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