Reprinted from *Colour Vision: Physiology and Psychophysics*
edited by J. D. Mollon and L. T. Sharpe,
Academic Press, 1983.

Microspectrophotometry of Human Photoreceptors

H. J. A. DARTNALL, J. K. BOWMAKER and J. D. MOLLON

Material

Through the co-operation of surgeon and patients we have obtained human eyes for examination by microspectrophotometry. The eyes were removed because of malignant growths. Only those cases in which a substantial portion of the retina (including fovea) was uninvaded by the tumour were suitable. We report results from the first seven such eyes.

One eye (number 1, see Table 1) was removed under red light, while two others (numbers 4 and 7) were exenterations, and were taken complete with eyelids sutured together. The remaining four eyes were removed under the full glare of the operating lights. The eyes were dissected under deep red light, and 1 mm^2 portions of the retina, close to (and including) the fovea, were taken and prepared in the usual way (Bowmaker, Dartnall and Mollon, 1980) for examination of the photoreceptors. Transverse measurements were made of the absorbance spectra of the outer segments of the rods and cones, using a Liebman microspectrophotometer (for description see Knowles and Dartnall, 1977).

From this material 173 records of different outer segments were obtained. It was easy to recognise the outer segments of the rods, and all 44 examples had spectra with λ_{max} (wavelength of peak absorbance) close to 496 nm. The outer segments of the cones, however, were assigned to three classes simply according to their λ_{max}, since we were unable to distinguish the classes morphologically. Sixty-nine of them peaked between 550 and 570 nm and were designated "reds", 49 were "greens" (peaking at 520–540 nm) and 11 were "blues" (415–425 nm).

COLOUR VISION
ISBN 0 12 000000 0

Precise values of λ_{max} were not calculated for 26 (15%) of the 173 records. This was either because their signal-to-noise ratios were very low (transverse density < 0.01) or because the traces were irregular through drifting of cells into or out of the beams. The remaining 147 acceptable records (see Table 1) gave the following mean results:

rods	λ_{max} = 496.3 ± 2.3 nm (n = 39);
"red" cones	λ_{max} = 558.4 ± 5.2 nm (n = 58);
"green" cones	λ_{max} = 530.8 ± 3.5 nm (n = 45);
"blue" cones	λ_{max} = 419.0 ± 3.6 nm (n = 5).

Absorbance Spectra of the Photoreceptors

The mean absorbance spectra of these four classes of photoreceptor are shown in Fig. 1. From left to right these four curves refer to "blue" cones (419 nm), rods (496 nm), "green" cones (531 nm) and "red" cones (558 nm).

It is appropriate here to emphasize the unfamiliar way in which the spectra of Fig. 1 have been plotted. It has become usual over the years to plot the spectra of visual pigments against a scale of frequency rather than one of wavelength. This practice stems partly from considerations of Quantum Theory and partly from a nomogram (Dartnall, 1953) which was constructed on the supposition that on the frequency basis all visual pigments had the same shape of absorbance spectrum. In 1968, however, Liebman and Entine showed that the spectra of frog and tadpole photoreceptors, as determined by the microspectrophotometric method, were progressively narrower on this scale as the peak wavelength (λ_{max}) advanced towards longer wavelengths. We confirmed this for the four pigments of the cynomolgus monkey and showed that the band-width of the spectrum was linearly related to the spectral location of the peak on a frequency scale (Bowmaker, Dartnall and Mollon, 1980). More recently Barlow (1982), also using our cynomolgus data, made the capital observation that the shapes of the spectra can be made very similar by plotting them in another way – namely to an abscissal scale of the fourth root of wavelength.

It is on this scale that the mean absorbance spectra of the four kinds of photoreceptor are plotted in Fig. 1. It is clear from the figure that Barlow's observation also holds closely for the human pigments, the continuous curves drawn through the data being of exactly the same shape in each case. This standard curve is, in fact, the mean of the four curves drawn through each set of points separately. Data for drawing the standard curve, and hence for constructing the absorbance spectrum of any visual pigment based on retinal are given in Table 2.

