The Molecular Basis of Visual Excitation

by
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In 1967 Professor Wald, together with Professors H. K. Hartline and R. Granit, received the Nobel Prize for Medicine. The article that follows consists of most of the lecture delivered by Professor Wald last December when he received the prize in Stockholm.

As a graduate student at Columbia University, I was introduced to vision by Selig Hecht in a particularly provocative way. Hecht was one of the great measurers of human vision, like Aubert, König and Abney before him. But he was not content merely to measure. He wanted to understand what lay behind the measurements, what was going on at the molecular level in vision.

There a door was opened for him, while still a graduate student at Harvard, by the great Swedish physical chemist Svante Arrhenius. Hecht has told me of the excitement with which he read Arrhenius's new book *Quantitative Laws in Biological Chemistry*[1]. It offered the hope of translating accurate measurements on whole organisms into the simple kinetics and thermodynamics of chemical reactions in solution.

In this vein Hecht applied his measurements and those of earlier workers to constructing a general conceptual model for the photoreceptor process. A photosensitive pigment, S, was dissociated by light into products, P + A, one of them responsible for excitation. In turn P + A, or a variant, P + B, recombined to regenerate S. In continuous light these opposed reactions achieved a pseudo-equilibrium, a photostationary state, that underlay the steady states of vision in constant illumination[2].

I left Hecht's laboratory with a great desire to lay hands on the molecules for which these were symbols. That brought me to Otto Warburg in Dahlem, where I found vitamin A in the retina[3]. There were good reasons to look for it there, as I found out later, and that is the way I wrote my paper. Dietary night blindness, a condition already known in ancient Egypt, had been shown in Denmark during the First World War to be a symptom of vitamin A deficiency[4]; and Fridericia and Holm[5] and Tansley[6] had shown that vitamin A deficient rats synthesize less rhodopsin than do normal animals. But vitamins were still deeply mysterious, and at that time one hardly expected them to participate directly in physiological processes. I think this was the first instance of so direct a connexion, though Warburg and Christian were already analysing the first yellow enzymes[7], and shortly their chromophore riboflavine would prove to be vitamin B_2 (ref. 8).

After that, things happened quickly. I went to Karrer in Zurich, who with Morf and Schöpp had the year before established the structure of vitamin A (ref. 9), to complete its identification in the retina. Then I went on to Meyerhof in Heidelberg, to do something else; but with a shipment of frogs that had gone astray, I found retinene, an intermediate in the bleaching of rhodopsin, on the way to vitamin A (ref. 10). Years later Ball, Goodwin and Morton in Liverpool showed that retinene is vitamin A aldehyde[11]. At Morton's suggestion the names of all these molecules have recently been changed, in honour of the retina, still the only place where their function is understood. Vitamin A is now retinol, retinene is retinal (Fig. 1); there is also retinoic acid.

That early Wanderjahr in the laboratories of three Nobel laureates—Warburg, Karrer, Meyerhof—opened a new life for me: the life with molecules From then on it has been a constant going back and forth between organisms and their molecules—extracting the molecules from the organisms, to find what they are and how they behave, returning to the organisms to find in their responses and behaviour the greatly amplified expression of those molecules.

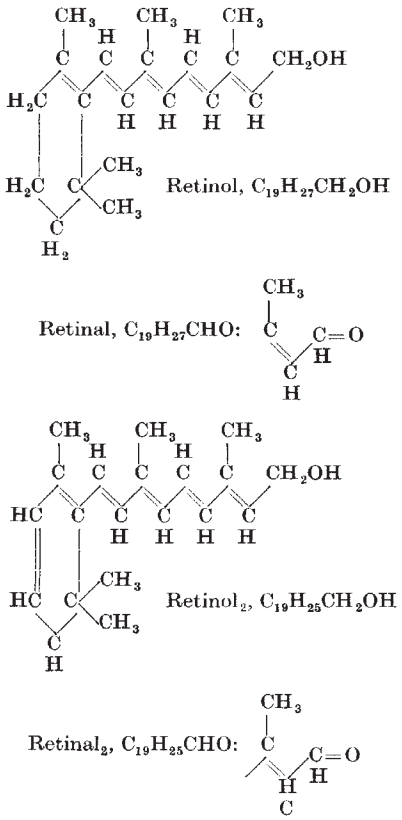

Fig. 1. Structures of retinol$_1$ and retinol$_2$ (vitamins A_1 and A_2) and their aldehydes, retinal$_1$ and retinal$_2$ (formerly retinines 1 and 2).

A basic characteristic of the scientific enterprise is its continuity. It is an organic growth, to which each worker in his time brings what he can; like Chartres or Hagia Sofia, to which over the centuries a buttress was added here, a tower there. Hecht's work was most intimately bound up with that of men who had worked generations before him: Hermann Aubert in Breslau[12], Arthur König in Berlin[13], Abney in England[14]. Now I entered into such a relationship with Willy Kühne of Heidelberg. Kühne had taken up rhodopsin immediately on Franz Boll's discovery of it in 1877[15], and in two extraordinary

years he and his co-worker Ewald learned almost everything known about it for another half-century[16]. It was largely on the basis of Kühne's observations that I could conclude that rhodopsin is a protein, a carotenoid-protein such as Kuhn and Lederer had just shown the blue pigment of lobster shells to be[17], that in the retina, under the influence of light, engages in a cycle of reactions with retinal and vitamin A (refs. 10 and 18).

I owe other such debts to past workers in far-off places. Köttgen and Abelsdorff had found the visual pigment from eight species of fish to have difference spectra displaced considerably toward the red from the rhodopsins of frogs, owls and mammals[19]. Trying to check this observation at Woods Hole, I was surprised to find the same rhodopsin–retinal–vitamin A cycle in fishes there as frogs[20]. It turned out that Köttgen and Abelsdorff had worked entirely with freshwater fishes. On turning to them, I found another visual pigment, porphyropsin, engaged in a cycle parallel with that of rhodopsin, but in which new carotenoids replace retinal and vitamin A (ref. 21). On the basis of these observations, it was suggested that the substance that replaces vitamin A in the visual system of freshwater fishes be called vitamin A_2 (ref. 22). In what follows I shall call it retinol$_2$, and its aldehyde retinal$_2$. These substances differ from their analogues in the rhodopsin cycle only in possessing an added double bond in the ring (Fig. 1)[23].

