J. Physiol. (1955) 127, 81–89

STUDY OF THE PHOTOSENSITIVE PIGMENTS IN THE PINK AND GREEN RODS OF THE FROG

By E. J. DENTON and J. H. WYLLIE

From the Physiology Department, University of Aberdeen

(*Received* 22 *June* 1954)

It is generally accepted that, of the light striking an animal's eye, the fraction useful for vision is that absorbed by the photosensitive pigments in its retinal receptors. Spectral sensitivity and absolute sensitivity depend on the absorption curves and optical densities of such pigments within the receptors.

The methods which have been most used to measure the absorption curves and densities of visual pigments are those in which the pigments are extracted from the retina and studied in solution. Recently, however, Rushton (1952), Hagins & Rushton (1953*a*, *b*) and Weale (1953*a*, *b*) have made measurements on retinae *in situ*.

One aim of the present experiments was to design a simple method for measuring densities of photosensitive pigments in individual receptors. The method evolved was applied to the study of frog retinae. Photomicrographs of a retina were made before and after bleaching its photosensitive pigments. From such photographs the following quantities were found:

(1) The density change on bleaching of the individual visual purple filled rods for light of wavelength 0·52 μ.

(2) The relative areas of the rods and the spaces between them.

(3) The average retinal density change on bleaching for wavelength 0·52 μ.

(4) The curve of average retinal density change on bleaching against wavelength.

Some information was also obtained about the numbers and spectral absorption of the green rods.

MATERIAL

Freshly dissected retinae from freshly killed, dark-adapted *Rana temporaria* were used. The frogs were caught near Aberdeen a few days before the experiments which were performed in May and June.

METHODS

Dissection and mounting of the retinae

Using only very dim light (usually red) the retina was dissected out of the eye under Ringer's solution. It was then floated rod-side upwards into a well on a microscope slide. This well had been made by fixing a ring of wax to the slide. The well was filled with Ringer's solution and the retina was covered by a cover-slip.

Light source, microscope and camera

The microscope light source was a 48 W, 8 V tungsten filament lamp. This was underrun at an accurately controlled voltage of 7·5 V. The current to run the lamp was taken from two large 6 V, 140 amp-hour car batteries in series. A holder for an opal screen and for colour filters was mounted in front of the light source. A holder for neutral gelatine filters was placed between the microscope mirror and the substage condenser. The neutral filters were therefore in the almost parallel beam of light coming to the substage diaphragm of the microscope.

The flashes of light used were of constant duration and were given by a shutter rotating between the light source and the microscope mirror. The shutter was driven by a governed gramophone motor run through a constant voltage transformer from the 50 c/s, 230 V mains. The constancy of this motor was checked from time to time during the experiments. A second hand-operated shutter could be interposed in the light beam.

The retina was focused in dim light (usually red), and great care was taken not to expose it to light other than that required for the experiment.

In all the experiments described here a $\frac{2}{3}$ in. objective NA 0·28 was used with critical illumination and the photographs were made on Ilford HP 3 35 mm roll film using a constant exposure of 10 sec. The film was developed in I.D. 11. The magnification from retina to film was 36 diameters. It is important not to use too high a magnification as so much light will have to pass through the preparation to take a photograph that the photosensitive pigments of the retina will be appreciably bleached.

The condenser was used with an effective NA very close to, but less than, that of the objective.

Methods of estimation

The density change of individual rods for one given wavelength. The principle of the method is best described in terms of a hypothetical experiment. A colour filter is used to isolate a narrow band from the spectrum. Three photographs are made before bleaching. These differ only in that neutral filters of density $D+0{\cdot}3$, $D+0{\cdot}2$ and $D+0{\cdot}1$ are placed in turn beneath the substage condenser. The preparation is now bleached for 2 min in the strong white light obtained by taking away both colour and neutral filters. This light is so bright that the preparation appears to be bleached completely in a few seconds. Ten minutes after bleaching, photographs are made with neutral filters in the series of densities $D+0{\cdot}1$, $D+0{\cdot}2$, $D+0{\cdot}3$, ..., $D+1{\cdot}5$.

