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RETINAL PHOTOPIGMENTS IN THE ALBINO RAT

By D. M. LEWIS*

From the Physiological Laboratory, University of Cambridge

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In recent years a number of workers have made studies of visual pigments *in vivo* by measuring the changes which occur in the amount of light reflected from the fundus oculi in living animals during the bleaching or regeneration of the pigments. Brindley & Willmer (1952) were the first to make quantitative measurements of this type in man. The methods have been improved since as described by Rushton, Campbell, Hagins & Brindley (1955), Campbell & Rushton (1955) and Rushton (1956*a*) in man and the albino rabbit, also by Weale (1953, 1955) in the cat and other animals using a different technique for measuring the reflected light.

The present measurements were made upon rats by a method similar in principle to that described by Rushton (1956*a*). Light reflected from the fundus oculi in various states of light and dark adaptation was measured by a photocell and the change in intensity due to differences in rhodopsin density was recorded. This was distinguished from intensity changes of instrumental origin by comparing the measurements in red light, which must be unaffected by the rhodopsin density since rhodopsin is transparent to red.

One object was to measure the maximum density and the difference spectrum in the eye of the living rat. Extracts of rats' retinas have already been studied by Wald (1938), Collins & Morton (1950) and Crescitelli & Dartnall (1953), and the spectral sensitivity of the rods has been measured by Granit (1941) using his micro-electrode technique. It seemed interesting to compare these results with rhodopsin measurements *in vivo* as has already been done for the cat by Weale (1953) and for man by Rushton (1956*a*, *b*).

With all measurements of the density of visual pigments made by opthalmoscopic techniques we must face the question: 'Does the light which passes twice through the retina suffer the same absorption at each passage?' If so, the density of the pigment will be half the double density measured. But we

* Present address: London Hospital Medical College, Turner Street, London E. 1.

know too little about the inward, and still less about the return, pathway to be able to say confidently that the two paths are the same, or if not, which offers most absorption. The excised eye of the albino rat, however, provides a direct answer, for the choroid and sclera are so translucent that a photocell placed behind the eye can measure the change in *transmissivity* on bleaching while the ophthalmoscopic arrangement measures the reflectivity change. Since the first involves one transit of the retina and the second two, the relative absorptions may be evaluated. This work has already been briefly presented (Lewis, 1956).

METHODS

The rats used were mostly males weighing 200–300 g, anaesthetized with 7·5 ml. of 25% urethane/kg body wt. given subcutaneously, with further small doses as required. They were atropinized (0·2–0·3 ml. 10% (w/v) atropine sulphate per rat) and a tracheal cannula inserted. They were kept warm (rectal temperature 37–38° C) on a heated box.

The heads of the animals were fixed in a holder based on one described by Beattie (1952). The holder (Fig. 1) provided three-point attachment to the head, with the incisor teeth (7) and with two metal, pointed ear plugs (1) held together with a wire and pushed firmly into the external meatuses. Attached to the Bakelite base board (2) were two vertical pillars (3, 4), threaded to receive screws (5, 6) which fitted into the hollowed-out ends of the ear plugs. One screw (5) was short and fixed tightly into the pillar (3) being flush with its outer side; this was the side of the eye on which observations were made and such an arrangement presented no obstruction to the light entering the eye. The other screw (6) was long (and threaded through a broad pillar (4) to obtain maximum stability) and could be screwed in until both ear plugs were firmly held. The third point of attachment was a small vice (7) notched to receive the upper incisors and tightly clamped on them. This was mounted upon the base board through two stages each slotted (8) to permit movements sideways and forwards respectively. Thus the tooth clamp could be moved in two dimensions to allow for variations in head sizes, and clamped in the correct position.

The whole base board was screwed down (9) on to the metal top of the heating box which supported the rest of the body. Thus the board (and head) could be rotated to change its angle to the direction of the measuring light. The heating box top was set an an angle of 45° to the horizontal so that rotation of the head about its horizontal axis brought the optical axis of the eye into a horizontal plane suitable for the measuring apparatus.

The holder was adaptable and easily adjusted to different animals but once set up the head was rigidly fixed. Eye movements relative to the head were reduced to a minimum by the deep anaesthesia used.

A small glass hemispherical contact lens, filled with Ringer–Locke solution, was placed on the front of the eye to correct for irregularities of the corneal surface. This further served to keep the cornea moist and to hold back the eyelids. Drops of atropine (10% solution) and cocaine (4% solution) were previously placed in the eye to reinforce the injected atropine in its effect of dilating the pupil.