TABLE 1 Details of the results. ''Mean density'' is the mean transverse optical density at λ_{max}

Eye number	1	2	3	4	5	6	7	All
Sex	male	male	male	male	female	female	female	
Age	46	43	58	70	34	74	63	
Rod outer-segments								
Number	11	5	1	13	1	8	0	39
Mean λ_{max}	496.5	497.2	496.0	496.2	494.0	495.9	–	496.3 ± 2.3 nm
Mean density	0.035	0.027	0.025	0.044	0.033	0.045	–	0.039 ± 0.011
''Blue''-cone outer-segments								
Number	3	1	0	1	0	0	0	5
Mean λ_{max}	419.3	419.0	–	418.0	–	–	–	419.0 ± 3.6 nm
Mean density	0.037	0.024	–	0.023	–	–	–	0.032 ± 0.011
''Green''-cone outer-segments								
Number	11	2	5	13	2	9	3	45
Mean λ_{max}	532.7	532.5	527.6	530.7	534.5	529.0	530.7	530.8 ± 3.5 nm
Mean density	0.033	0.026	0.040	0.036	0.032	0.040	0.030	0.035 ± 0.008
''Red''-cone outer-segments								
Number	20	5	3	8	2	9	11	58
Mean λ_{max}	560.9	561.4	552.0	553.1	556.0	556.3	560.0	558.4 ± 5.2 nm
Mean density	0.027	0.021	0.029	0.033	0.029	0.034	0.023	0.028 ± 0.007

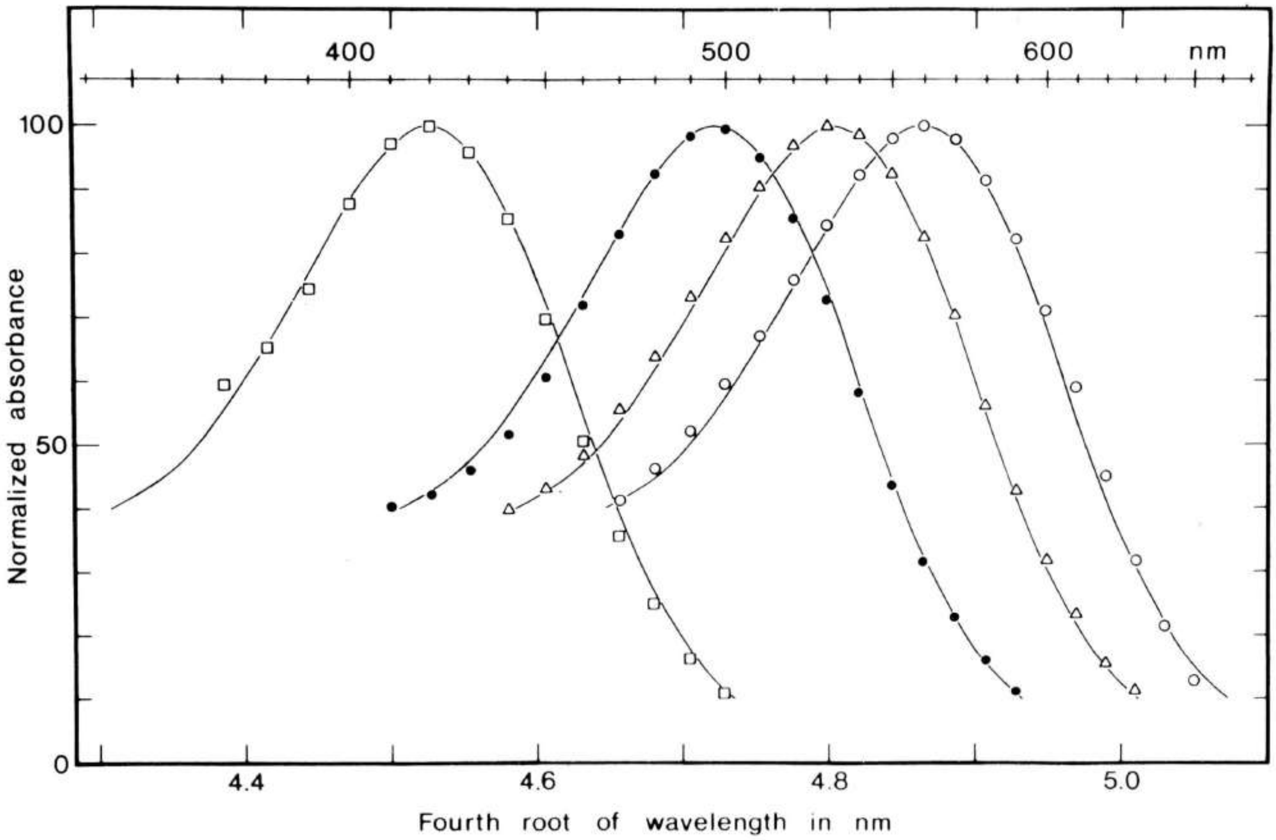

FIG. 1 The mean absorbance spectra of the four human photoreceptors. Squares, the "blue" cones (λ_{max} = 419.0 ± 3.6 nm, mean of 5); filled circles, the rods (λ_{max} = 496.3 ± 2.3 nm, mean of 39); triangles, the "green" cones (λ_{max} = 530.8 ± 3.5 nm, mean of 45); plain circles, the "red" cones (λ_{max} = 558.4 ± 5.2 nm, mean of 58). The curves are all exactly the same shape and were calculated from the data tabulated in Table 2. Note inset scale of wavelengths.

