Light-Induced Free Radicals of Retinal, Retinol, and Rhodopsin*

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ABSTRACT: Light-induced electron paramagnetic resonance signals that disappeared upon melting were elicited from trapped paramagnetic photoproducts of all-*trans*-, 9-*cis*-, and 13-*cis*-retinal, all-*trans* retinol, and rhodopsin. Solutions of these substances in organic solvents or 2% aqueous digitonin were irradiated by visible light at −196°. At about 1 mM concentration of the three retinals and from rhodopsin, electron paramagnetic resonance singlet spectra were obtained at $g \simeq 2.004$, with line widths of about 23 gauss and with some suggestion of further unresolved spectral detail. In general the electron paramagnetic resonance signal was greater with increasing illumination and with decreasing polarity of the solvent. At higher concentrations an electron paramagnetic resonance photosignal also was obtained from retinol, evincing an over-all, roughly symmetrical three-line spectrum plus additional hyperfine structure, with a total width of about 210 gauss but unaccompanied by a half-field signal. Room temperature fluorescence was observed from retinol but not from the aldehydes. The data are taken to indicate strong localization of excitation energy and of unpaired electron distribution in retinol, with relative delocalization in the retinaldehydes. Retinal and rhodopsin free radicals are viewed as probable products of the light-induced triplet states previously noted by others, and they are discussed as possible participants in the first (isomerizing) step in the photochemical conversion of rhodopsin. Photochemical creation of radical ions also is proposed as the initial step in the charge separation that appears to be associated with at least some components of the early receptor potential of the electroretinogram (recorded from illuminated retinae *in vivo*).

Whereas the sensitivity of the cells to electrical stimuli and the fundamental role of electrochemical processes in the nervous transmission of information in biological systems have both been amply documented, no evidence has been reported previously in support of a *mechanism* for the translation of a primary sensory chemical event into an electrical signal.

The photochemical changes which occur on exposure of rhodopsin to visible light have been reviewed by Hubbard *et al.* (1965) and Hubbard and Kropf (1967). Briefly, the first step, the conversion of rhodopsin into prelumirhodopsin, was identified by Yoshizawa and Wald (1963) at liquid nitrogen temperature (−196°), and it is currently believed that this step represents the isomerization of the retinal present as 11-*cis* (neo-b) in rhodopsin to the all-*trans* form in prelumirhodopsin. This is believed to be the only light-requiring step in the normal physiological bleaching of rhodopsin, the remainder of the cycle consisting of so-called dark reactions (Hubbard and Kropf, 1958, 1967; Wald, 1965). Accordingly, the demonstration of a free radical from illuminated rhodopsin at this temperature may shed some light on the mechanism of the only light-sensitive step in the visual process. The presumptive involvement of electron transfer in the photochemical formation of free radicals also is of interest with regard to its possible etiological role in the charge separation thought to give rise to the early receptor potential of the electroretinogram, which has received so much attention recently (Brindley and Gardner-Medwin, 1965, 1966; Brown and Murakami, 1964; Cone, 1964, 1967; Hagins and McGaughy, 1967; Pak and Ebrey, 1965; Pak and Cone, 1964).

It is well known that visible light can isomerize retinal (the aldehyde of vitamin A) both in solution and when the retinal molecule is part of rhodopsin, the light-sensitive pigment of the rod cells of the eye (Hubbard and Wald, 1953; Hubbard *et al.*, 1953; Hubbard, 1956). When any single isomer of retinal is irradiated in solution, an equilibrium mixture of *cis–trans* isomers is produced, of which the all-*trans* form predominates (Jurkowitz *et al.*, 1959) (Figure 1). This can occur even in rigid glasses at low temperature (Jurkowitz *et al.*, 1959).

Jurkowitz had observed that when he irradiated solutions of all-*trans*-retinal at −185 to −192°, the peak extinction at 387 mμ fell as much as 80% after 1-hr irradiation, and two smaller peaks arose at 350 and 412 mμ (Jurkowitz *et al.*, 1959). On rewarming to room temperature, the above changes were reversed, except for a 1.3% decrease in extinction attributed to some isomerization to *cis* isomers, which have lower extinction coefficients. Jurkowitz postulated that the spectral change was due to production of a metastable state, possibly a pair of radicals or ions.