For the experiments on *isolated* eyes, the rats were dark-adapted during the night and, after atropinization, killed with a large dose of urethane injected intraperitoneally, draining the head of blood. During this operation the head was covered by black cloth. Then, working in deep red light, the eyeballs were removed, cleaned, and finally mounted between two glass cups (similar to the corneal glass) filled with Ringer–Locke solution. The cups were cemented into holes in two metal plates, which were screwed together and fixed in the measuring apparatus. No special precautions, other than keeping moist, were taken to maintain the eyes in good condition, but the measurements were taken quickly and at the end of each experiment the eye was examined to see that it had remained clear. If not, the result was rejected. In most cases after

the short period of the experiment (15–20 min) the eye media remained clear and the eye appeared normal to direct observation, save that there was some shrinkage and contraction of the pupil probably due to the loss of intra-ocular fluid. If left for longer periods, opacities developed in the eye media, especially the lens, but these were seen in only a few of the eyes examined immediately after an experiment.

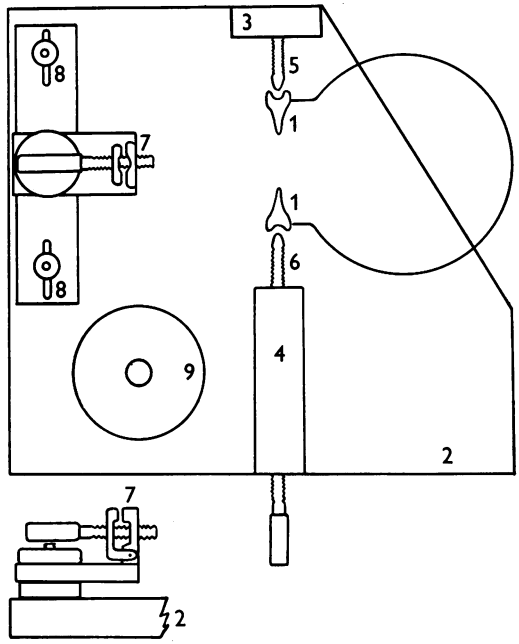

Fig. 1. Holder used for immobilizing the rat's head shown in plan. Inset, side view of the tooth clamp. For description see 'Methods'.

Method of measurement

Briefly, the light which fell upon the retina flickered between red and some other wave-length at which measurements were to be taken (say green). The light after reflexion from the fundus was measured by a photomultiplier tube.

The intensity of the green light reflected depended on the amount of rhodopsin in the retina. That of the red light was independent of rhodopsin but could be varied by a neutral density photometric wedge. The wedge was set so that the currents produced by the two component lights in the photocell became equal. A change in the density of rhodopsin required a new wedge setting to regain a balance—the change in wedge density was then equal to the change in rhodopsin density to the green light.

The two flickering components had each a sinusoidal time course and a phase-sensitive rectifier, described by Rushton *et al.* (1955), was employed to exclude extraneous light and other noise and to determine the balance point accurately.

The apparatus used to produce light flickering between red and various other monochromatic lights was different from that used by Rushton (1956*a*, *b*). It is shown in Fig. 2 and described below.

Apparatus

The source of light (*I*) was a 6 V 18 W straight, coiled filament car headlamp bulb run from a mains transformer and housed in a light-proof metal cylinder with a vertical exit slit opposite the filament.

The beam of light passed through a heat absorbing filter (F_0) and a polaroid (P_1). The polaroid was mounted in a ball-race driven by an electric motor controlled by a rheostat. A lens (L_1) rendered the beam of light parallel, whence it fell on a sheet of plain glass (G_1) which split the beam into two. Finally the two beams were combined into one by a second plate (G_2), the recombined beam being focused by L_2 on to the corneal lens forming an image of the filament. Great care was taken in adjusting G_1 and G_2 so that the two beams were recombined exactly, forming only one filament image on the corneal lens. G_1 was set at the polarizing angle so that the reflected light contained about 25% of the incident light polarized entirely in the vertical plane. Thus