The film is developed and enlargements are printed. Prints to be compared must be processed similarly at all stages after exposure of the film.

From the prints it is possible to see what change in neutral filter exactly replaces the density change caused by bleaching. If this density change on bleaching were 0·5, then the rods on the photograph made with the neutral filter $D+0{\cdot}1$ before bleaching would match those made with $D+0{\cdot}6$ after bleaching. Similarly, $D+0{\cdot}2$ before bleaching would match $D+0{\cdot}7$ after bleaching, and $D+0{\cdot}3$ before bleaching would match $D+0{\cdot}8$ after bleaching.

The comparisons are made by cutting across a line of rods in a print of retina before bleaching, and laying the half rods at the edge of the cut against their corresponding halves on the prints of retina after bleaching. On these prints it is easy to compare the same individual rods before and after bleaching. The two halves of rod to be compared are isolated from their backgrounds with a small diaphragm and examined under a low-power binocular microscope.

The average retinal density change on bleaching. The method is similar to that described above except that the comparisons are made by a simple photo-electric densitometer. A small part of the

film is interposed between a constant light source and a photo-electric cell. The output voltage of this cell is fed through a cathode follower to a voltmeter. From the series of photographs made after bleaching a graph is plotted of meter reading against the density of neutral filter which was under the substage when the photograph was made.

If now the meter reading given by a film made before bleaching with neutral filter $D+0{\cdot}1$ corresponds to one of $D+0{\cdot}67$ (read from the graph) after bleaching, then the density change on bleaching is 0·57.

The estimate of average retinal density change can be made more accurately than the estimate of the change of density of individual rods because the graph allows accurate interpolation between the density steps used.

In two early experiments the measurements were made with the retina both in focus and far enough out of focus to give a uniform field. The values given in these two conditions were so close to one another that it was clear that very small errors would be introduced by using only in-focus photographs. In this way estimates of individual rod density changes and average retinal density changes were made using the same photographs.

To determine the approximate spectral sensitivity curve of the principal pigment present. The method used is adapted from that described above for determining the average retinal density changes on bleaching.

TABLE 1. Correcting factors for change in contrast of film with wavelength

Mean wavelength transmitted by filter	Correcting factor
0·365 μ	1·16
0·400	1·06
0·424	1·02
0·474	1·02
0·496	1·01
0·519	1·00
0·554	1·00
0·585	0·95

A calibrating series of photographs of the bleached retina is made at one wavelength (usually $\lambda = 0{\cdot}52\,\mu$) and a curve of meter reading against density constructed. Photographs are taken before and after bleaching for a series of colour filters covering the spectrum to be used. White light was used to bleach the retina.

For each filter are found the meter readings for corresponding negatives made before and after bleaching. The density change to which these readings correspond is read from the graph. In this way the density changes for the lights transmitted by a series of filters can be found and a curve showing density change against wavelength can be plotted. The constrast of the film changes with wavelength. The corrections for this were found relative to the light transmitted by the Ilford filter 604. For each other filter used a calibrating series of photographs was made along with a corresponding series for the Ilford filter 604. All photographic conditions were the same as those used in the experiments apart from the absence of a retina. Plots of galvanometer deflexion against density of neutral filter used were made as described above. These were used to find factors correcting for change in contrast of film with wavelength. These factors are shown in Table 1.

Estimation of proportion of total area covered by visual purple rods. This was made on enlarged prints of retina by measuring the diameters of many rods to give an average value and then counting the number of rods in a given area of the retina. The apparent diameter of a rod is not altered by bleaching.

Time. The time required after bleaching to complete the measurements was longest for the measurements of spectral absorption curves. Here the measurements were complete 30 min after bleaching.