Since exposure to light results in *cis–trans* isomerization of retinal, and since this must require some kind

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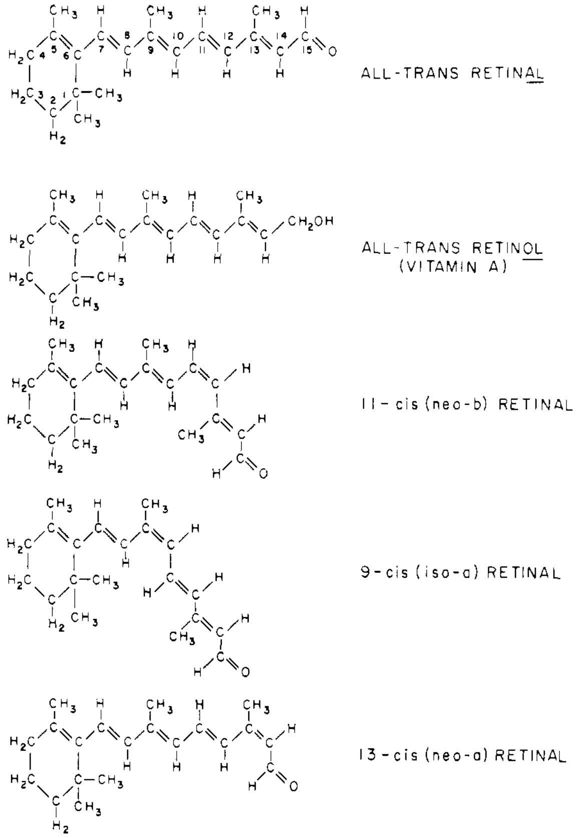

FIGURE 1: Geometrical isomers of retinal and retinol.

of temporary partial loss of the double-bond character of the bonds to be isomerized, there seemed to be a reasonable possibility that absorbed light energy might be sufficient to remove an electron, thus producing a free-radical intermediate which should allow isomerization to occur quite readily. The possibility that a free radical might play a role in the rhodopsin system had also been raised before by others for various reasons (Wald *et al.*, 1950; Collins and Morton, 1950). Therefore, we have undertaken an electron paramagnetic resonance study of the effect of visible light upon retinal, retinol, and rhodopsin at low temperatures.

Polis and Wyeth (1962) had elicited a free-radical, electron paramagnetic resonance signal from rod outer segments and from digitonin solutions of rhodopsin by light exposure at room temperature, but this was an extremely weak signal, requiring summation of several hundred time curves by means of a computer of average transients to bring it out of the background noise, and even then they considered the signal too weak to scan an actual electron paramagnetic resonance spectrum (B. D. Polis, personal communication). Interestingly, they were unable to demonstrate a signal from retinal or retinol under similar conditions. Among the possible reasons for a weak signal are that very few free radicals are produced, or perhaps that a great many are produced, but their decay is so rapid that at any given instant the steady-state population is small.

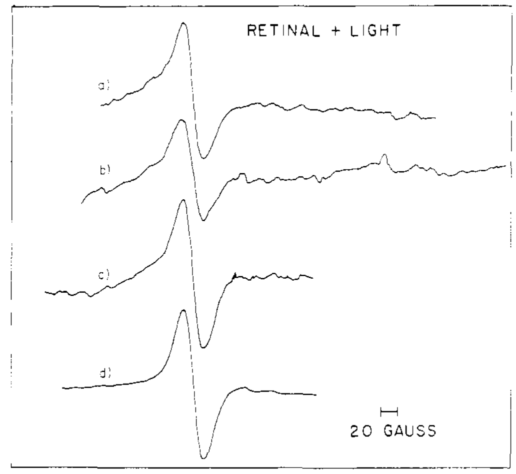

FIGURE 2: Epr spectra of 10^{-3} retinal in heptane at $-196°$ after brief exposure to light: (a) all-*trans*-retinal, (b) 13-*cis*-retinal, (c) 9-*cis*-retinal, and (d) the signal from any one isomer (here 9-*cis*) becomes more symmetrical after prolonged irradiation. Modulation amplitude used was 16 gauss at 37 mW of incident microwave power. Spectroscopic g value $= 2.004 \pm 0.001$.

We therefore decided to examine the effect of light on these substances, employing the "freezing-in" technique which Wald (Yoshizawa and Wald, 1963) had utilized so successfully in determining the intermediates involved in the rhodopsin cycle, *i.e.*, the labile species is "trapped," because the activation energy which it needs to decay is not available to it. Thus, as the labile species continues to be generated, its concentration can build up to a point where it can be more easily detected.