Spectral Distribution of the Photoreceptors

As already mentioned the values of λ_{max} in each of the four groups of photoreceptors were spread over considerable ranges of wavelength. In Fig. 2 these distributions are shown in histogram form for each of the seven eyes separately, and also as totals. The eyes 1, 2, 3 and 4 were from male patients, and 5, 6 and 7 from females. There is no obvious difference between the sexes. The absence of the uncommon "blue" cones in females is only apparent – not real. In fact in one female eye (no. 6) two examples of bleachable "blue" cones were found, but the records were not good enough for the precise computation of λ_{max}, and consequently do not appear in Fig. 2.

In Fig. 2 the mean values of λ_{max} for the four kinds of photoreceptor are shown by the four vertical dashed lines. There are only five examples of the "blue" cone, the λ_{max} ranging from 414–424 nm. With so few examples, there is little one can say about the distribution of λ_{max}. In the case of the other receptors, however, there are perhaps sufficient numbers of observations to warrant some cautious remarks about distribution. Thus it is at once clear

TABLE 2 Data for constructing the Absorbance Spectrum of any visual pigment, based on retinal and of known λ_{max}

Absorbance *A* (% maximum)	$\lambda_A^{1/4} - \lambda_{max}^{1/4}$
40	−0.216
50	−0.157
60	−0.128
70	−0.098
80	−0.073
90	−0.048
95	−0.031
95	+0.033
90	+0.047
80	+0.068
70	+0.085
60	+0.099
50	+0.114
40	+0.130
30	+0.149
20	+0.174
10	+0.211

Example. Suppose λ_{max} = 535 nm. Consequently $\lambda_{max}^{1/4}$ = 4.809 and 80% (say) absorbance on the short-wave side of the maximum occurs at a wavelength λ_A given by $\lambda_A^{1/4} - 4.809 = -0.073$, i.e. at 503 nm.

from Fig. 2 that whereas the mean λ_{max} for rods coincides with the most frequent value this is not so with the "green" and "red" cones. In both these cases, the mean values of λ_{max} occur where there is a dip in the distribution frequency.

In order to take the examination of λ_{max} distribution a stage further the histograms for the rod, "green" cone and "red" cone populations have been re-plotted on a larger scale in Fig. 3, together with interpretative statistical functions.

The 39 rod records give a mean λ_{max} value of 496.3 nm and a standard deviation of ± 2.3 nm. The dashed curve in Fig. 3 is what one would expect from these statistics when the distribution is normal. The normality of a distribution can be conveniently assessed, when n is small, by Shapiro and Wilk's (1965) analysis of variance test. Application of the test to our rod data showed no evidence of non-normality ($W = 0.969$, $p = \sim 0.5$).

The 45 "green" cone records give a mean λ_{max} of 530.8 nm and a standard deviation of ± 3.5 nm. The distribution (if normal) to be expected from these values is given by the dashed curve in Fig. 3; it does not seem to be a satisfactory