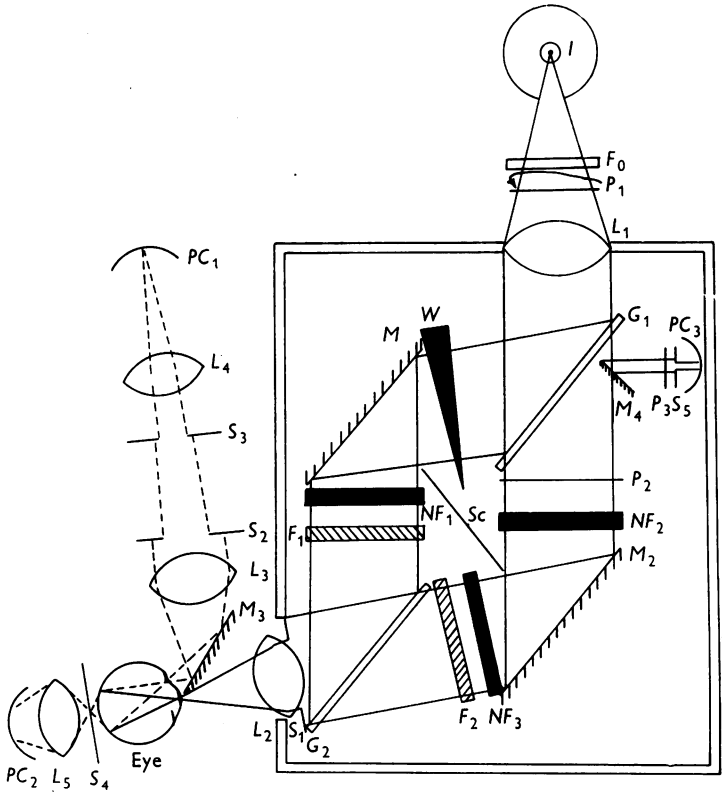

Fig. 2. Diagram of the apparatus used for measuring visual pigments: not to scale. For description see 'Methods'.

as the polaroid P_1 rotated, the intensity of this reflected beam varied sinusoidally. The light passed through a neutral density wedge (W) and was reflected from a mirror (M) surface-silvered (so as not to affect its polarization) through a red filter (F_1) on to G_2, where again about 25% was reflected. An additional neutral density filter could be included at NF_1. The 'red' filter was usually an interference filter, with peak transmission at 618 mμ, with an accessory Ilford 204 gelatine filter. If more light was required (as in the experiments on isolated eyes) an Ilford 205 filter was used that transmitted all light of wave-length greater than 620 mμ and some light between 600 and 620 mμ.

The light transmitted by G_1, which was partly polarized in a horizontal plane, was made completely polarized by a polaroid P_2. It was reflected by a second surface-silvered mirror (M_2) on to G_2 where it was completely transmitted. An interference filter (plus an accessory filter where

necessary) was put in this beam together with neutral density filters (F_2 and NF_3). This beam was very much stronger than the reflected one and a neutral density 2 filter had to be used in the central region of the spectrum, though none was needed at the far ends—light of longer wave-length than 618 mμ could be used in order to check how much absorption occurred at 618 mμ. A white bleaching light was made by using this beam after removal of the filters. The principle of the phase-sensitive rectifier demands that a comparison sine wave be supplied in phase with the signal, in terms of which the signal is accepted and 'noise' rejected. This comparison wave was supplied by a second photocell (PC_3) which received a little light from the main beam reflected in the mirror (M_4) adjusted for phase by the polaroid (P_3) and for intensity by the triangular slit (S_5).

The large diagonal screen (Sc) stopped light scattered by the holders of filters, etc., from entering the main beams. All the holders were painted matt black and the whole apparatus between L_1 and L_2 enclosed in a blackened box.

The Maxwellian lens L_2, stopped down by S_1, formed a uniformly illuminated patch on the retina. The light reflected back was caught by a silvered cover-slip (M_3) fixed with one edge touching the corneal lens and the edge of the filament image. It was set at 45° to the light beam and covered half the pupil, the other half being filled with the magnified image of the filament. These are the optimal conditions for collecting light reflected from the fundus.

The retinal light reflected in M_3 was focused by a short focus lens (L_3) into photomultiplier tube, 931A (PC_1). In the pathway two stops were placed. S_2 was in the plane of the image of the retina; it has a hole the same shape but smaller than the image of the illuminated retinal patch and was centred on that image. This prevented light from other parts of the retina reaching the photocell. The position of the head was usually arranged so that the illuminated patch did not include the optic disk (identified as the origin of blood vessels) but if this could not be done satisfactorily a piece of 'Plasticene' was put over the image of this spot.

S_3 was in the plane of the image of the half pupil covered by M_3. The stop was a semicircle coinciding with this image, thus permitting the passage of all the fundal light reflected in M_3 but excluding light scattered by the iris or mirror edge. Light reflected from the optical surface (e.g. cornea or lens) could be seen as bright sources of light and seen to be excluded by these stops.

In addition to this, when isolated eyes were used, the light *transmitted* by the translucent choroid and sclera was focused by L_5 on to a second photocell (PC_2). In this case a stop (S_4) was placed immediately behind the eye with a pin-hole aperture corresponding to the illuminated patch of the retina. This cut off oblique rays of light.

