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ON THE MODE OF ACTION OF VISUAL PURPLE ON THE ROD CELL

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This paper deals with some unexpected results obtained in parallel measurements of visual purple concentration and size of the dominant initial *b*-wave of the retinal electrical response. Some general correlations between the properties of visual purple and the *b*-wave have already been established. Thus we know that the size of the *b*-wave reproduces the absorption curve for visual purple (see, for example, a summary by Granit [1938]). But more important for our purpose than this static criterion is a dynamic one, based on the work of Tansley [1931] and Charpentier [1936]. The former measured the concentration of visual purple, the latter the size of *b*-wave of the retinal electrical response during dark adaptation of white rats previously adapted to light. It was found that the rise in concentration of visual purple during regeneration was accompanied by an equivalent rise in size of the *b*-wave. The same conclusion is also emphasized by the work of Wrede [1937] and Riggs [1937]. It would seem therefore that the size of *b*-wave, at least during regeneration of visual purple, might be directly determined by the total amount of visual purple available.

So far, however, no strictly parallel measurements of the retinal electrical response and the density of visual purple extracts have ever been made in any laboratory, and, though the results referred to are very definitely established, it is not known, for instance, whether a reduction of the electrical response due to moderate light adaptation is accompanied by a parallel reduction in the concentration of visual purple. For this purpose it was found necessary to develop a technique for measuring the concentration of visual purple from single eyes (see below). Having done so we soon found that adaptation to different monochromatic lights which caused a considerable reduction in size of

the *b*-wave, elicited by a test light, did not lead to a measurable reduction of the amount of visual purple obtainable from the eye used. The evidence for this conclusion is given below.

As we have pointed out in a preliminary note [1938], this lack of correspondence during moderate light adaptation between the behaviour of the *b*-wave and the amount of visual purple during regeneration is of great theoretical interest. It is important to know how accurately visual purple of individual eyes can be determined. Below we give details of our technique and procedure.

Technique and procedure

Photoelectric technique. The requirements of the technique for measuring V.P. (visual purple) densities are: (i) low intensity of the light reaching the cell in order to avoid bleaching the photosensitive substance;

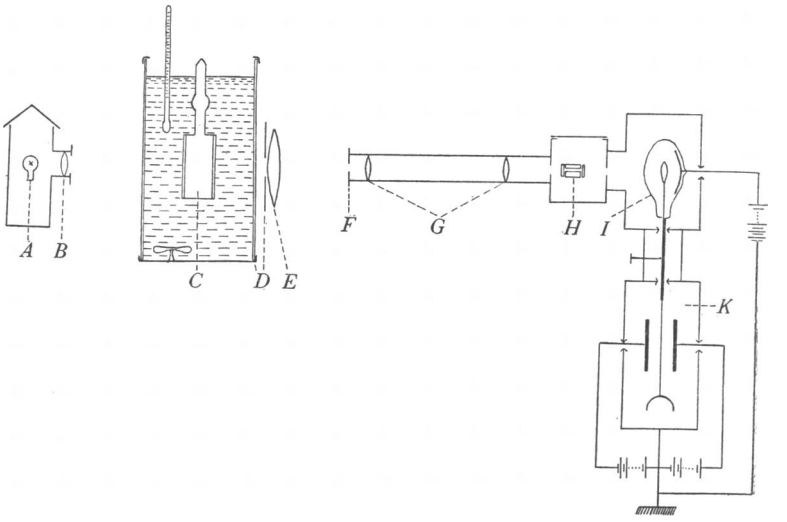

Fig. 1. Diagram of apparatus for photoelectric absorption measurements. *A*, lamp; *B*, condenser lens (10 cm. focal length); *C*, Christiansen dispersion filter in water bath fitted with electric thermo-regulator; *D*, diaphragm; *E*, lens of focal length 75 cm.; *F*, diaphragm; *G*, lenses required by optic cell; *H*, light-tight case for optic cell; *I*, photocell in dried case; *K*, string electrometer with connexions. Not to scale.

(ii) reasonably monochromatic light adjusted to match the highest point of the V.P. absorption curve around 0·500 μ.; (iii) enough sensitivity to enable accurate working with about 0·4 c.c. of V.P. extract. These requirements were fulfilled by the apparatus illustrated diagrammatically in Fig. 1.