There was no sign of regeneration of visual purple in these experiments. In several experiments measurements were made immediately after bleaching and 30 min later. In the green, $\lambda = 0{\cdot}52\mu$, the greatest change in this time was a small increase in density of the retina of 0·03 log unit. This could not however be attributed to regeneration of visual purple for a similar change took place at other wavelengths. This change probably represents a slight increase in the general opacity or scattering properties of the retina.

RESULTS

For six retinae the average retinal density change, the individual rod density change, the proportion of the total area covered by visual purple rods, and the proportion of green rods were all measured. These results are given in Table 2. The density changes are given for the light transmitted by the colour filter Ilford 604. In making measurements on the individual visual purple rods in a retina these were not found to be all equally dark before bleaching but the density change on bleaching was very uniform, the dark rods before bleaching being the darker ones after bleaching.

TABLE 2. Information about retinal receptors obtained from photomicrographs

No. of expt.	Individual rod density change	Average density change	% area covered by pink rods	% green rods
1	0·65	0·42	55	5·9
2	0·67	0·48	64	7·5
3	0·57	0·44	59	7·1
4	0·71	0·46	50	14·5
5	0·83	0·49	67	6·5
6	0·50	0·38	58	7·5
Av. for retinae 1–6	0·66	0·45	59	8·2

Accuracy

The mean of the measurements of the average density change for one retina has a standard error in density of 0·022.

The accuracy of the measurements of density change of individual rods on bleaching is less than this. When comparing one photograph before bleaching with a series of photographs made with different intensities of light after bleaching it can be said, to take one example, that the density change is certainly more than 0·74 and certainly less than 0·96 and near to 0·85. The values 0·74, 0·96 and 0·85 are the accurate densities of the neutral filters used in the experiment.

The maximum error of three such comparisons cannot be much greater than $\pm 0{\cdot}05$ in density.

Spectral density difference curve for the retina

The spectral density difference curve on bleaching was determined for two retinae (Fig. 1). The values given are corrected for the change in contrast of the film with wavelength.

This experiment shows that the principal photosensitive pigment involved in these measurements is visual purple and that the difference curve on bleaching is very much like that obtained with extracts in solution. An accurate fit would not be expected in the blue because in these preparations the process of bleaching is complex (Denton, 1954*b*).

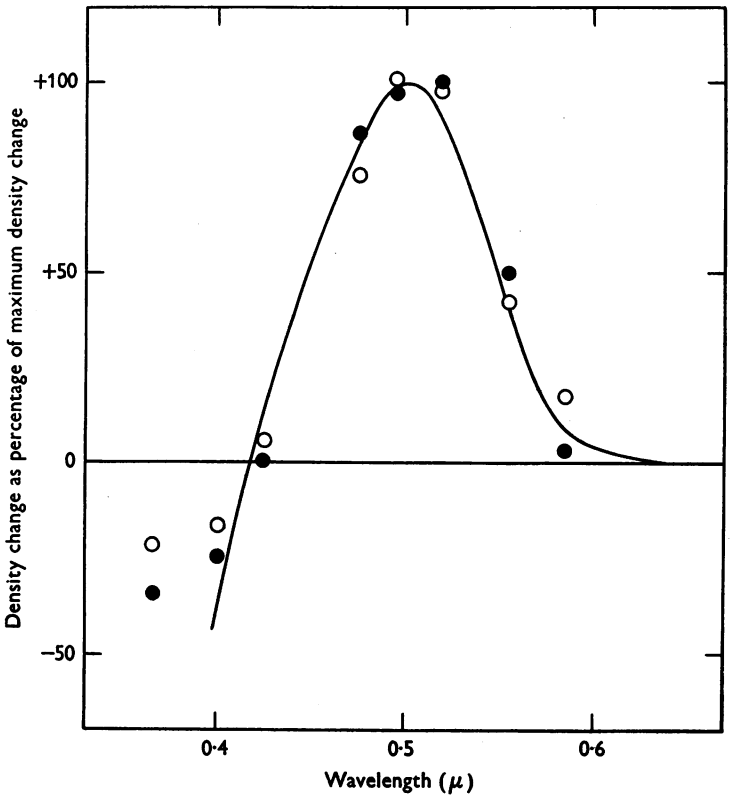


Fig. 1. Comparison of the difference spectrum of frog visual purple in solution with that in intact retinae. The continuous line is the difference spectrum of frog visual purple in solution at pH 8·5 (Lythgoe, 1937). The points (●, and ○) give the spectral density change of two intact frog retinae bleached with white light.