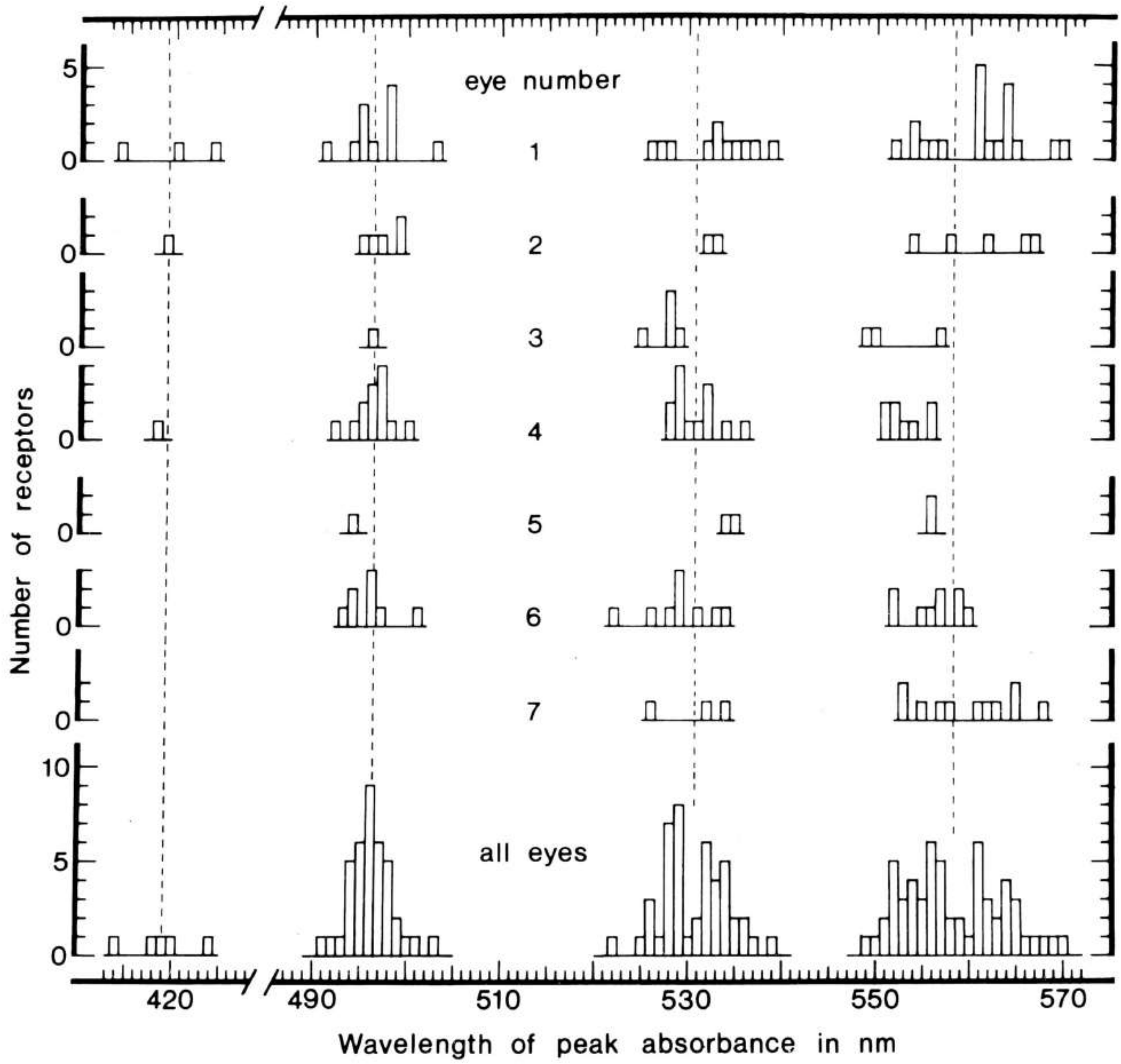

FIG. 2 Spectral distributions of the human photoreceptors. The vertical dashed lines give the mean values of λ_{max} for the four kinds of receptor. See also Table 1.

description. Does this mean that the distribution is not normal? Are there subpopulations of "long" and "short" green cones?

If we divide the 45 "greens" into two groups according to whether λ_{max} lies above or below 530.5 nm we obtain 23 "longs" with a mean λ_{max} of 533.7 nm and standard deviation of ± 2.1 nm, and 22 "shorts" with a mean of 527.8 and standard deviation of 1.8 nm. The expected distribution for *two* such normal populations is given by the double-hump continuous curve in Fig. 3.

This might be thought to describe the actual distribution of λ_{max}-values better than the single-hump (dashed) curve. But when Shapiro and Wilk's test is applied to the "green" cone data the *W* value of 0.976 is obtained, close to the value of 0.973 given for the 0.5 probability level. Thus, perhaps surprisingly, there is no evidence for non-normality. It is clear that when the mean values of two normal populations are close in relation to their standard