The light source was a small bulb of the automobile headlight type run off a storage battery below its rated voltage. This light was passed through the Christiansen dispersion filter (Schott and Gen. Jena). This filter contains minute particles of glass in an organic fluid (an ester of benzoic acid) and has to be used in conjunction with the diaphragm (*F*) and lens (*E*) forming an image of the filament on the adjustable aperture of the diaphragm. For each temperature of the bath containing the filter the refractive indices of the glass and the fluid inside the filter are identical only for a narrow spectral region. In the system used, therefore, the image on the aperture of the diaphragm is monochromatic. In our experiments the water bath was kept at 33·00° C. with the aid of a mercury thermoregulator. Spectroscopic tests showed that the maximal intensity then fell at 0·498 μ. With a diameter of 35 mm. of lens *E* and an aperture of 4 mm. of the diaphragm the total spectral range covered was 0·008 μ. It proved to be important to warm up the filter and keep the bath at the wanted temperature for not less than 1 hr. before taking measurements.

The cross-sectional area of the absorption trough was only 17 mm.^{2}, its length, however, 20 mm. A great deal of care had, therefore, to be devoted to the adjustment of the optical system. Further lenses (*G*) and an additional diaphragm in front of the container holding the trough (*H*) were carefully adjusted to give a slightly convergent beam passing through the axis of the column of fluid in the optic cell (*H*).

The intensity of the light beam passing through the trough was measured with the aid of a vacuum photocell (potassium cathode). This cell did not show any of the troublesome fatigue effects of the slightly more sensitive gas-filled type of photocell. Worked in conjunction with the Wulff electrometer it proved to be sufficiently sensitive for our purposes. The anode of the photocell was connected to the string of the electrometer through electrostatically shielded leads isolated with amber, the surrounds being kept dry by means of phosphorus pentoxide. This degree of insulation proved sufficient to enable us to measure the photoelectric current by observing with a stopwatch the time necessary for the development of a given charge corresponding to a deflexion of 20 points on the scale in the eyepiece of the electrometer. The value obtained is inversely proportional to the intensity. Controls were made with six carmine solutions, diluted in the ratios of 1 to 1/2, 1/4, 1/8, 1/16, and 1/32. Single measurements gave the ratio of their densities as 2·01 instead of the theoretical value 2·00. In the experiments the same solutions were often repeatedly measured. The maximum difference from

the mean value amounted to about 1%, but quite often successive readings gave identical values. Some practice with the stopwatch guaranteed an accuracy of about 0·5%. It was held unnecessary to develop a better method of timing.

The visual purple extracts. The important point was to obtain *all* the visual purple from a given retina. A great deal of attention has recently been devoted [Lythgoe, 1937; Chase & Haig, 1938] to purification of the extracts in order to obtain the true absorption curve for visual purple, but such methods are too elaborate for our purpose owing to the greater risk of unevenly distributed losses in the different stages of purification. The presence of impurities is of little importance when the amount of V.P. has to be measured as a difference between the readings for an unbleached and a completely bleached solution at a single wave-length.

The method chosen for extraction was the one used by Tansley [1931], but it was improved upon by paying more attention to centrifugation. We shall show below that it was adequate for our purpose.

All frogs to be used were dark-adapted over night at room temperature. Removal of the retinae had to be carried out with extreme care as the success of this operation, other conditions being equal, ultimately determines the accuracy of our conclusions. If the retinae do not come out easily too low values for V.P. concentration are obtained. At times some pigment clings to the retina, but this fact, against expectation, was found not to influence the measurements. Removal of the retinae took place in red light of wave-lengths longer than 0·615μ. Each retina was put in 1 c.c. of a 2% digitonin solution, left standing for 40 min. and then centrifuged for 20 min. at a rate of 3800 rev./min. The digitonin solution was prepared fresh every day by boiling. The centrifuged V.P. extracts are perfectly clear. When extraction was complete (see below) the retinal debris at the bottom of the test-tube has lost its red colour.

The measurements. The absorption in the solution of visual purple could be measured photoelectrically without any bleaching taking place during this procedure. Thus, in one experiment the initial density was 0·442; after 17 min. of continuous exposure to the monochromatic light it was 0·437, and finally, after 34 min., 0·436. Density is defined as $D = \log_{10} I_0/I_t$, where I_0 is the intensity of the light incident on the solution, I_t the light transmitted to the recording instrument. The amount of visual purple is given as the difference between the density of the solution immediately after extraction and its density after bleaching of the visual purple for 5 min. with a 1000 W. lamp at a distance of 40 cm.