Shortly afterwards, it emerged that such familiar euryhaline and hence potentially migratory fishes as salmon, trout and the "freshwater" eel possess mixtures of rhodopsin and porphyropsin, in which the system commonly associated with the spawning environment tends to predominate. Other such euryhaline fishes as the white perch and alewife possess this system virtually alone[24]. The bullfrog has porphyropsin as a tadpole, and changes to rhodopsin at metamorphosis[25]. The sea lamprey, *Petromyzon marinus*, possesses mainly rhodopsin on its downstream migration to the sea, but on going upstream as a sexually mature adult to spawn has changed to porphyropsin[26]. Denton and Warren having shown that the rhodopsins of deep sea fishes have spectra displaced to shorter wavelengths than those of surface forms (that is, to λ_{max} about 480 mμ)[27], it developed that the European eel at sexual maturity, preparatory to migrating to the Sargasso Sea to spawn, transfers from its previous mixture of rhodopsin and porphyropsin to deep-sea rhodopsin[28]. The chemical pattern of visual systems maintains close relationships with the evolution, development and way of life of these and other animals[29]. I am glad to say that the pursuit of molecules has not taken me out of biology, but led me more deeply into it.

Let me now leave this history and say where it has brought us.

All the visual pigments we know are built on a common plan. All of them consist of retinal bound as chromophore to a type of protein, called an opsin, found in the outer segments of vertebrate rods and cones and the analogous rhabdomeres of invertebrate eyes. In vertebrates, the two retinals, 1 and 2, join with two great families of opsins, those of the rods and those of the cones, to form the four major pigments of vertebrate vision[30]:

		Usual λ max (mμ)
Retinol$_1$ $\overset{DPN^+}{\underset{DPN-H}{\rightleftarrows}}$ Retinal$_1$	+ Rod opsin $\overset{light}{\rightleftarrows}$ Rhodopsin	500
	+ Cone opsin $\overset{light}{\rightleftarrows}$ Iodopsin	562
(Alcohol dehydrogenase)		
Retinol$_2$ $\overset{DPN^+}{\underset{DPN-H}{\rightleftarrows}}$ Retinal$_2$	+ Rod opsin $\overset{light}{\rightleftarrows}$ Porphyropsin	522
	+ Cone opsin $\overset{light}{\rightleftarrows}$ Cyanopsin	620

The retinols are oxidized to the corresponding retinals by alcohol dehydrogenases. The first such enzymes we examined, those of frogs and fishes, used DPN as coenzyme[31]. Other systems have since been found that prefer TPN as coenzyme[32a], and recent work, as now seems inevitable, is multiplying the numbers of such enzymes, some of which may act preferentially on retinol among the alcohols (hence retinol dehydrogenases)[33].

In physiological conditions the equilibrium between retinol and retinal lies far over toward reduction, so far that retinol is oxidized to retinal only to the degree that the latter is trapped out of the system. *In vitro* that can be done with hydroxylamine, which condenses spontaneously with retinal to form retinal oxime: $C_{19}H_{27}HC{=}O + H_2NOH \rightarrow C_{19}H_{27}HC{=}NOH + H_2O$. In the retina, opsin performs this function, trapping retinal as fast as it appears, to form the visual pigments[34]. Thus it is opsin that regulates how much retinol is oxidized, and visual pigment synthesized.

Along with this parallelism of structure, the visual pigments exhibit an extraordinary parallelism of chemical behaviour. The reactions one finds with any of them are usually found, with minor variations, in all the others. The opsins, like other proteins, are species specific, and often are multiple within a species, as in man. With the different opsins go differences in absorption spectrum, stability, the kinetics of bleaching and regeneration, and other properties. Yet all the visual systems we know represent variations on a central theme. The vertebrates play this out to the end. The invertebrates tend to cut it short at various levels. The action of light on the known invertebrate pigments ends with the production of retinal, in most instances still attached to opsin. Also all the known visual pigments of invertebrates have retinal$_1$ as chromophore[29a].

Some years ago Collins, and Morton and Pitt[35], provided good evidence that in rhodopsin, retinal is bound to opsin in Schiff base linkage, by the condensation of the aldehyde group of retinal with an amino group of opsin: $C_{19}H_{27}HC{=}O + H_2N\text{-opsin} \rightarrow C_{19}H_{27}HC{=}N\text{-opsin} + H_2O$. Bownds in our laboratory has recently identified this amino group in cattle opsin as the ε-NH_2 group of lysine. He has also analysed the neighbouring covalently bound amino-acids; together with lysine they constitute a decapeptide segment of the composition: ala_3 phe_3 thr pro ile ε-*N*-retinyl lysine[36]. Cattle rhodopsin as usually prepared has a molecular weight of about 40,000 (ref. 37). If the molecule is spherical, its diameter is about 40 Å. The chromophore, though its molecular weight is only 282, is about 20 Å long. It looms surprisingly large therefore in the structure of rhodopsin.

To make visual pigments demands not only retinals 1 or 2, but the right *shapes* of these molecules[37]. The retinals possess four carbon-to-carbon double bonds in the side-chain, each of which might potentially exist in either *cis* or *trans* configuration (Fig. 2). The most stable and prevalent form is the all-*trans*. The first double bond at C 7 is always *trans*, since hindrance between the methyl groups on C 1 and C 9 prevents the 7-*cis* linkage from forming. The 9 and 13-*cis* forms are common; but the 11-*cis* linkage was recognized to be highly improbable, since it too involves a large overlap, between the methyl group on C 13 and the hydrogen on C 10. A *cis*-linkage always represents a bend in the chain; but because of this steric hindrance the 11-*cis* molecule is not only bent but twisted at the *cis* linkage. This departure from planarity, by interfering with resonance, was expected to make the molecule so unstable that one hardly expected to find it[37,38].

Nevertheless it has turned out that 11-*cis* retinal, once prepared, is reasonably stable provided it is kept dark; and all the visual pigments we know that have been analysed to this degree possess as chromophore 11-*cis* retinal$_1$ or retinal$_2$ (ref. 39).