The green rods

No accurate curve was determined for the change in density of the green rods on bleaching. Photographs were made with lights of different wavelengths before and after bleaching with white light. Examples of such photographs are shown in Pl. 1. There are two standards of reference in these photographs, first the spaces between the rods, and secondly the visual purple filled rods. It is therefore possible to make an estimate of both the absorption spectrum before bleaching and its changes on bleaching. When unbleached,

the green rods absorb strongly in the blue (filters with mean transmissions 0·4–0·44 μ), moderately in the yellow and red (filters with mean transmissions 0·56–0·68 μ) and they are very transparent in the green (filters with mean transmissions 0·49 and 0·52 μ). After 'bleaching' in strong white light they become much paler in the blue and darker in the green, while in the yellow and red there is little or no change. This is why when observed in white light the green rods become grey on bleaching.

Using as a criterion of bleaching the large change in absorption in the blue, we have found that the green rods are easily bleached in blue light but are very insensitive to green light. They are also insensitive to the red light transmitted by the Corning cut-off filter 2408 which transmits wavelengths greater than 0·65 μ. This last observation does not support the observation by Kühne (1878) that the green rods are especially sensitive to red light. An exposure of this light which was sufficient to bleach the visual purple rods seemed to have no appreciable bleaching action in the blue or in the red on the green rods.

DISCUSSION

The values given above are for density change on bleaching for the light transmitted by the Ilford filter 604. The light transmitted by this filter is not all of the wavelength which visual purple absorbs best. If the accurate values for the spectral absorption curve of frog visual purple are those given by Hecht, Shlaer & Pirenne (1942) as an average of the results of Lythgoe (1937), Wald (1938) and Chase & Haig (1938) then the density change for the wavelength which is best absorbed would be 15·7 % higher than that for the light transmitted by the Ilford filter 604.

If this correction is applied, the value of 0·66 for the density change of the individual rods for the light transmitted by the Ilford filter 604 is equivalent to a density change of 0·76 for the wavelength (0·5 μ) which is absorbed best. The bleaching in these experiments is to a product which probably has at 0·52 μ some residual absorption of its own. If we assume that this product is that given by bleaching in neutral solution then the values given for the density changes will be some 8 % lower than the absolute densities of visual purple (G. Wald, private communication).

These values are much higher than those usually given by the method of extracting the visual purple, measuring its density in solution, and calculating the absolute density *in situ* on the assumption that the visual purple was originally spread uniformly over the retinae from which it was extracted. Using this method, Wald (1938) gives a value of 0·21 for the bullfrog, and Dartnall (1953) gives 0·25 for *Rana temporaria*.

Hubbard (1954) has calculated, on the basis of measurements on extracted visual purple, that the density of visual purple along the axis of the outer limb of a rod of *R. esculenta* is 0·50.

The difference between the values given in this paper and those given by the above authors is mainly accounted for thus: the visual purple rods cover only 59% of the retinal surface; the molecules of visual purple are orientated in a regular fashion in the rods so that they absorb light coming down the axis of the rod more heavily than they would if the same molecules were arranged at random in the rods. The rods are known to be dichroic (Schmidt, 1938; Denton, 1954*a*); when laid on their side the visual purple in them absorbs polarized light heavily when the electric vector is across the rod axis and very slightly when it is along the axis of the rod. If we assume that the absorption of a molecule of visual purple depends on the angle between it and the plane of polarization of the incident light and falls off as the square of the cosine of this angle, then in the rods in their natural position the orientated visual purple molecules absorb $1\frac{1}{2}$ times more light than they would do if they were arranged at random. Taking these two factors into account, we can multiply the values of 0·21 (Wald, 1938) and 0·25 (Dartnall, 1953) by $(1/0{\cdot}59) \times 1\frac{1}{2}$ to give the values 0·53 and 0·63. The value of 0·50 (Hubbard, 1954) multiplied by $1\frac{1}{2}$ gives 0·75. These values for absolute densities are close to those given here for the density change of individual rods.