RESULTS

Difference spectra

Fig. 3 shows the difference spectrum obtained from intact anaesthetized rats. In each experiment readings were taken at a number of wave-lengths, repeating the series two or three times. Unless the sets agreed reasonably well the results were rejected (usually any small discrepancies were consistent with their being due to slight bleaching by the measuring light). The retina was bleached with white light for 20 sec, after which further series of readings were taken to obtain the difference spectra. In Fig. 3 the vertical bars represent the standard error of the mean (derived from eight experiments) when the results had been scaled to coincide at 530 mμ. Where no standard error is shown the points show the results of a single experiment where the preparation was sufficiently stable to allow readings at twelve wave-lengths

instead of the usual six. An interrupted line is drawn freehand through the points. The smooth curves show the difference spectrum of a solution of frog's rhodopsin measured 8 min after bleaching (Wald, 1938, Fig. 3*A*). All the curves and experimental points are scaled at 530 mμ where it was considered that there would be little absorption by possible bleaching products.

Fig. 4 shows difference spectra obtained in isolated eyes measuring both the transmitted and the reflected light before and after bleaching. Ten eyes

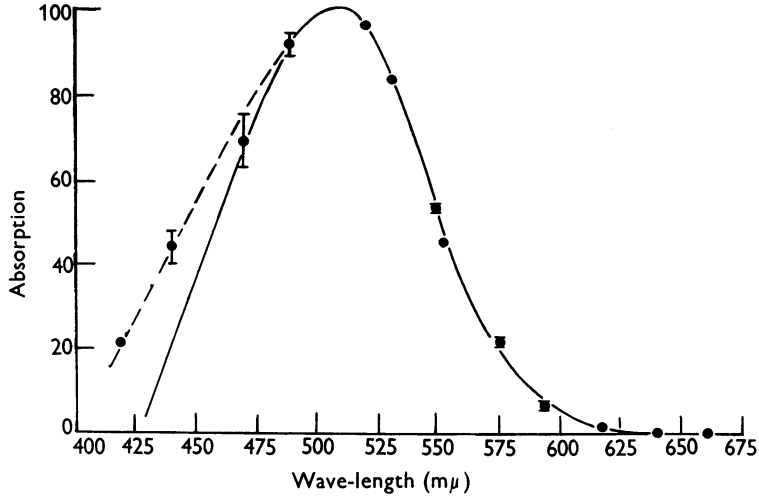

Fig. 3. Difference spectra: ——, frog's rhodopsin (Wald 1938); ●———●, intact rat's retina, the vertical bars show ±S.E. of the mean. All scaled at 530 mμ.

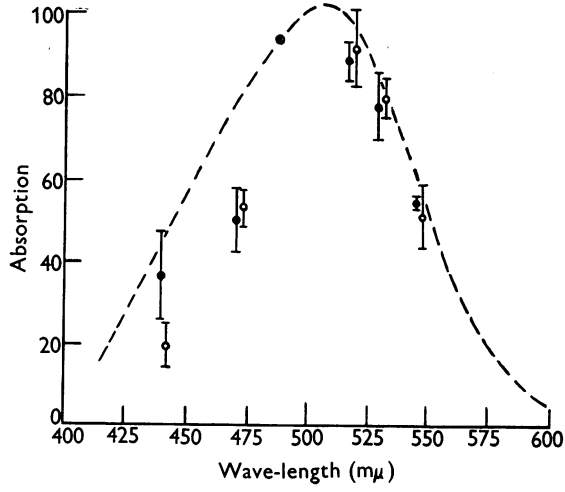

Fig. 4. Difference spectra of rat's visual pigment: ○, measured by reflected light, and ●, by transmitted light in the isolated eye, vertical bars show ±S.E.; ----, by reflected light in the living animal (from Fig. 3). All scaled at 488 mμ.

were used and measurements made at three or four wave-lengths in each, including a reading at 488 mμ in every eye. It was inadvisable to measure more than four wave-lengths on one excised eye as speed was essential to prevent opacity developing. Moreover, since the excised eye had a much smaller pupil than when intact, the amount of light reflected was less, hence the intensity of the measuring light had to be increased and too much bleaching occurred to take a long series of readings. The stability of these excised preparations compensated for this in that only two readings had to be taken at each wave-length. The results are scaled at 488 mμ and compared with those in living animals (the broken line in Figs. 3 and 4).

Maximum density

Anaesthetized animals were dark-adapted for 6–8 hr in the apparatus or set up in red light after remaining in the dark during the night. On bleaching, the wedge change was found to be between 0·37 and 0·40 density units in most cases at 488 mμ when the control wave-length was 662 mμ. The *retinal* density corresponding to this change is discussed below.