When, however, a visual pigment is bleached by light, the retinal that emerges is all-*trans*. It must be re-

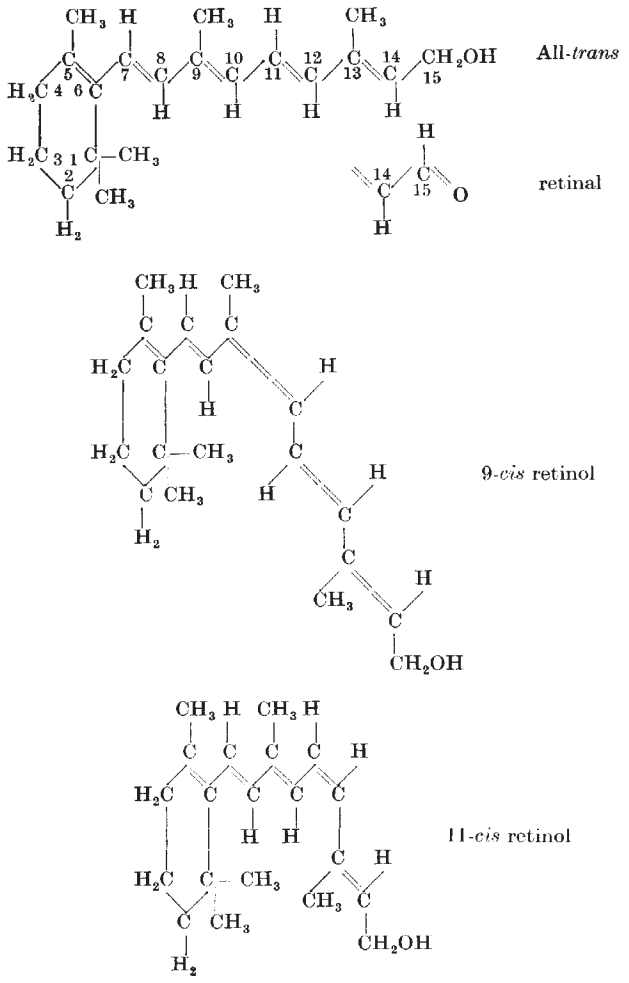

Fig. 2. Structures of the all-*trans*, 9-*cis* and 11-*cis* isomers of retinol and retinal.

isomerized to the 11-*cis* configuration before it can take part again in regenerating the visual pigment. Hence a cycle of *cis-trans* isomerization is an intrinsic part of every visual system we know (Fig. 3).

The 9-*cis* isomer, which is closest in shape to 11-*cis* retinal, also combines with the opsins to yield light-sensitive pigments that behave much as do the visual pigments[37]. We call them the iso-pigments: isorhodopsin, isoporphyropsin[39], and so on. None of them has yet been identified under physiological conditions in a retina. So far as we yet know, the iso-pigments are to be regarded as artefacts.

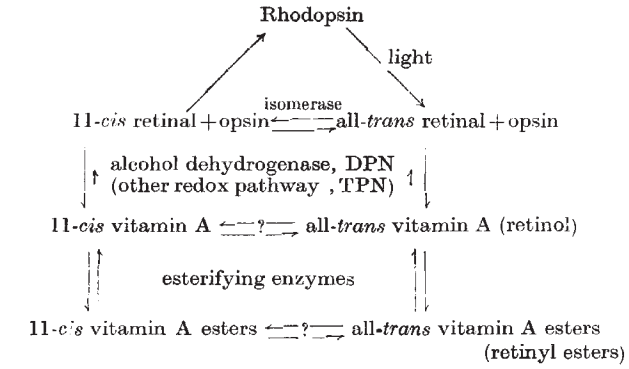

Fig. 3. Diagram of the rhodopsin system, showing the isomerization cycle. The bleaching of rhodopsin by light ends in a mixture of opsin and all-*trans* retinal. The latter must be isomerized to 11-*cis* before it can regenerate rhodopsin. While that is happening, much of it is reduced to all-*trans* vitamin A, most of which in turn is esterified[18,32b]. These products must be isomerized to or exchanged for their 11-*cis* configuration before engaging in the resynthesis of visual pigments.

As a prelude to what is about to be said, it should be understood that when any single geometrical isomer of retinal in solution is exposed to light, it rapidly isomerizes to a steady state mixture of all the possible isomers, in proportions that depend on the wavelength of the light, and even more on the polarity of the solvent[37,38b]. In such a homopolar solvent as hexane, about 95 per cent of the final, steady state mixture is all-*trans*; whereas in such a polar solvent as ethanol, about 50 per cent is distributed among *cis* configurations, and a surprisingly large fraction, about 25–30 per cent, ends as 11-*cis* retinal. Though thermodynamically improbable, this is one of the most favoured configurations of retinal[38b,40].

A few years ago Hubbard and Kropf showed that the only action of light in vision is to isomerize the chromophore of a visual pigment from the 11-*cis* to the all-*trans* configuration[41]. Everything else that happens—chemically, physiologically, indeed psychologically—represents "dark" consequences of this one light reaction.

This photochemical step can be isolated by bringing a visual pigment to very low temperatures. For example, if rhodopsin in a 1 : 1 mixture of water and glycerol is brought to liquid nitrogen temperature (about −190° C), at which the solvent vitrifies, its absorption spectrum narrows, rises and shifts slightly towards the red (Fig. 4)[42]. Exhaustive irradiation with blue light shifts the spectrum farther towards the red, ending with the production of a steady state mixture, in which part of the chromophore is still 11-*cis*, hence still rhodopsin, and the rest has been isomerized to all-*trans* to form the photoproduct, prelumirhodopsin (λ_{max} 543 mμ). At this point irradiation with orange light drives the spectrum back to its original position: the orange light, by re-isomerizing the all-*trans* chromophore to 11-*cis*, has re-converted all the prelumirhodopsin back to rhodopsin. Long irradiation with green light can drive the spectrum to still shorter wavelengths, by isomerizing the 11-*cis* chromophore to 9-*cis*; the pig-