Recently, Arden (1954) has made measurements on a suspension of rods and gives the value of 0·31 for the density of visual purple in the individual rods; this becomes 0·46 on correcting for the dichroism of the rods.

The average absorption of light by the visual purple in the retina

The average retinal density change on bleaching was taken as a measure of this quantity. It was found to be 0·45 for light transmitted by Ilford filter 604 ($\lambda = 0{\cdot}52\mu$). This figure is the average of six values given in Table 2. The density of 0·45 for the light transmitted by the Ilford filter 604 means probably a density of 0·52 for light of wavelength $0{\cdot}50\mu$. This means that the retina absorbs about 70% of light of wavelength $0{\cdot}50\mu$ striking it.

The visual purple rods which before bleaching are darker than the spaces between them become on bleaching more transparent than these spaces. The spaces after bleaching are about 0·2–0·3 log unit darker than the rods. Two simple explanations suggest themselves: first, that the spaces contain substances which absorb light more strongly than the contents of the bleached rods; and secondly, that the rods, because of their higher refractive index, collect light from the spaces. In the conditions of these experiments light is converging with a maximum angle to the axes of the rods of 16°. If the refractive indices of the materials in rods and spaces are respectively 1·47 and 1·33 as assumed by Helmholtz (1896), all the light entering the end of the rod will be totally reflected from the inside of the walls and will reach the other end of the rod. Most of the light falling on the spaces between the rods will strike the outside surfaces of rods once or several times, and at each reflexion

a proportion will be refracted into a rod. This process would make the visual purple more effective in the eye than might be suggested by its density in the individual rods and the fraction of the area covered by them. No attempt has been made to estimate the relative importance of such factors as these.

The green rods

The green rods were first described by Boll (1876) and by Kühne (1878).

The green rods show their major photosensitive change on bleaching in the blue. At wavelengths around 0·43 μ they become much paler, and it seems likely that whatever else their function, they will act as blue-sensitive receptors (Granit & Munsterhjelm, 1937).

The darkening in the green is more surprising since none of the photosensitive pigments so far studied in solution shows on bleaching an increase in absorption for wavelengths longer than those at which it absorbs best when unbleached.

The absence of a change in the absorption of red light by the green rods when they are 'bleached' suggests that this absorption is made by a light stable pigment in these rods.

It is clear, however, that frogs could have dichromatic colour vision using only their retinal rods. There is therefore no certainty for other animals that colour vision will necessarily be cone vision.

SUMMARY

1. Simple photomicrographic methods of determining, on freshly dissected retinae, the average retinal density change on bleaching and the individual retinal receptor's density change on bleaching are described.

