Densitometry of pigments in rods and cones of normal and color defective subjects

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Hecht’s Photochemical Theory may fairly claim to have been the most influential visual theory of recent times. In one form or another it is to be found in most textbooks of vision, and has certainly played a major part in forming the physiological views of most investigators in vision, whether clinical or academic. The strength of Hecht’s theory was that a wide range of visual phenomena could be quantitatively explained by the physics of light and the chemistry of the visual pigments which absorb it. These quantities were in principle within reach of independent objective measurement, as opposed to nerve signals and sensations which at that time were not. The weakness of Hecht’s theory rested in the fact that he never quite succeeded in making those objective measurements and showing that the postulated bleaching and regeneration of rhodopsin matched the chemistry that actually occurred. This, indeed, was not easy to do with the resources then available, but it is noteworthy that the great body of investigators who for some 20 years have “explained” their findings in terms of the hypothetical kinetics of rhodopsin and of still more hypothetical cone pigments seem seldom to have stopped to ask whether these speculations were within 100-fold of the actual kinetics of real visual pigments.

In order to answer the photochemical question raised by the psycho-physical observations of ophthalmology and psychology, what is really needed is a technique that will measure rhodopsin and cone pigments in the living eye of normal and abnormal human subjects. Such a technique we have developed and used for the past 10 years and have measured the pigment in rods and foveal cones of a large number of subjects—mainly students of the Cambridge University Medical School.

The principle of the ophthalmoscope is the principle of this method. As is well known, abnormalities of the choroid are visible in this instrument, and hence light must return from behind the retina and consequently must have passed through it twice. If this reflected light signal is received not upon the observer’s eye but upon a photo-multiplier tube, the output of the tube will be greater the more the pigment has been bleached away, since less pigment will then be present to absorb the signal in its double passage. In this way it is possible to measure the amount of pigment bleached and regenerated—for those changes will be strictly correlated with the size of output from the photo-tube.

The measuring light used must not be too bright or it will bleach away the pigment that should be measured intact, and our measuring light does not bleach more than 2 per cent. But the light signal re-
turning is then so weak that a good deal of attention must be given to the design of the equipment, so that it is self-compensating against various kinds of error. Into this we shall not enter here, hoping that readers may be satisfied with a brief account of the performance, and will be content to consult the references for more detailed descriptions.

The first observations were made upon rhodopsin, and Fig. 1 shows measurements of its bleaching and regeneration performed by Dr. F. W. Campbell on my eye (Campbell and Rushton). The white circles show the effect upon the rhodopsin level (ordinates) when a steady bleaching light was applied, first the moderate retinal illumination of 20,000 trolands, then 100,000, and finally 2,000,000 that bleached away all the pigment. The black circles show the regeneration in the dark during the next 18 min. To this figure I have added (triangles) some results of dark adaptation recently obtained. By a special application of the Aguilar and Stiles technique it has proved possible to measure the dark adaptation curve of rods over a range of 5 log units, and the triangles show the dark adaptation curve so obtained in my eye (taken from Fig. 1 of Rushton). The triangles fit the curve of rhodopsin recovery obtained from my eye 10 years ago, the scale showing fraction of pigment bleached (on left) being suitably graded to match the scale (on right) showing the log threshold during dark adaptation. This justifies Hecht's conjecture that rod dark adaptation is linked with the regeneration of rhodopsin, shows what in fact the link-age is—namely that log threshold is proportional to the fraction of rhodopsin bleached—and confirms on normal man what Dowling had already shown on rats by E.R.G. threshold and Rushton on a rod monochromat by visual threshold.

Fig. 1 strongly suggests that our technique in fact measures rhodopsin, and further tests of various kinds confirmed this conclusion. The Bunsen-Roscoe law of photochemistry $I = k$ was found to hold up to $t = 45$ sec. (after which regeneration becomes noticeable). Lights of various wavelengths that appeared equally bright in twilight vision were each increased some 10,000 times and applied in separate experiments as bleaching lights. They all bleached our "rhodopsin" at the same rate, and this actual rate was more or less what was to be expected from the measurements by Dartnall, Goodeve, and Lythgoe. When measured in lights of different wavelength our "rhodopsin" had nearly the same difference spectrum as true rhodopsin, and when measured at different places on the retina the amount present varied hand in hand with the variation in the population density of the rods (Østerberg), and in particular showed no "rhodopsin" on blind spot or fovea.