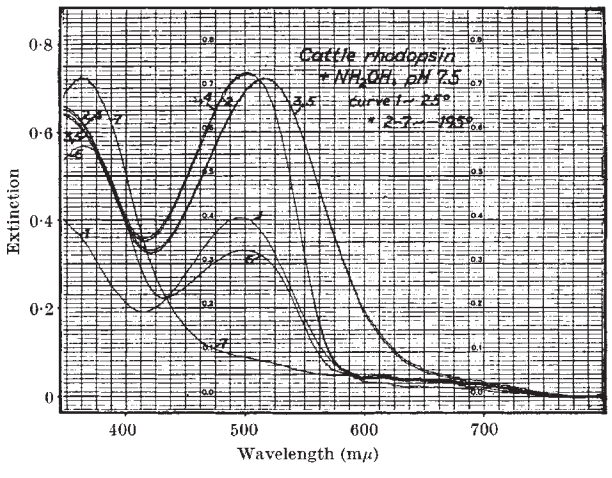

Fig. 4. Interconversion of rhodopsin and prelumirhodopsin by light at liquid nitrogen temperature. Cattle rhodopsin in 1 : 1 glycerol-water (1) is cooled to −195° C (2). On long irradiation with blue light (440 mμ) the spectrum moves to (3). The light has isomerized the 11-*cis* chromophore of rhodopsin to a steady state mixture of rhodopsin and prelumirhodopsin (all-*trans*). Irradiation with orange light (600 mμ) re-isomerizes all the all-*trans* chromophore back to 11-*cis*: it is now again all rhodopsin (4). Reirradiation with blue light brings it back again to the steady state mixture (5). On warming in the dark to 25° C, the prelumirhodopsin in this mixture bleaches to all-*trans* retinal and opsin, the retinal condensing with hydroxylamine to yield retinal oxime (λ_{max} 367 mμ); the rhodopsin in the mixture remains unchanged. This product was re-cooled to −195° and its spectrum recorded (6). Finally it was warmed again and bleached completely to all-*trans* retinal oxime and opsin (7). From ref. 42.

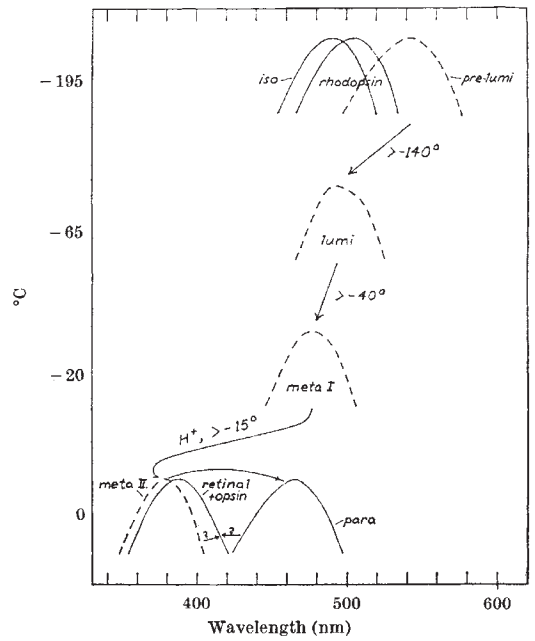

Fig. 5. Intermediates in the bleaching of cattle rhodopsin as they appear by irradiating at liquid nitrogen temperature and then gradually warming in the dark. Irradiation at −195° C yields steady state mixtures of rhodopsin (11-*cis*) with prelumirhodopsin (all-*trans*) and isorhodopsin (9-*cis*) in proportions that depend on the wavelength of irradiation. On warming in the dark, prelumirhodopsin goes at certain critical temperatures over a succession of all-*trans* intermediates to a final mixture of all-*trans* retinal and opsin.

ment is now isorhodopsin. Irradiation of the isorhodopsin with blue light again yields the same steady state mixture of rhodopsin and prelumirhodopsin as before. In this way one can go back and forth without loss as often as one likes among rhodopsin (11-*cis*), prelumirhodopsin (all-*trans*) and isorhodopsin (9-*cis*). At liquid nitrogen temperature this is a perfectly reversible system.

Comparable changes occur in squid rhodopsin[43], chicken iodopsin[44], and, as I have just learned from Yoshizawa, now back in Osaka, in carp porphyropsin. It is interesting that in all these cases the prelumi photoproduct, though the first step in bleaching, is a more intense pigment than the visual pigment itself, its spectrum both shifted towards the red and considerably taller than that of the visual pigment.

As already said, the irradiation of rhodopsin with blue light at liquid nitrogen temperature produces a steady state mixture of rhodopsin and prelumirhodopsin. On gradual warming in the dark, the latter goes, at specific critical temperatures, through a progression of intermediate stages—lumirhodopsin, metarhodopsin I, metarhodopsin II—representing stepwise changes in the conformation of opsin (Fig. 5). Finally, the Schiff base linkage hydrolyses to yield all-*trans* retinal and opsin[45]. In the course of these transformations new groups on opsin are exposed: two sulphydryl (−SH) groups per molecule[46], and one proton-binding group with pK about 6·6, perhaps imidazole[47].

Literal bleaching in the sense of loss of colour occurs mainly between metarhodopsins I and II. Visual excitation must have occurred by the time meta II is formed (Fig. 7). This stage is reached within about 1 ms at mammalian body temperature[48]. All subsequent changes are much too slow to be involved in excitation.

Up to metarhodopsin II, the all-*trans* chromophore has remained attached to opsin at the same site. So long as that is so, the absorption of a photon, by isomerizing the all-*trans* chromophore to 11-*cis*, immediately regenerates rhodopsin (Fig. 4).

We have been in the habit of saying that light bleaches visual pigments. What it does, however, is to isomerize the chromophore. The end of this process, if it is allowed to go to completion, is a steady state mixture of isomers of the chromophore, in proportions that depend on the wavelength of irradiation and the relative quantum efficiencies of the photoreactions. A first photon absorbed by rhodopsin can isomerize its 11-*cis* chromophore to all-*trans*, the initial step in bleaching. The absorption of a second photon, however, by any of the all-*trans* intermediates of bleaching can re-isomerize the chromophore to 11-*cis*, regenerating rhodopsin; or to 9-*cis*, forming isorhodopsin. Light not only bleaches visual pigments, but can regenerate them or the iso-pigments, depending on the circumstances.