In isolated eyes measurements were made by transmitted and reflected light at 488 mμ in twenty eyes. The experiments may be divided into two groups; in ten eyes readings were taken at only the wave-length 488 mμ and in the other ten at three or four wave-lengths (these are the ten described above in the section on difference spectra). This division has been made since the results of the first group show greater consistency, the mean ratio of density by reflected light to that by transmitted being 1·87 (S.E. ± 0·03). In the second group the mean was nearly the same (1·85) but the scatter greater (S.E. ± 0·08).

DISCUSSION

In all measurements of visual pigments by ophthalmoscopic densitometry there arise two difficulties in interpretation. The first is how much of the 'double-density' measured is due to absorption of the ingoing light and how much to that of the outgoing. The second is how much of the light returned to the photocell has not been twice through the visual pigment of the retina. This *stray light* dilutes the signal and makes the pigment density appear less.

A great advantage of the measurements by transmitted light in the excised albino eye is that practically all the light received upon the photocell has passed once through the retina, so the above difficulties are less serious. Measurements by transmitted light, therefore, provide a standard by which the simultaneous measurements by reflexion may be assessed. In the experiments it was found that the ratio of the 'double-density' by reflexion to the 'single-density' by transmission was 1·87. This is consistent with the supposition that light was absorbed equally by rhodopsin on both passages, but the reflected signal contained some 7% of stray light.

The difference spectra measured by transmitted and reflected light (Fig. 4) are in reasonable agreement. The standard error is rather large because only a few observations contribute to each measurement, but the two difference spectra coincide except at wave-length 441 mμ. That is a light which is strongly scattered in the eye media, but poorly reflected from the fundus, so the low value of rhodopsin density by reflexion at this wave-length is to be expected. On the whole, the two spectra from the isolated eye show a good agreement not only with each other but also with the results from the living eye (broken curve Fig. 4) and from rhodopsin solution (Fig. 3) for wave-lengths greater than 488 mμ.

Maximum rhodopsin density

The maximum change in 'double-density' on bleaching eyes which had been in the dark for a very long time was 0·4 unit at 488 mμ wave-length. Now the relation between true density (of transmission) and the 'double-density' (of reflexion) was shown earlier to be 1/1·87, so the maximum density difference of rhodopsin is 0·21 at 488 mμ. It has been seen that the results of Fig. 3 coincide well with those of Wald (1938), where the difference spectrum of purified rhodopsin solution was measured about 8 min after a rapid bleach. So, despite the fact that he was investigating frog's rhodopsin, we shall not be far out in accepting his density figures. From Wald's curves one can read off the initial rhodopsin density which would be required to produce at 488 mμ the difference of 0·21 which was found in the rat's eye. The initial density (at 500 mμ) must have been 0·32, so that more than 50% of green light falling upon the retina is absorbed.

The amount of rhodopsin which can be *extracted* from the dark-adapted rat's retina is also described in this same paper (Wald, 1938). If spread uniformly over the retinal surface it would absorb 13% of incident green light, which corresponds to a mean density of 0·06. This formal way of expressing the amount of rhodopsin present gives a figure very much less than the actual density of the rhodopsin in the central region of the retina, for the orientation of rhodopsin molecules in the rods (Schmidt, 1937; Denton, 1954), the funnelling of light from inner to a narrower outer segment (Rushton, 1956*a*, *b*), and the poverty of rhodopsin at the periphery of the retina must be taken into account. These factors, taken together, would probably raise Wald's formal figure of 0·06 to about 0·2 for the actual rhodopsin density of the central region of the retina, where in the present paper it has been found to be 0·3. A similar 2:3 discrepancy has been noted by Denton & Wyllie (1955) in the frog and by Rushton (1956*b*) in man.

SUMMARY

1. Methods are described for measuring photopigment densities and difference spectra in the intact and excised eyes of the albino rat.

2. In isolated eyes the ratio of densities by reflected and transmitted light is 1·87/1, which is consistent with the density to the double passage through the retina taken by the reflected light being exactly twice the density in the path of the transmitted light but diluted by 7 % of stray light.

The difference spectra are similar in the two cases save in the blue, where stray light has the strongest effect.

3. The difference spectrum *in vivo* has a maximum at 505 mμ compared with 497 mμ obtained in extracts. The measurements *in vivo*, however, were taken some 5 min after bleaching. When these are compared with the difference spectrum of (frog's) rhodopsin at a comparable interval the results are similar.

4. At the maximum 50 % of the light incident on the retina is absorbed.

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