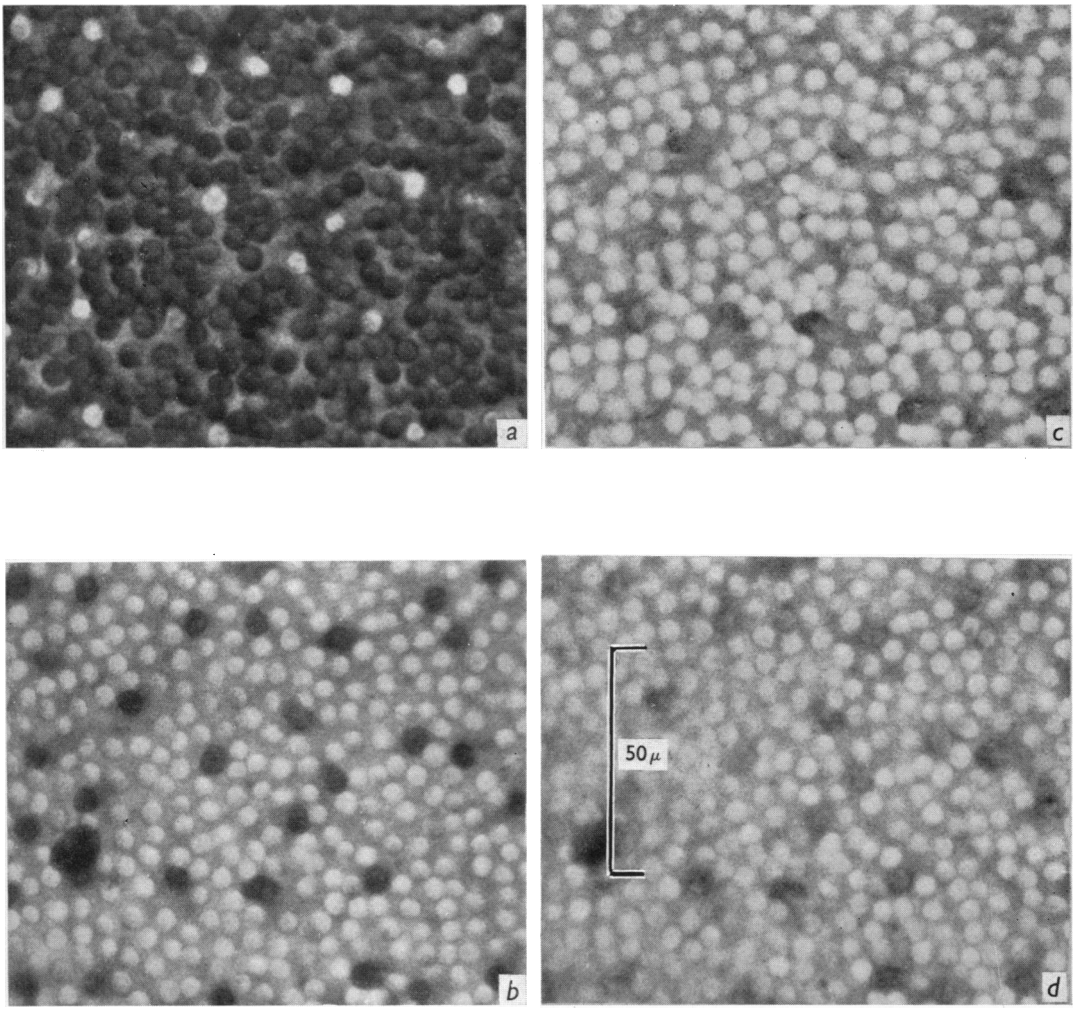
2. For the retina of *Rana temporaria* the visual purple rods occupy about 60% and the green rods about 8% of the total area.

3. The green rods which before bleaching absorb heavily in the blue ($\lambda = 0{\cdot}4$–$0{\cdot}44\,\mu$), slightly in the yellow to red ($0{\cdot}56$–$0{\cdot}68\,\mu$) and are very transparent in the green ($0{\cdot}49$–$0{\cdot}52\,\mu$), become on bleaching with white light much paler in the blue and darker in the green while in the yellow and red there is little or no change.

4. The individual visual purple rods show on bleaching a density change of about 0·66 for the light transmitted by the Ilford filter 604 ($\lambda_{mean} = 0{\cdot}52\,\mu$).

5. The average retinal density change on bleaching which is shown to be dominated by that of the visual purple rods is about 0·45 at $\lambda = 0{\cdot}52\,\mu$.

6. The values of 0·45 and 0·66 for density change for $\lambda = 0{\cdot}52\,\mu$ would presume still higher absolute densities of visual purple in the unbleached retina for $\lambda = 0{\cdot}5\,\mu$. They are, nevertheless, high compared with estimates of these quantities made on the basis of studies of extracted visual purple. The reason for this is discussed.