The final stages of bleaching still present problems. Under conditions that promote a surge of metarhodopsin II—notably the exposure of a rhodopsin solution or dark adapted retina to a short, intense irradiation and recording its consequences in the dark—one finds it flowing in part back into the form of a more highly coloured product, so that the absorption in the visible region, having fallen (metarhodopsin II), rises again (Figs. 5 and 7). I saw this happening long ago in solutions of frog rhodopsin[49]; and we have recently followed this change in cattle rhodopsin solutions and frog retinas[45]. This seems also to be what Hagins observed after flashing excised rabbit eyes (his product C)[48]. This product (λ_{max} about 465 mμ) resembles metarhodopsin I in spectrum and has sometimes been confused with it. It may be called "pararhodopsin". It is formed from metarhodopsin II in the dark, and more rapidly in the light. Light also drives it back to metarhodopsin II, so that, depending on the wavelength of irradiation, one can push this intermediate back and forth

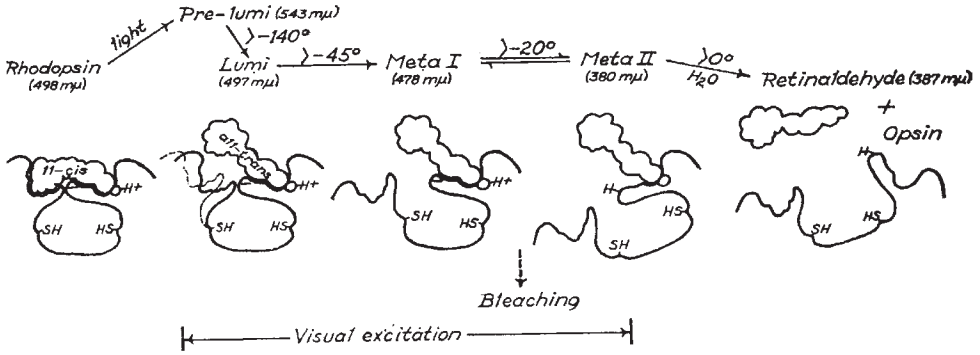

Fig. 6. Stages in the bleaching of rhodopsin. The chromophore of rhodopsin, 11-*cis* retinal, fits closely a section of the opsin structure. The only action of light is to isomerize retinal from the 11-*cis* to the all-*trans* configuration (prelumirhodopsin). Then the structure of opsin opens progressively (lumi and the metarhodopsins), ending in the hydrolysis of retinal from opsin. Bleaching occurs in going from metarhodopsin I to II; and visual excitation must have occurred by this stage. The opening of opsin exposes new chemical groups, including two —SH groups and one H^+-binding group. The absorption maxima shown are for prelumirhodopsin at −190° C, lumirhodopsin at −65° C, and the other pigments at room temperature.

between these two states. In the dark, pararhodopsin decays to retinal and opsin[45]. Whether it is an essential intermediate or a bypass in the bleaching of rhodopsin, and whether on irradiation it can directly regenerate rhodopsin, all require further analysis[50].

I must confess to having become somewhat bored with such minutiae of the final stages of bleaching, which come much too late to have anything to do with visual excitation. They have suddenly taken on a new interest, however, owing to an astonishing development. Having had nothing to do with this, I can praise it freely. For generations past the chemistry of vision and its electrophysiology have travelled separate paths. Suddenly many of the details of the bleaching and regeneration of visual pigments are emerging in a new class of electrical responses.

The arrangements are familiar by which one measures an electroretinogram (ERG) (Fig. 8). An active electrode on the cornea and an indifferent electrode elsewhere on the eye or on the body connect through an amplifier to an oscilloscope. On exposing the eye to a flash of light there is a silent period lasting at least 1·5 ms even in a mammal, and much longer at lower temperatures; then a biphasic fluctuation of potential, the characteristic *a* and *b* waves of the ERG. One would suppose that the latent interval before the response represents the time needed to bleach the visual pigment to the critical stage, and for secondary events, including the large build-up of amplification, that lead to the response.

About three years ago K. T. Brown and Murakami found a new electrical response that fills in this interval—the early receptor potential (ERP) (Fig. 8)[51]. It has no measurable latency. Ordinarily it takes a flash about 1 million times as intense as would produce a moderate ERG to evoke an ERP of about the same amplitude. The ERP also is biphasic, consisting of a rapid cornea-positive wave (R1) followed by a slow cornea-negative wave (R2). In the cold (5° C and below), R1 appears alone[52].

It is becoming increasingly clear that the ERP has its source in the action of light on the visual pigments themselves. One can get an ERP in retinas cooled to −30° C, heated to 48° C, bathed in glycerol solutions, or fixed in glutaraldehyde. All it requires is intact—and oriented—rhodopsin. In the membranes that compose the rod outer segments, rhodopsin is almost perfectly oriented[53]. If the orientation is destroyed by heating, the ERP goes with it[54].

A recent experiment demonstrates the immediacy of these relationships. A flash of light acting on the rhodopsin of a dark adapted retina having evoked the usual ERP, another flash acting on an intermediate stage of bleaching (probably para and metarhodopsin II) so as to photoregenerate rhodopsin produces a biphasic ERP of reversed polarity (Fig. 9)[55a]. That is, the isomerization of the rhodopsin chromophore from 11-*cis* to all-*trans* having triggered reactions that produce the normal ERP, the reverse isomerization from all-*trans* to 11-*cis* induces similar changes of potential reversed in sign.

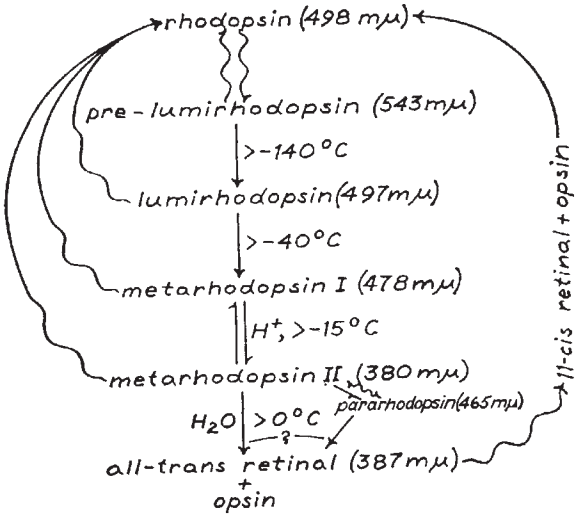

Fig. 7. Intermediates in the bleaching and regeneration of rhodopsin. Wavy arrows represent photoreactions, straight arrows thermal ("dark") reactions. The interrelationships of pararhodopsin with the final products of bleaching still present problems.