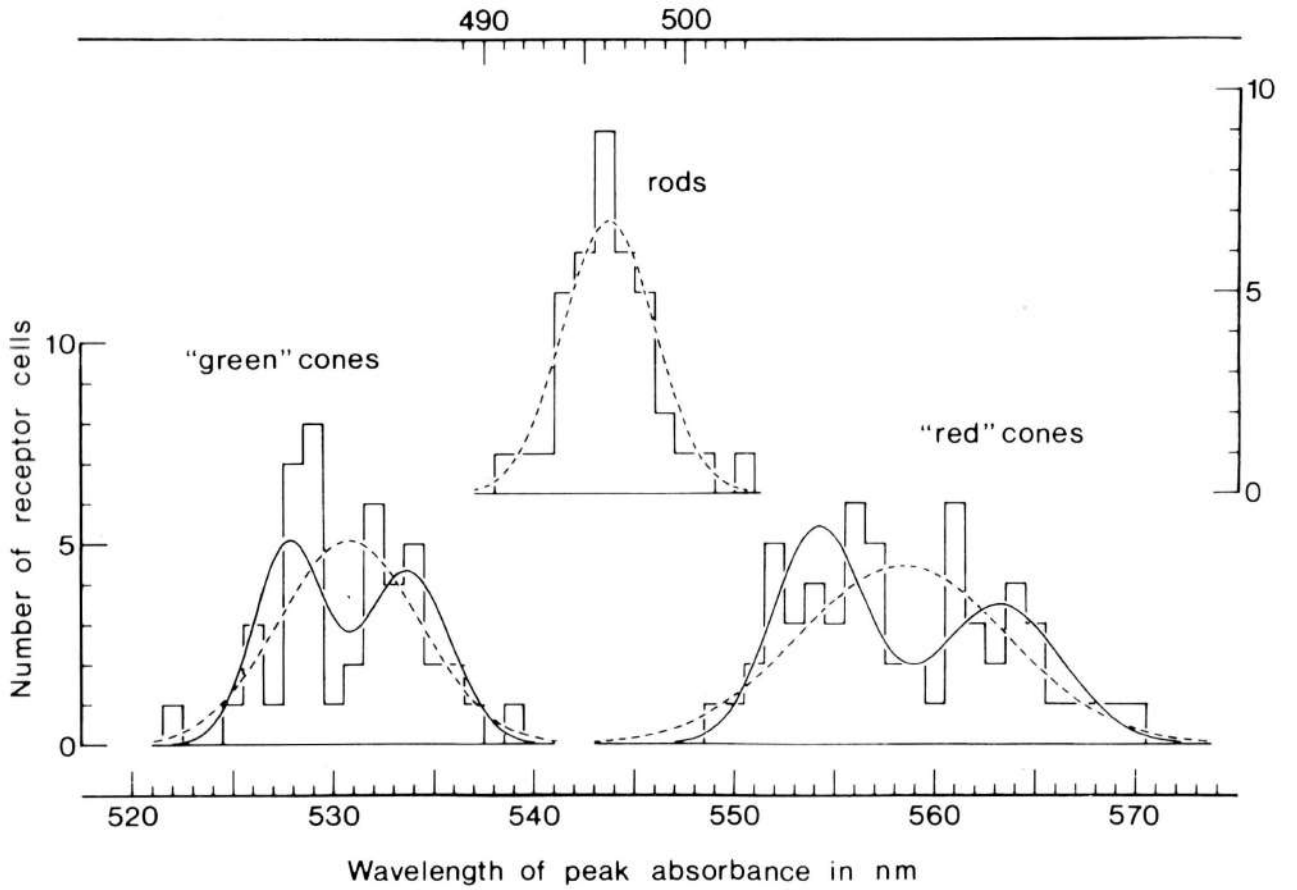

FIG. 3 Spectral distributions of the human rods, "green" cones and "red" cones interpreted in terms of their statistics. The dashed-line curves give the normal distributions expected from the relevant means and standard deviations. The double-hump continuous curves drawn through the "green" and "red" cone data give the expectations if the respective populations are regarded as containing "long" and "short" subpopulations. For further explanation see text.

deviations the test will not easily distinguish the combination from a single normal distribution having its mean between the two.

The 58 "red" receptors yield a mean λ_{max} of 558.4 nm with a standard deviation of $\pm$ 5.2 nm. The normal distribution corresponding to these statistics is given by the dashed curve in Fig. 3. It seems to be a poor description of the population distribution, even worse than in the case of the "green" cones. Application of the Shapiro and Wilk test confirms that distribution of the "red" cone population is significantly non-normal ($W = 0.940, p = \sim 0.02$).

If we divide the 58 "reds" into two groups according to whether the λ_{max} lies above or below 558.4 nm we obtain 27 "longs" with mean $\lambda_{max} = 563.2 \pm 3.1$ nm and 31 "shorts" with mean $\lambda_{max} = 554.2 \pm 2.3$ nm. The expected distribution for such a population is given by the continuous double-hump curve for "reds" in Fig. 3. It seems to be a fair approximation to the actual distribution.