The electrophysiological technique and the bleaching of the eyes. The electrophysiological material on which the comparison is based consists of seventy experiments performed with twenty-four opened, excised and dark-adapted eyes of Hungarian frogs. The electrical response of these eyes to a constant test flash (1 sec.) of wave-length 0·500 μ. was measured with the aid of a directly coupled amplifier and a cathode ray before and after 5 min. of adaptation to certain wave-lengths of a Tutton monochromator. The results obtained have been evaluated elsewhere from other points of view [Granit, Therman & Wrede, 1938]. Here we shall use certain averages and compare them with V.P. absorption measurements from experiments which were carried out in exactly the same manner up till the moment when, after bleaching, the retina was put into digitonin instead of being tested for its electrical response. A very brief description of the technique of adaptation to monochromatic light should therefore suffice, a detailed account being found in the paper referred to.

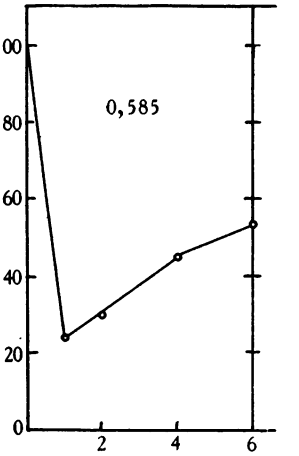

Fig. 2. Bleaching *in vivo* (or adaptation) to wave-length 0·585 μ. Ordinates: relative amounts of visual purple calculated from the size of the *b*-wave of the electrical response. Zero abscissa shows amount of visual purple before adaptation. Abscissae from 1 to 6 min. refer to time after cessation of adaptation.

Initially a number of electrical responses to wave-length 0·500 μ. are measured with the dark-adapted eye. Size of *b*-wave is then of the order of 0·7 mV., but the initial values are averaged and reduced to the arbitrary unit of 100. All effects of adaptation are then conveniently given as percentage reduction of the response to the test light. The initial value having been fixed, a filter of 1·3 log units is removed from the monochromator and the eye exposed to some wave-length of the equal energy spectrum for 5 min. Then the filter is reinserted and the response to the test light during recovery measured at intervals of 1 and, later, 2 min. A typical recovery curve is illustrated in Fig. 2. Generally the eyes were exposed à second time to the adapting light for a new period of 5 min. In fact, all the eyes used for V.P. extraction were bleached twice for two periods of 5 min.

Instead of testing for size of *b*-wave with wave-length 0·500 μ. we now removed the retina after the adaptation and placed it in the digitonin solution. The operation lasted on an average 75 sec. From the

curves of Granit *et al.* [1938] the amount of reduction of the retinal response at this moment could be ascertained.

Results

Effect of bleaching as measured electrophysiologically. Table I shows the effect of adaptation to different wave-lengths in terms of concentration of visual purple as calculated from the size of the *b*-wave elicited by the test

Table I. The table illustrates reduction in concentration of visual purple 75 sec. after cessation of adaptation, as it should be if calculated from the reduction in size of the *b*-wave of the electrical response. The wave-lengths of the adapting lights used are given in the upper horizontal row.

Wave-lengths in μ.	0·585	0·560	0·540	0·500	0·470	0·450	0·430
Percentage reduction	74	69	77	59	56	35	27

light 75 sec. after the period of bleaching. The figures indicate percentage reduction from the original level of 100 before adaptation. V.P. concentration is calculated on the basis of the equation of Chaffee & Hampson [1924] [see Granit *et al.* 1938]. The table illustrates the surprising discovery made by the latter authors that the effect of bleaching is not proportional to the absorption curve for visual purple; the long wave-lengths cause a much greater depression of the response to test light 0·500μ. than the short wave-lengths. The actual amount of bleaching produced by the bleaching light is, however, very small. If the absorption trough containing the V.P. solution is placed in the beam of this light (0·500μ) for 10 min. the effect on the absorption measured by the photo-electric colorimeter does not exceed 2–3%. This indicates extreme sensitivity of the dark-adapted eye to light.

Comparison of visual purple concentration in right and left eyes. Assuming the two eyes of the same frog to contain identical amounts of visual purple, the first experiments carried out were to determine the accuracy of our method by comparing measurements of visual purple densities in right and left eyes. The outcome of this experiment is shown in Table II for fourteen frogs. Despite variations from animal to animal the final averages for this number of frogs are identical to within 1%. The first eight frogs were from an earlier delivery. Their averages were respectively 0·401 and 0·413 and thus differed by 3%. The six remaining frogs (later obtained from Hungary) had eyes containing more visual purple, for right and left eye respectively 0·564 and 0·538, the difference being 4%. Thus it can be seen that with a sufficient number of experimental animals right and left eyes contain amounts of visual purple

identical to within 1% and that an accuracy of 4–6% can be obtained with averages from only four to six eyes.