This wide and fairly good correlation left little doubt that it was rhodopsin that was being measured, and it encouraged us to see whether on the fovea it was possible even to detect pigments in the cones.

On the face of it that prospect was poor. Wald, Brown and Smith in their well-known paper on Iodopsin concluded "that the ratio of the sensitivities of rod and cone
vision in the intact animal is of the same order as the ratio of absorptions of visual pigments in the rods and cones. Since human cone vision is 100 to 1000 times less sensitive than rod vision it was to be expected that cone pigments would be utterly undetectable by retinal densitometry.

Fortunately the facts proved quite contrary to their conclusion and the density of pigments in foveal cones turned out to be about the same as that of rhodopsin in rods. Thus in this same year it was possible to show9 that the normal fovea contains two photosensitive pigments in the red-green range whereas protanopes were found to lack the red-sensitive one. This was exactly 100 years after Clerk Maxwell9 at the age of 23 proved that protanopes lacked the red primary mechanism of normal trichromacy.

The most satisfactory way to find whether only one photosensitive pigment is present on the fovea in the red-green spectral range is to bleach first with a red light of such a strength that about 50 per cent of the pigment is bleached; then, after full regeneration (7 min.), bleach again, this time with a blue-green light of such a strength that about the same amount is bleached as before. If two pigments are present, that which is more red-sensitive will be more bleached by the red light; the other will be more bleached by the blue-green light. If, on the other hand, only one pigment is present, naturally only one kind of change is possible, and thus when the red and the blue-green lights are adjusted to bleach equally, the two difference spectra will coincide at all wavelengths.

This is what is seen to be the case in Fig. 2. Both for protanopes (triangles) and deutanopes (circles) there is no significant difference between white and black symbols—the bleaching produced by blue-green or red light respectively.

It has sometimes been urged, though always on weak evidence, that deutanopes have the same "red" and "green" cone pigments as normal subjects, but that their nerve signals or their sensations are some-how "fused" to give a single quality of color over the whole red-green range. If this were true, the effect of bleaching seen in Fig. 2 as black and white circles would also be found in the normal subject who does not differ as to pigment (on this
view) and whose nerves and sensations are not involved in these measurements of reflected light. Fig. 3 shows the results of bleaching a normal fovea with the same red and blue-green lights as were used on the deuteranope to give the results of Fig. 2, B. On the normal fovea (Fig. 3), the two lights no longer match. The blue-green bleaches more in amount and differently in kind, for the excess preponderates in the greener half of the effective spectrum. This clearly proves that deuteranopes do not have the two normal “red” and “green” pigments, and indeed the coincidence of black and white symbols in Fig. 2 shows that both protanopes and deuteranopes have but a single pigment in the red-green range. But it will be noticed that this single pigment is not the same in protanope and deuteranope, the deuteranope’s pigment being more red-sensitive than the protanope’s. In fact these are the two normal cone pigments, present together in a mixed population of cones on the normal fovea where they serve to distinguish red lights from green. In these dichromats only one pigment is present forming a homogeneous population in the red-green range. As they have only one pigment they can only appreciate one feature of light, namely its brightness; color (as distinct from brightness) cannot be discriminated.

It is useful to have names for these pigments. Kühne set a bad example when he used “rhodopsin” or sehpurpur which characterizes a pigment by the color of light that was not absorbed and to which the rods were insensitive. The hypothetical “red” (or “green,” or “blue”) photopigments of color vision theory has always meant the “red-sensitive” pigment, and this seems a more natural nomenclature. I have therefore labelled the red-sensitive normal cone pigment, that is also present in the deuteranope, erythrolabe (= red taking); the normal green-sensitive pigment that is also present in the protanope, chlorolabe (= green taking); and the blue sensitive pigment that is present in the normal, protanope, and deuteranope but not in the tritanope, cyanolabe. It was a great satisfaction to find that this nomenclature received support from Dr. Wald who himself has contributed so many names to the chemistry of vision. “Among various sets of terms we have lately been offered for the colour vision pigments, we think those proposed by Rushton to be the most apt and beautiful.”