Spectral Location of "Red" Cones, and Red Sensitivity

When the microspectrophotometric examination of the first four eyes (see Fig. 2) had been completed it was noticed that the results obtained with the first and fourth eyes showed differences as regards the average spectral locations of the "red" cones. It was thought possible that these differences were large enough to have visual significance. Consequently the vision in the remaining eyes of patients 1 and 4 was tested psychophysically. Each patient completed a number of clinical tests of colour vision comprising: (a) pseudoisochromatic plates, including the Ishihara (9th edn), the Okuma (Amoriex Co., Tokyo), the Farnsworth tritan plate, and an unpublished tritan plate kindly provided by J. Birch-Cox; (b) the City University test, a booklet version of the Farnsworth Panel D15 test; (c) the Farnsworth-Munsell 100-hue test; and (d) the Nagel anomaloscope. For details of several of these tests see Pokorny, Smith, Verriest and Pinckers (1979).

A reduced version of Stiles' field-sensitivity method (Stiles, 1978) was also used. Detection thresholds were first measured for 666-nm targets presented for 10 msec in Maxwellian view to the dark-adapted fovea. When the absolute threshold had been established the 666-nm target was set to be one $\log_{10}$ unit above the absolute threshold and a steady adapting field, subtending 6.25 degrees of visual angle was introduced. In successive runs the wavelength of the adapting field was either 650 nm or 555 nm and in each run the radiance of the field was adjusted, according to a single staircase procedure, in order to estimate the radiance at which 50% of targets were detected (see Mollon and Polden (1977) for general details of the apparatus).

Both patients gave normal responses on all the pseudoisochromatic plates, and neither made any errors on the City University test. On the Farnsworth-Munsell test, patient 1 had a total error score of 89 on first test and 40 on second; for patient 4 the corresponding values were 115 and 51. These scores are within the ranges for normal observers of the appropriate age groups (Verriest, 1963). Neither patient showed the clustered pattern of errors characteristic of colour defective patients.

On the Nagel instrument the anomalous quotients for patient 1 were 1.09 for a 3.1-deg field and 1.04 for a 1.2-deg field; for patient 4 the corresponding values were 0.98 and 1.08. The range of anomalous quotients for normal trichromats is given as approximately 0.74 to 1.33 by Pokorny *et al.* (1979).

However, although both patients were classified as normal trichromats by all clinical tests of colour vision, there was a clear difference in their relative sensitivities to long-wave fields under the conditions designed to isolate the long-wave pigment. Table 3 shows for each patient the $\log_{10}$ psychophysical ratio, that is the difference between the $\log_{10}$ field sensitivities at 555 nm and 650 nm. The range of values in normal subjects is not known but two other normal trichromats, tested concurrently, gave values of 0.83 and 0.70.

TABLE 3

Patient	$\log_{10}$ psychophysical ratio	
	Measured	Estimated from microspectrophotometry
1	0.56	0.58
4	0.75	0.71

The table also shows for comparison the values of the $\log_{10}$ psychophysical ratio calculated from the absorbances at 555 nm and 650 nm of the "red" cones of the two patients. The estimated values were obtained by assuming that receptors in the central fovea have a length of 35 μm and a specific absorbance of 0.015 μm^{-1}, giving an axial absorbance at λ_{max} of 0.525 (see Bowmaker and Dartnall, 1980). It was also assumed that there was no difference in the pre-receptoral absorption at the two wavelengths. The agreement between the measured and estimated values of the $\log_{10}$ psychophysical ratios is satisfactory, and supports the conclusion from the microspectrophotometric measurements that the "red" cones of patient 1 peak, on average, at longer wavelengths than those of patient 4.

Comparison of a Deutan and a Normal

Some considerable time after completion of the microspectrophotometric examination of the seven eyes described above we were fortunate in being able to examine a 75-year-old deutan the day before enucleation of his right eye. The examination was confined to the left eye as he was not able to perform the tests with his right. This patient (No. 9) made 22 errors with the Ishihara Plates, indicating severe red–green deficiency. On the four diagnostic plates (nos 22–25) he gave three deutan responses and one erroneous but unclassified response ("8" to plate 22). On the screening cards of the Okuma test, he gave 7/7 responses characteristic of the "red–green blind". On the diagnostic cards the classification was "severe deutan".