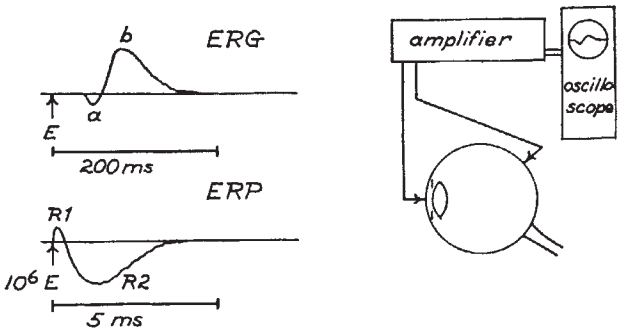

Fig. 8. Diagram to show the essential hook-up for observing an electroretinogram (ERG) or early receptor potential (ERP) in a dark adapted vertebrate eye. Each of these responses is a biphasic fluctuation of potential, involving cornea-positive (upward) and cornea-negative (downward) components. Unlike the ERG, the ERP has no measurable latency. For both types of response to be comparable in amplitude, the flash that stimulates the ERP must be of the order of 1 million times more intense.

Such experiments with rat and squid retinas have begun to identify individual components of the ERP with the photoreactions of specific intermediates in the bleaching of rhodopsin[55a,b]. It is hard to see how intramolecular changes of this kind, presumably involving charge displacements, can generate changes of potential between the front and back of the eye; but there is no doubt that they do, and we can only hope eventually to understand them. There is as yet no evidence that the ERP generated by rhodopsin itself is part of the mechanism of excitation; obviously the action of flashes of light on intermediates of bleaching, though they generate various forms of ERP, do not excite. At the least, the ERP offers a new and powerful tool for studying the reactions of the visual pigments *in situ*.

For many centuries man was the only object of visual investigation. Recently he has again become for many purposes the experimental animal of choice. In certain ways experimenting with man offers unique advantages. With other animals one can pursue biophysics, but only with man also psychophysics. Moreover, human genetics is by now probably better understood than that of any other animal; and with man one does not have to seek out mutant forms—their curiosity and anxiety bring them in.

A few years ago Paul Brown designed and built a recording microspectrophotometer in which one could measure the difference spectra of visual pigments in small fragments of retina[56]. With this one could record the difference spectrum of the visual pigments in the rod-free area of the human fovea. Just as the spectrum of human rhodopsin agrees with the spectral sensitivity of human rod vision[57], so the spectrum of the foveal pigments accounts for the spectral sensitivity of human cone vision[58].

The human cones, however, are the receptors of colour vision. Since Thomas Young it has been recognized that normal human colour vision is trivariant; it involves the interplay of three independent variables. A prevalent thought for many years past has been that it involves three types of cone, each with its own visual pigment.

By irradiating human and monkey foveas with deep red light in the microspectrophotometer, we were able to bleach the red-sensitive pigment alone and measure its difference spectrum (λ_{max} about 565 mμ). When red light

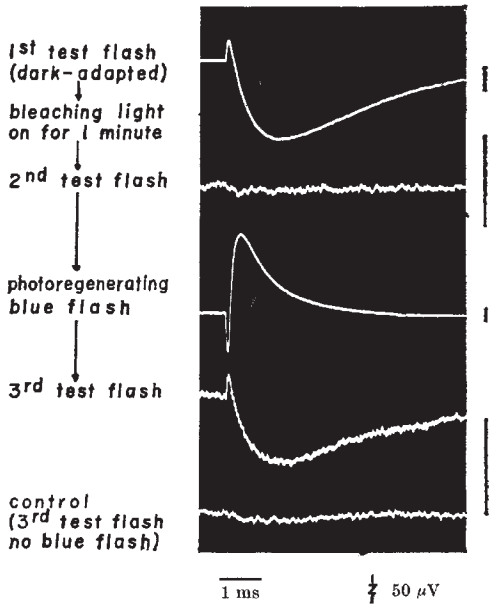

Fig. 9. Photoregeneration of the early receptor potential (ERP) in the eye of an albino rat. Both the test flash and the bleaching light were composed of long wavelengths primarily absorbed by rhodopsin. The blue photoregenerating flash contained wavelengths absorbed by longer-lived intermediates of bleaching (apparently mainly metarhodopsin II and pararhodopsin). The control trace was obtained from a second eye subjected to the same bleaching light and test flashes, but without the interpolated blue flash. 27° C. The first test flash yields a normal ERP. The bleaching light having removed rhodopsin, a second test flash yields nothing, even though the amplification is increased (see gain index at right). A blue flash, photoregenerating rhodopsin, yields an ERP of reversed polarity. The third test flash, yielding again a normal ERP (high gain!), shows that the blue flash did regenerate rhodopsin. The control flash shows that without the interpolated blue flash, no response is obtained (high gain). From ref. 55*a*.

caused no further change, yellow light induced a renewed bleaching that yielded the difference spectrum of the green-sensitive pigment (λ_{max} about 535 mμ)[58]. Presumably there was also a blue-sensitive pigment that could not be measured adequately with these arrangements.

Shortly afterwards it proved possible to measure in the microspectrophotometer the difference spectra of the visual pigments in single parafoveal rods and cones of human and monkey retinas. Such measurements were made simultaneously and independently in our laboratory and by Marks, Dobelle and MacNichol at Johns Hopkins University[59*a*,*b*]. They showed that primate retinas possess, in addition to rods with their rhodopsin, three kinds of cone—blue, green and red-sensitive—each containing predominantly or exclusively one of three colour-vision pigments, with λ_{max} at about 435, 540 and 565 mμ (Fig. 10).

The red and green-sensitive pigments, after being bleached by light, are regenerated by adding 11-*cis* retinal in the dark[58]. The same seems to be true of the blue-sensitive pigment. Since all the visual pigments of the primate retina, rod and cone apparently have the same chromophore, 11-*cis* retinal, they must differ in their opsins.

The iodopsins, with λ_{max} near 560 mμ, and cyanopsins, with λ_{max} near 620 mμ, are widely distributed among animals. Iodopsin is the major, perhaps the only cone pigment in the chicken, pigeon[30*b*], cat, snake and frog[60]; just as cyanopsin seems to be the cone pigment of such retinol$_2$ animals as the tench and tortoise[61], a freshwater turtle and frog tadpoles[62]. In the few vertebrates whose colour vision systems have been analysed, these pigments are apparently the red-sensitive components. So in man and the monkey, the red-sensitive pigment of colour vision is apparently iodopsin[58]; just as it is cyanopsin in the goldfish[63] and carp[64]. Recently, pairs of visual pigments resembling in spectrum rhodopsin and iodopsin have been found in two species of crayfish[65]; and here again the "iodopsins" seem to be the red-sensitive components in systems of colour discrimination[66].