The experiments of Fig. 2 established firmly that the dichromats have only one photosensitive pigment in the red-green range, but they did not prove that this was a visual pigment nor were they designed to measure at all accurately what the spectral sensitivity was. There are two tests by which we can show that a pigment is a visual pigment, i.e., one by means of which visual responses arise. They both are simple when the pigment appears alone as in the red-green range of these dichromats.

(1) Lights of various wavelengths adjusted in intensity to look equally bright (and hence appearing identical to the dichromat) should all bleach the visual pigment at the same rate. (2) If a bleaching exposure is followed by a period in the dark, the recovery of log threshold, i.e., the dark adaptation curve should have the same time course as the regeneration of the visual pigment.

Test 1. It turns out that retinal densitometry is very suitable for accurate measurements of bleaching rates. It is only necessary to apply a steady bleaching light to the fovea and the pigment will soon be brought to equilibrium (as was shown in Fig. 1 for rhodopsin) where the rate of bleaching is exactly balanced by the rate of regeneration. Diminish slightly the bleaching light and regeneration gains; increase the light and the equilibrium level of bleaching rises. Change to light of a different wavelength but of suitable intensity and the level will remain exactly unchanged. This intensity is naturally the one that bleaches at the same rate as did the former light. In this way we may match bleaching lights correct to 0.06 log unit of intensity, taking 1 min. for each measure-
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Fig. 4. The continuous curve is the log quantum sensitivity of protanopes, the dotted curve of deuteranopes (Pitt, 1944). The black and white circles show plotted as difference from these curves the log action spectrum of chlorolabe and erythrolabe measured by densitometry from bleaching rates. Size of circle gives S.D. of results.

ment, but repeating the procedure a few times.

When this has been done, the same lights are matched in the eye of the same subject by flicker photometry. As there is very little color discrimination in dichromats this may be done correct to 0.01 log unit. We need not trouble about macular pigmentation or the retinal directional effect of color, for both bleaching and flicker measurements use the same optical path and hence these factors do not affect the comparison. In Fig. 4 the curves are the log spectral sensitivity curves of protanopes and deuteranopes taken from Pitt12; the points show by how much the bleaching rates of chlorolabe and erythrolabe deviate from this. In Fig. 4 the accuracy of the measurements is ± 0.06 log unit and these results measure (and those of Fig. 2 do not measure) the spectral sensitivity of erythrolabe and chlorolabe.

Test 2. The relation between dark adaptation and regeneration is shown in Fig. 5 represented in the same way as for rhodopsin in Fig. 1. Fig. 5A, squares, show the dark adaptation of a protanope following a full bleach (scale on the left); circles show the regeneration of chlorolabe, 2 runs (scale on the right). With suitable ratio of the two vertical scales all the experimental points fall upon the same curve. Fig. 5B shows precisely similar results for the deuteranope and proves that chlorolabe and erythrolabe regenerate at the same rate. The identity in the time course of recovery of pigment and of log sensitivity is exactly what was found for rhodopsin, and strengthens the view that the pigments chlorolabe and erythrolabe that we measure are responsible for cone vision.

It follows as an obvious and rather trivial conclusion from these results that in protanopes and in deuteranopes adaptation to lights of different colors will have no differential effect. This was verified some time ago. Rushton13 has applied this result (observed independently) to give a useful routine test for abnormal color vision.

The full proof that the chlorolabe and erythrolabe of the dichromats are the "green" and "red" pigments found in normal eyes is too involved to give here, but a strong indication is shown in Fig. 6, taken from Baker and Rushton14 where other evidence is given. Black triangles show the transmissivity difference spectrum of erythrolabe in the deuteranope, black circles of the "red" pigment in a normal (Dr. H. D. Baker whose fovea is rich in red sensitive cones). White triangles show the same for chlorolabe in the protanope and white circles for the "green" cones of Dr. Baker.