To face p. 89

We wish to thank Dr M. H. Pirenne for useful criticism and for the loan of colour filters bought with a grant from the Medical Research Council. We are grateful to Mr A. E. A. McLeod for excellent technical assistance.

REFERENCES

ARDEN, G. B. (1954). Density of visual pigment in the frog retina. *Nature, Lond.*, **173**, 360–361.

BOLL, F. (1876). Sull' anatomia e fisiologia della retina. *Mem. Accad. Lincei*, **1**, 371–393.

CHASE, A. M. & HAIG, C. (1938). The absorption spectrum of visual purple. *J. gen. Physiol.* **21**, 411–430.

DARTNALL, H. J. A. (1953). The interpretation of spectral sensitivity curves. *Brit. med. Bull.* **9**, 24–30.

DENTON, E. J. (1954*a*). A method of observing the dichroism of the visual rods. *J. Physiol.* **124**, 16–17*P*.

DENTON, E. J. (1954*b*). On the orientation of molecules in the visual rods of *Salamandra maculosa*. *J. Physiol.* **124**, 17–18*P*.

GRANIT, R. & MUNSTERHJELM, A. (1937). The electrical reponses of dark adapted frog's eyes to monochromatic stimuli. *J. Physiol.* **88**, 436–458.

HAGINS, W A. & RUSHTON, W. A. H. (1953*a*). Measurement of the rhodopsin density in the eye. *J. Physiol.* **122**, 9*P*.

HAGINS, W. A. & RUSHTON, W. A. H. (1953*b*). The measurement of rhodopsin in the decerebrate albino rabbit. *J. Physiol.* **120**, 61*P*.

HECHT, S., SHLAER, S. & PIRENNE, M. H. (1942). Energy, quanta, and vision. *J. gen. Physiol.* **25**, 819–840.

HELMHOLTZ, H. (1896). *Handbuch der physiologischen Optik*, p. 205. Hamburg and Leipzig: Leopold Voss.

HUBBARD, R. (1954). The molecular weight of rhodopsin and the nature of the rhodopsin-digitonin complex. *J. gen. Physiol.* **39**, 381–399.

KÜHNE, W. (1878). *The Photochemistry of the Retina and on Visual Purple.* Translated by M. Foster. London: Macmillan.

LYTHGOE, R. J. (1937). The absorption spectra of visual purple and indicator yellow. *J. Physiol.* **89**, 331–358.

RUSHTON, W. A. H. (1952). Apparatus for analysing the light reflected from the eye of the cat. *J. Physiol.* **117**, 47*P*.

SCHMIDT, W. J. (1938). Polarisation optische Analyse eines Eiweiss-Lipoid-Systems, erläutert am Aussenglied den Sehzellen. *Kolloidzschr.* **85**, 137–148.

WALD, G. (1938). On rhodopsin in solution. *J. gen. Physiol.* **21**, 795–832.

WEALE, R. (1953*a*). Slow and rapid regeneration in the living cat's retina. *J. Physiol.* **122**, 11*P*.

WEALE, R. (1953*b*). Photochemical reactions in the living cat's retina. *J. Physiol.* **122**, 322–331.

EXPLANATION OF PLATE

Photomicrographs all of the same part of one frog's retina

(*a*) This photograph was taken with green light before bleaching the preparation. The numerous visual purple rods absorb this light heavily and appear dark. The occasional rods which appear light do so because they transmit the green light very well. These are the green rods; when viewed in white light they appear grass green while the others are pink.

(*b*) Like (*a*) this photograph was taken before bleaching. Blue light was used. The visual purple rods are almost transparent while the green rods absorb heavily.

(*c*) This photograph is identical with (*a*) in all respects except that it was taken after bleaching the retina with strong white light.

(*d*) This photograph is identical with (*b*) in all respects except that it was taken after bleaching the retina with strong white light.

The mean wavelengths used were 0·52 μ for the green light and 0·42 μ for the blue light.