In the Farnsworth-Munsell 100-hue test his total error score was 220 on the first test and 305 on second. The mean results for this test are shown in Fig. 4 and provide a classical deutan pattern. In fact all tests carried out on this patient were consistent with a deutan defect. The acceptance of all R/G ratios on the anomaloscope (and the setting of a neutral point (at 502–504 nm) in the tungsten spectrum) strongly suggests that he is dichromatic.

The results of the microspectrophotometric examination of portions of the retina of the enucleated eye are equally striking and are also shown in the lower part of Fig. 4. Of the 17 receptors examined 2 were "blue" cones, 5 were rods and 10 were "red" cones. No "green" cones were found. The results obtained

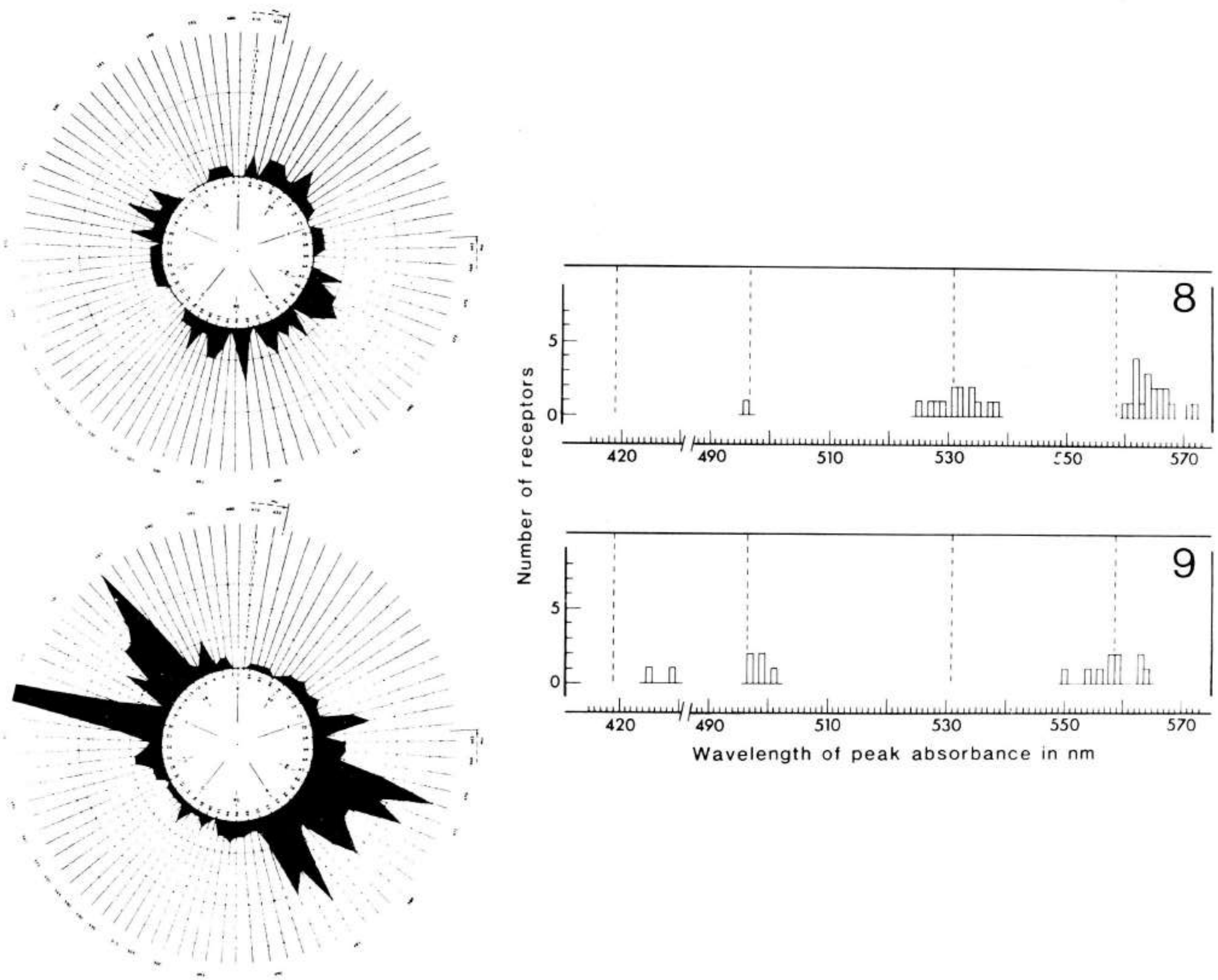

FIG. 4 Correlation between psychophysical and microspectrophotometric measurements on human patients. Results are shown for patient No. 8 (above), a normal trichromat, and for patient No. 9 (below), a deutan. In each case, performance on the Farnsworth-Munsell 100-hue test is shown to the left, and the spectral distribution of photoreceptors is shown to the right. The vertical dashed lines in the right-hand plots have the same spectral locations as those of Fig. 2. Note the absence of ''green'' cones in the case of patient No. 9.