A simple psychophysical procedure has recently been developed for measuring the spectral sensitivities of the three groups of cones in human subjects[67]. This is a highly simplified extension of the procedure by which W. S. Stiles first measured such sensitivity curves[68]. In my application of this procedure, the eye is continuously adapted to bright coloured lights, each of which so lowers the sensitivities of two of the three colour visior mechanisms that the measurements report primarily the properties of the third system. So, for example, exposure of the eye to a brilliant yellow light so lowers the sensitivities of the green and red-sensitive systems that measurements of the spectral sensitivity are dominated by the blue-sensitive system. Similarly exposure to wave bands in the blue and red, hence purple light, isolates the green-sensitive system; and exposure to bright blue light isolated the red-sensitive system. The sensitivity curves measured in this way come out much as do the difference spectra of the pigments measured in the microspectrophotometer (Fig. 12).

With this simple procedure one can inquire into mechanisms of colour blindness[69]. Just as normal human colour vision is trivariant (trichromatic), that of the usual congenital colour blind is divariant (dichromatic). Theoretically the reduction from three variable to two could occur in many ways, and at any level in the visual pathways. By now, however, I have examined a reasonably large number of dichromats with this procedure; and each of them apparently lacks one of the three colour mechanisms, the other two remaining normal and fully functional (Fig. 11). Depending on which component is lacking, the three major classes of dichromat can be characterized as blue, green or red blind.

Every schoolboy learns that colour blindness is caused by a sex-linked recessive mutation. That is true, however, only of red and green-blindness. About 1 per cent of men are red-blind, about 2 per cent green-blind, whereas both conditions are very rare in women. Blue-blindness also is very rare, and not sex-linked, affecting only about 1 in 20,000 persons, about 40 per cent of whom are women[70].

There is another, more widespread congenital colour defect, red or green anomaly, closely related to red and green-blindness. Persons with this condition have

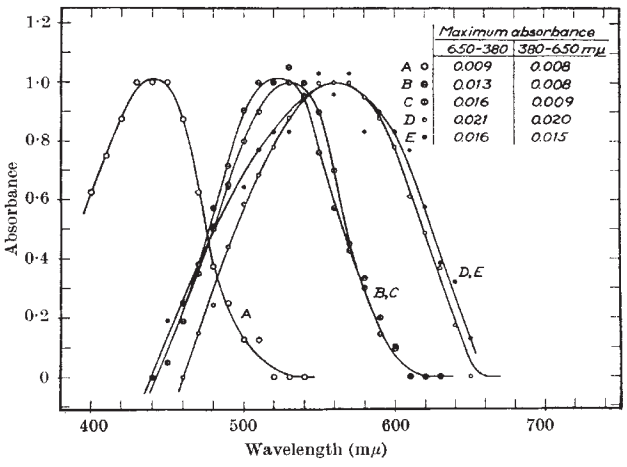

Fig. 10. Difference spectra of the visual pigments in five human parafoveal cones: one blue-sensitive, two green-sensitive and two red-sensitive cones. Each of these difference spectra was obtained by recording in the microspectrophotometer the "dark" and then the "bleached" spectra from 650 to 380 mμ and again in the reverse direction. Then the "bleached" spectra were subtracted from the "dark" spectra, and both curves averaged. The absorbances at λ_{max}, shown at the upper right, indicate the amounts by which these preparations bleached in the course of the two recordings. The second recording, from 380 to 650 mμ, always displays a somewhat smaller absorbance, owing to bleaching. From ref. 59*c*.

three colour vision, but make abnormal colour matches. Examined by my procedure, they yield the same type of result as red or green-blinds, showing that one of their three colour mechanisms is abnormally insensitive, so much so that my procedure does not detect it; in addition, to account for their abnormal colour matches this aberrant system must be displaced in spectrum. In one instance, that of a blue-blind subject who was also green-anomalous, the displaced sensitivity curve of the green-receptor could be measured[69]. It lay about midway between the normal green and red-sensitive curves, a position that accounts well for the abnormal colour matches.

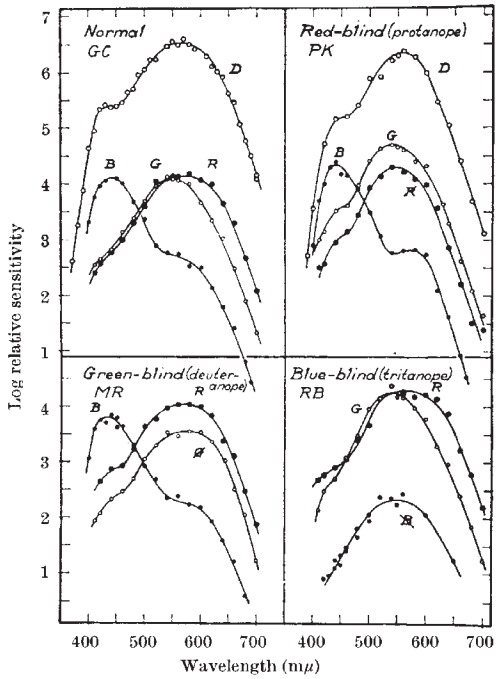

Fig. 11. Measurements of spectral sensitivity with a standardized selective adaptation procedure in a normal subject (trichromat), and typical subjects representing each of the three main classes of colour blindness (dichromats). In the normal eye this procedure displays the spectral sensitivity of the dark adapted fovea (D), and those of the blue, green and red-sensitive systems (B, G, R), as measured respectively on intense yellow, purple or blue backgrounds. Each of the colour blinds reveals the operation of only two of the three colour vision pigments; in each case the attempt to measure the third pigment reveals only one of the other mechanisms at a lower level of sensitivity. The crossed out symbols B, G and R represent such unsuccessful attempts to measure the missing systems in the colour blind eyes.

One of the triumphs of modern biology is to have shown that the usual business of a gene is to specify the amino-acid sequence of a protein. For many years geneticists have been unable to decide whether red and green-blindness involve one or two gene loci on the *X*-chromosome. I think the demonstration that two different proteins, two opsins, are needed to form the red and green-sensitive pigments settles this issue[58].