TABLE II. Comparison of visual purple content in right and left eyes of fourteen frogs

Frog no.	Density in one eye	Density in the other eye	Difference %
I	0·216	0·236	+ 9
II	0·409	0·405	− 1
III	0·484	0·524	+ 8
IV	0·574	0·518	−10
V	0·429	0·486	+ 8
VI	0·403	0·387	− 4
VII	0·315	0·332	+ 5
VIII	0·379	0·416	+ 9
IX	0·709	0·687	− 3
X	0·538	0·526	− 2
XI	0·480	0·509	+ 6
XII	0·570	0·538	− 5
XIII	0·541	0·426	−21
XIV	0·545	0·541	− 1
Averages	0·471	0·467	1

Concentration of visual purple in bleached eyes. In the first set of experiments one eye of a frog served as dark-adapted control, the other eye was bleached with one of the wave-lengths shown in Table III.

TABLE III. Bleaching of one eye with different monochromatic lights, the other eye serving as fully dark-adapted control

	Control	0·430 μ.	Control	0·450 μ.	Control	0·500 μ.	Control	0·585 μ.
	0·522	0·440	0·550	0·526	0·592	0·560	0·530	0·458
	0·531	0·530	0·628	0·668	0·345	0·335	0·481	0·509
	0·454	0·502	0·442	0·492	0·429	0·445	0·330	0·366
	0·542	0·492			0·254	0·319	0·415	0·314
Averages	0·512	0·491	0·540	0·562	0·405	0·415	0·439	0·412

The table shows that for the four wave-lengths used the densities of visual purple of the bleached eyes differ from the values of the controls by −4, +4, +2, and −6% respectively. Averaging separately all the fifteen dark-adapted controls and the fifteen bleached eyes, irrespective of wave-length used for adaptation, the average density of the controls is 0·470, that of the bleached eyes 0·464. The values are thus identical to within 1%, as in Table II. Thus adaptation to monochromatic light has led to no reduction whatsoever of the concentration of visual purple measured in the photoelectric colorimeter with a light of wave-length 0·498 μ. Nevertheless, the electrical response to a test light (0·500 μ.) of practically the same wave-length flashed on to the living retina has been greatly reduced by adaptation, as shown by Table I.

Wave-lengths 0·430 and 0·585 μ. cause approximately equal responses with the low intensity rod spectrum [Granit & Munsterhjelm, 1937].

Actually the response to 0·585 μ. is larger at the higher level of intensity used in these experiments, but this is due to cones taking part in the reaction in the long wave-lengths [Granit & Wrede, 1937]. From the point of view of V.P. absorption the presence or absence of active cones should be immaterial. On the other hand, the bleaching experiments of Table I have shown that V.P. concentration calculated from the reduction of the *b*-wave (for test light 0·500 μ.) is 74 % for wave-length 0·585 μ. as adapting light and only 27 % for wave-length 0·430 μ. as adapting light. In view of this result it was decided to perform a series of experiments in which one eye would be exposed to wave-length 0·585 μ., the other eye to 0·430 μ.

TABLE IV. Bleaching of one eye with wave-length 0·430, of the other eye with 0·585 μ.

0·430 μ.	0·585 μ.		0·430 μ.	0·585 μ.
0·432	0·416		0·439	0·506
0·342	0·412		0·416	0·414
0·483	0·548		0·452	0·397
0·437	0·545		0·417	0·364
0·331	0·504		0·316	0·452
0·359	0·263		0·530	0·486
0·388	0·367	Averages	0·411	0·436

The outcome of this experiment is shown in Table IV. The difference between the average densities is 0·025 or 6 %. It is too small to be regarded as significant, though greater than the previous series would have led one to expect.

DISCUSSION

The facts presented are simple and clear cut. On the one hand earlier work has shown that the size of the *b*-wave elicited by a constant test light increases during regeneration of visual purple, on the other hand we have found above that a considerable reduction in size of the electrical response caused by adaptation to monochromatic light does not coincide with a measurable diminution of the total quantity of visual purple. In a way our result is disappointing because it means that a number of electrically measurable changes must now be regarded as being beyond reach of direct photochemical control, among them the interesting fact that bleaching with long wave-lengths reduces the response to test light 0·500 μ. far more than bleaching with short ones. Nevertheless, it is a positive and significant result that unmeasurably small changes in the total concentration of visual purple are engaged in excitatory processes not far from the upper limit of maximal rod capacity.