from a normal patient (No. 8) of age 56 examined by similar procedures just over a month before the deutan are also shown in Fig. 4 (upper part) and provide a useful comparison. On the Farnsworth-Munsell test this patient had a total error score of 102, which is close to the mean for colour normals of this age group (Verriest, 1963), and, indeed, all other tests were consistent in suggesting that he is a normal trichromat. Of the 33 receptors examined by the microspectrophotometer, one was a rod, 13 were "green" cones and 19 were "red" cones. No example of the relatively uncommon "blue" cone was found in this patient, but this is not unusual (cf. Fig. 2). The values of λ_{max} for the "red" cones of patient 8 lie at the long-wavelength end of the distribution of "red" cones in Fig. 2.

The microspectrophotometric results for patient 9 provide direct evidence against the hypothesis that in deuteranopia although both "red" and "green" cones are present in normal proportions, their signals are mixed at a post-receptoral level. This hypothesis – which originates from Fick (1879) – has been resuscitated relatively recently (e.g. Wright, 1967). The present results, however, indicate the absence of "green" (middle-wave sensitive) cones in our deuteranope.

The strength of this conclusion can be approximately assessed in the following way. The first seven (normal) eyes we examined yielded 49 "green" cones and 69 "red" cones. If we assume that these numbers roughly represent the relative proportions of "green" and "red" cones in the foveolar region, then the chance that 10 non-"blue" cones would all be "red" is $(69/118)^{10} = 0.0047$, or less than one in two hundred. Thus the evidence is fairly strong that the deuteranopic eye (in which the 10 non-"blue" cones were indeed all "red") is lacking a cone type. Moreover, the mean λ_{max} of the residual class is 558.4 ± 4.4 nm, precisely the same as the mean value, 558.4 ± 5.2 nm, for the "red" receptors of the seven normal eyes of Table 1. Our conclusions are concordant with those drawn by Rushton (1965) and by Alpern and Wake (1977) from reflection-densitometry measurements.

References

Alpern, M. and Wake, T. (1977). Cone pigments in human deutan colour vision defects. *J. Physiol., Lond.* **266**, 595–612.

Barlow, H. B. (1982). What causes trichromacy? A theoretical analysis using comb-filtered spectra. *Vision Res.* **22**, 635–643.

Bowmaker, J. K. and Dartnall, H. J. A. (1980). Visual pigments of rods and cones in a human retina. *J. Physiol., Lond.* **298**, 501–511.

Bowmaker, J. K., Dartnall, H. J. A. and Mollon, J. D. (1980). Microspectrophotometric demonstration of four classes of photoreceptor in an Old World primate, *Macaca fascicularis. J. Physiol., Lond.* **298**, 131–143.

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

Fick, A. (1879). Die Lehre von der Lichtempfindung. In *Handbuch der Physiologie*, Vol. 3 (ed. Hermann, L.), pp. 139–234. Vogel, Leipzig.
Knowles, A. and Dartnall, H. J. A. (1977). *The Photobiology of Vision*, Vol. 2B of *The Eye* (ed. Davson, H.). Academic Press, London and New York.
Liebman, P. A. and Entine, G. (1968). Visual pigments of frog and tadpole *Rana pipiens. Vision Res.* **8**, 761–775.
Mollon, J. D. and Polden, P. G. (1977). An anomaly in the response of the eye to light of short wavelengths. *Phil. Trans. roy. Soc. B* **278**, 207–240.
Pokorny, J., Smith, V. C., Verriest, G. and Pinckers, A. J. L. G. (1979). *Congenital and Acquired Color Vision Defects.* Grune and Stratton, New York.
Rushton, W. A. H. (1965). A foveal pigment in the deuteranope. *J. Physiol., Lond.* **176**, 24–37.
Shapiro, S. S. and Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika* **52**, 591–611.
Stiles, W. S. (1978). *Mechanisms of Colour Vision.* Academic Press, London.
Verriest, G. (1963). Further studies on acquired deficiency of color discrimination. *J. opt. Soc. Am.* **53**, 185–195.
Wright, W. D. (1967). *The Rays are not Coloured*, p. 78. Hilger, Bristol.