Normal human vision requires the synthesis of four different opsins: one in the rods, to make rhodopsin, and three in the cones, for the colour vision pigments. Each of these must be specified by a different gene. It seems reasonable to assume that two such genes, lying close together in the *X*-chromosome, specify the opsins of the normal red and green-sensitive pigments (Fig. 12)[69]. Mutations in these genes that result in the failure to form either pigment probably account for red or green-blindness. Other mutations at the same loci, resulting in the formation of abnormally small amounts of visual pigments with displaced spectra, may account for red and green-anomaly. All these conditions breed true, the normal condition being dominant to colour anomaly, which in

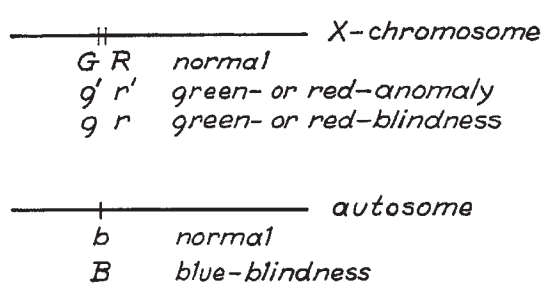

Fig. 12. Diagram showing a hypothetical arrangement of genes that specify the three opsins which, with 11-*cis* retinal, form the blue, green and red-sensitive pigments (B, G, R) of normal human colour vision. Two such genes on the *X*-chromosomes are assumed to determine G and R. Mutations in these genes that result in the production of less effective pigments, displaced in spectrum, are responsible for colour anomaly; and other mutations that result in the failure to form functional visual pigments result in colour blindness. The normal condition is dominant to red or green-anomaly, which in turn is dominant to red or green-blindness. The location of the gene for the opsin of the normal blue-sensitive pigment is not known; but the mutation responsible for blue-blindness seems clearly to be autosomal, since this condition is almost equally distributed between males and females; and it may be dominant to the normal condition.

turn is dominant to colour blindness as expected. The mutation responsible for blue-blindness must be autosomal; and there is some evidence that it is inherited as an irregular dominant, though too few blue-blind genealogies are yet available to characterize the genetics reliably[71].

A recent investigation, taking off from earlier observations by König[72] and Willmer and Wright[73], introduces an altogether different aspect of colour blindness. A small, central patch of the normal fovea, subtending a visual angle of about 7 to 8 min and hence hardly larger than the fixation area, is blue-blind in the sense of lacking functional blue-sensitive cones (Fig. 13)[74]. This is a matter of retinal topography, not of size of field, for blue-sensitive cones are well represented in a field of this size fixated elsewhere in the fovea or in the peripheral retina. Blue-blindness, though by far the rarest form of congenital colour blindness, appears to be the usual condition of the fixation area of the fovea.

This intrusion of colour blindness at the centre of the normal fovea recalls the old and often repeated observation that other, more peripheral areas of the normal retina are red or green-blind; and that areas still further out are totally colour blind (Fig. 14). It is difficult to make good colour vision experiments in such outlying areas, and much remains to be done. Nevertheless it

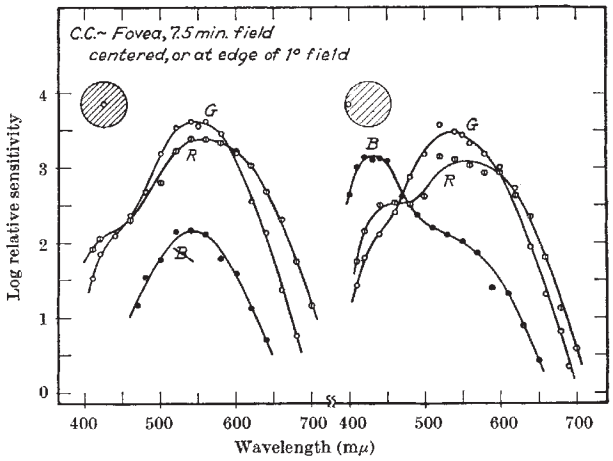

Fig. 13. Blue-blindness of the fixation area of the normal fovea. Spectral sensitivities of the blue, green and red-sensitive cones (B, G, R), measured in a 7·5 min field fixated either centrally or 7/16° from the fixation point. At the centre of the fovea, though G and R are well represented, all attempts to measure B result only in finding G or R at lower levels of sensitivity. As soon as the field is moved away from the fixation area, B appears; simultaneously G and R decline somewhat in sensitivity, owing probably to smaller densities of cones. From ref. 74.

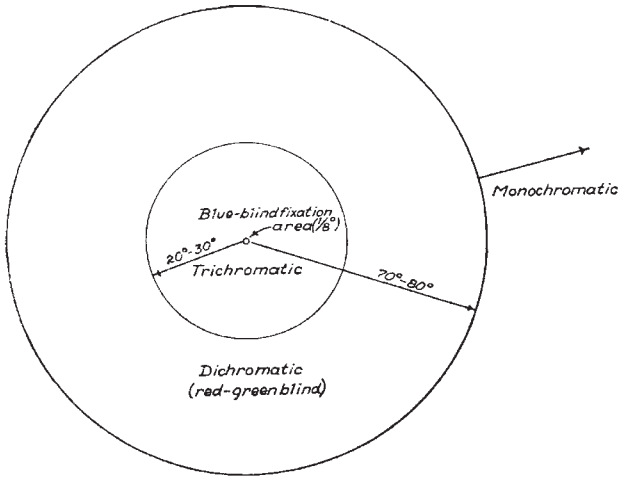

Fig. 14. Diagram to indicate approximately the distribution of colour function over the normal human retina. The fixation area is blue-blind, in the sense of lacking functional blue-sensitive cones. From there to about 20°–30° from the fixation point is trichromatic. Beyond this range, to perhaps 70°–80° out, the retina behaves as though red or green-blind; and still further out as totally colour blind (monochromatic). The nature of peripheral colour blindness and its mechanisms are still to be explored; yet it seems possible that all the classic forms of colour blindness are represented in various zones of the normal retina.

now seems possible that all the classic forms of colour blindness, including total colour blindness, are represented in the normal retina.

What we regard as normal trichromatic vision appears now to be only the special property of a broad annulus of retina stretching from the blue-blind fixation area at the centre to about 20°–30° out (Fig. 14). Most of the normal retina is colour blind. The mechanisms of colour blindness in the peripheral retina—whether the lack of certain classes of cone or the reduction or fusion of nerve channels from the cones—must still be explored.

However this comes out, it raises an altogether different kind of problem from that of congenital colour blindness: not what pigments the organism can make, but how it distributes those pigments and the cells that contain them. That takes us from molecular genetics to questions of embryogeny and cell differentiation. The human retina, with its complex topography and radial zonation, may be a particularly fortunate place to study such problems.

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