How then are we to explain that the size of the electrical response of the retina in one type of experiment—regeneration—is some simple function of V.P. concentration, in another type of experiment—our own—does not correlate in the least with density measurements of visual purple? Our own choice of a working hypothesis is contained in the assumption that most of the visual purple is a store which although it is bleachable is nevertheless physiologically inactive and incapable of initiating nerve impulses. The concentration of the inactive visual purple must be high because by simple diffusion or otherwise it determines how much photosensitive material is available at the locus where excitation takes place. Thus we have accounted for the necessity of a high concentration of visual purple for large electrical responses, as well as for the absence of measurable photochemical changes accompanying the reduction of the *b*-wave after adaptation to moderately strong monochromatic stimuli. In the latter case only the decomposition in the relatively small fraction directly concerned in the process of excitation is physiologically significant.

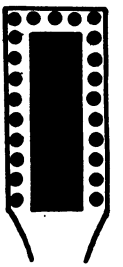

Fig. 3. The diagram illustrates the outer limb of the rod containing a store of inactive visual purple inside the cell and a layer of active stimulating photosensitive material (filled circles) along the surface.

This scheme is pictured by the diagram of Fig. 3 illustrating the outer limb of the rod cell. We have assumed that the active visual purple is active because of its particular manner of distribution, say, at the surface of the cell. The surface charge may be kept up, as suggested, by diffusion from the store of active material inside the cell. Illumination may be assumed to lead to a depolarization of the surface spreading electrotonically and giving rise to the *b*-wave of the electrical response of the retina. In this scheme excitation takes place along the whole surface of the rod cell, but this particular assumption is by no means essential. It is quite possible that excitation is initiated at some other point of still smaller dimensions, and that V.P. has to be bleached in its neighbourhood in order to excite. In fact, such a state of affairs is indicated by the presence of the "directional sensitivity" of the retina, discovered for cone vision by Stiles & Crawford [1933, 1937].

The idea of a store of photosensitive material from which the active material at the locus initiating excitation is replenished owes a great deal of its attractiveness to its simplicity combined with the many perspectives it suggests both with regard to theory and further experimental progress. Thus there is the well-known phenomenon of a heavy outburst of im-

pulses associated with the *b*-wave which appears as an initial "explosion" leaving "refractoriness" in its wake. There is also the "silent period" following the initial discharge [Hartline & Graham, 1932; Hartline, 1938], both of which suggest the hypothesis just outlined.[1] It is also worth while pointing out that on this view the time constant of the process of regeneration of visual purple may be of relatively little immediate significance for the actual process of excitation. The "rate of diffusion" to the locus of excitation may by comparison be more important. It is also of interest for the general problem of cone vision to see that exceedingly small quantities of a photosensitive substance suffice for excitation of a visual cell.

Finally we cannot avoid mentioning a fundamentally different line of thought capable of explaining our results. This is the possibility that the visual purple of the living rod may exist in some relatively stable form comparable to its state in the "artificial retinae" which Weigert & Nakashima [1929, 1930] made by dissolving visual purple in gelatine. If this were so—a possibility by no means excluded—our results would necessitate a still more radical revision of current concepts with regard to how visual purple mediates sensations.

Summary

Two methods have been used in parallel: (i) measurements of the size of the electrical retinal response with a cathode-ray oscillograph and a directly coupled amplifier, (ii) measurements of the total quantity of visual purple of single eyes (digitonin extracts) with a photocell and a Christiansen filter adjusted for wave-length 0·498μ. The preparation was the dark-adapted excised eye of the Hungarian frog.

The aim of the work was to find out whether a reduction of the electrical response to a constant test light (of wave-length 0·500μ.), caused by means of adaptation to monochromatic light, is accompanied by a measurable reduction of the amount of visual purple obtainable from such eyes.

Initially it is shown that the total quantities of visual purple from right and left eye of the same frog are identical to within 1% (averages of fourteen eyes). Thus one eye may be used as dark-adapted control when the other eye is illuminated.

The experiments demonstrate that the total quantity of visual purple obtained from eyes in which the retinal electrical response to wave-length

[1] Dr H. K. Hartline in a discussion with one of us drew attention to the applicability of this idea as a possible explanation of the "silent period".

0·500μ. has been reduced by one-third to one-half by adaptation is the same as in the control eyes. The rods may therefore be forced to nearly maximal activity without incurring measurable losses in their content of visual purple.

Since, on the other hand, the retinal electrical response increases during regeneration of visual purple and thus somehow is determined by the total amount of photosensitive material available, it has been necessary to suggest a hypothesis to account for the apparent discrepancy between these two sets of results.

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