The dotted curves show the theory of Hsia and Graham15 regarding the spectral sensitivity for deuteranopes and protanopes; the continuous curves show the theory of Stiles16 regarding the spectral sensitivities of the red mechanism πr and the green mechanism πg, measured in the normal eye by increment thresholds. Thus both by densitometry and by psychophysics there is a very close resemblance between the pigments found isolated in protanopes and deuteranopes, and those found in conjunction in normal subjects.
These results were completed and presented to The Physiological Society more than 2 years ago.\textsuperscript{17-21} They show without reasonable doubt that protanopes and deuteranopes contain only one cone pigment in the red-green range and that the action spectrum corresponds in each case to the subject's spectral sensitivity of vision. They also show with a little less accuracy, but with high plausibility that the "red" and "green" cone pigments on normal foveas may be identified with the erythrrolabe and chlorolabe of those dichromats. And in all subjects, whether dichromat or normal, the regeneration of cone pigments was seen to follow very closely the course of dark adaptation.

There remained, however, two important questions that could not be answered by our technique of retinal densitometry in man. (1) Is there a demonstrable cone pigment cyanolabe whose spectral sensitivity corresponds to the π mechanism of Stiles\textsuperscript{22} and to the blue cone "achromatopsia" described by Blackwell and Blackwell\textsuperscript{23} (2) Are the three cone pigments separated so that each lies in a different cone?

It is beyond my scope, and probably my competence, to describe adequately the fine work by which Marks, Dobelle, and MacNichol\textsuperscript{24} and Brown and Wald\textsuperscript{25} have, by another technique, answered these remaining and very difficult questions. But the achievement may simply be stated. Both sets of investigators have worked with the excised retinas from man and monkeys and both have been able to measure pigments in single cones. They have succeeded in finding a few cones that contain cyanolabe.

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**Fig. 5A. Dark adaptation curves (scale on left) and pigment regeneration curves (scale on right) after a full bleach. Results with a protanope. Each 30 per cent of pigment bleached raises the threshold about 1 log unit.**
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Fig. 5B. Dark adaptation curves (scale on left) and pigment regeneration curves (scale on right) after a full bleach. Results with a deuteranope. Each 30 per cent of pigment bleached raises the threshold about 1 log unit.

Fig. 6. Transmissivity spectra for dichromats and normal subjects. Black symbols erythrrole in the deuteranope (triangles) and the normal (circles). White symbols chlorolabe in the protanope (triangles) and the normal (circles). Dotted lines, spectral sensitivity of dichromats (Haia and Graham), continuous lines Stiles' red and green mechanisms \( \pi_0, \pi_1 \), in the normal. Erythrrole—the missing pigment with maximum absorption in the blue.

Fig. 7 taken from Marks and associates\(^5\) shows the “corrected” difference spectra plotted by their computer in the analysis of 9 primate cones. It is seen that 3 “red” curves run close together and 3 “green” curves run close together, and the two “blue” curves are clearly distinguished from the rest. Thus there seem to be 3 distinct classes of cone as judged by pigment content.

It is not easy to know simply from these results whether the “red” curve (say) is due to a single pigment or to some red + green mixture. Perhaps at this point our results with retinal densitometry may help. The difference spectrum of erythrrole (Fig. 6 black circles and triangles) has been re-
plotted as black circles in Fig. 7 scaled up to the maximum of that figure; the difference spectrum of chlorolabe (Fig. 6 white symbols) has been similarly plotted as white circles in Fig. 7. Now we have proved in the deuteranope that erythrolabe is a single pigment, and similarly that in the protanope, chlorolabe is a single pigment. The pigments in the red and green cones of Fig. 7 exhibit the same difference spectra as erythrolabe and chlorolabe, consequently those cones are very likely to contain those same single pigments.

Thus, though a mass of detail still awaits analysis, the broad basis of color vision at the pigment level now seems pretty clear and confirms almost exactly what Thomas Young proposed over 150 years ago. Each cone contains one and only one of his "resonators" that responds to various frequencies of light vibration as shown by the curves of Fig. 7. These resonators are the 3 cone pigments—erythrolabe, chlorolabe, and cyanolabe—and lack of one of them, as Young suggested, results in dichromacy.

Retinal densitometry has played a useful part in establishing this. It afforded the first and the only objective evidence that dichromats lack one pigment, and has shown that the erythrolabe or chlorolabe that they possess is the "red" or "green" pigment of normal cones. Moreover, by discovering 10 years ago the then surprising fact that pigments are about as dense in cones as in rods, it has opened the way for the fine new and very sophisticated computer analysis of single cones themselves.

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REFERENCES