Methods and Materials

Solutions of 1 mM all-*trans*-retinal (Sigma) were made up in heptane, carbon tetrachloride, acetone, acetonitrile (all reagent grade), and 2% aqueous digitonin. 11-*cis*-Retinal was not available to us, but 1 mM solutions of 9-*cis*- and 13-*cis*-retinal (Sigma) were also made up in heptane, while all-*trans* vitamin A (retinol) (Sigma) was dissolved in acetone and acetonitrile to a final concentration of 0.1 M. All of the above solutions were carefully flushed with nitrogen for 10 min before examination because Abrahamson *et al.* (1959) have shown that oxygen quenches the triplet state of retinal. A 0.2-ml aliquot of each solution was then placed in a quartz sample tube of 3-mm i.d. which was, in turn, placed in a Varian liquid nitrogen filled dewar with a clear quartz finger through which the sample could be irradiated.

The rhodopsin preparations were made by the method of Collins *et al.* (1952), *viz.*, the retinas from 50 cattle eyes which had been iced and dark adapted since enucleation were removed in dim red light. The retinas were then lightly ground in a 40% sucrose–

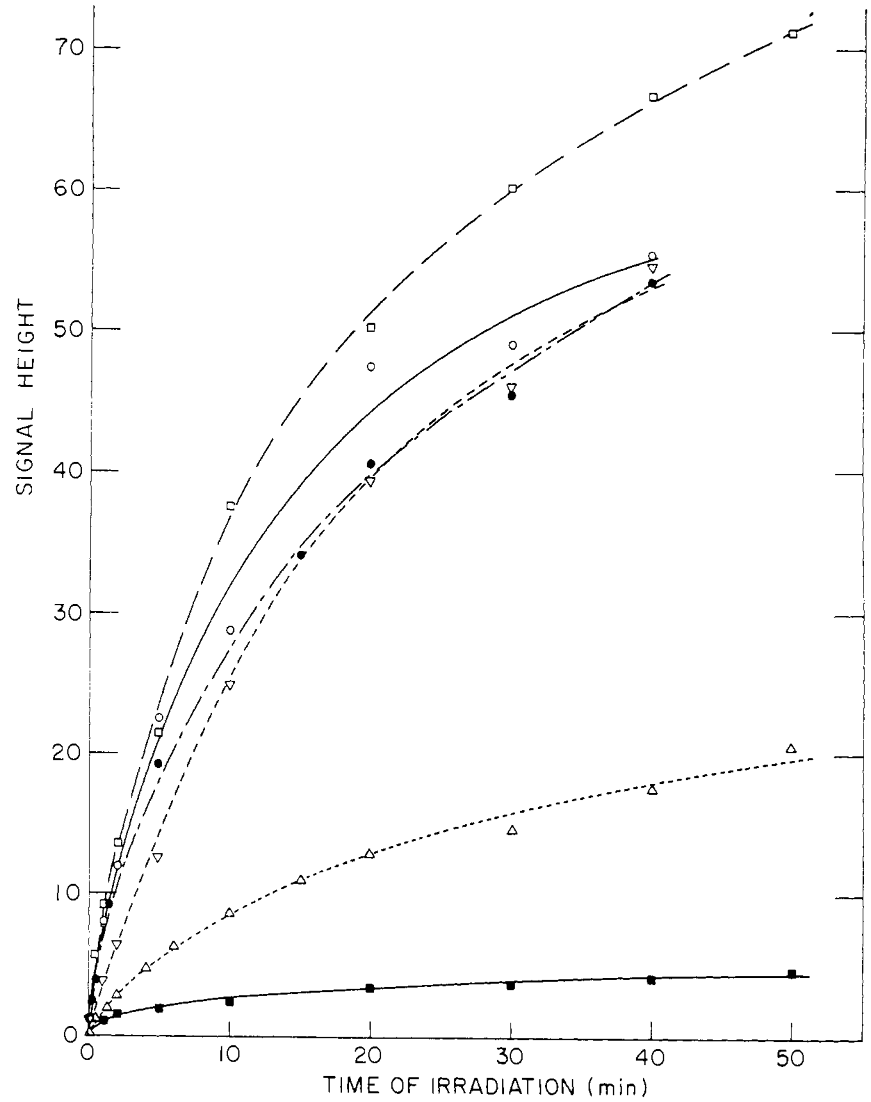

FIGURE 3: Increase in the height of the epr signal from 1 mM retinal isomers upon continued exposure to light in boiling nitrogen (−196°). (– – – –●) all-*trans*-retinal in heptane; (——○) all-*trans*-retinal in CCl_4; (——△) all-*trans*-retinal in acetone; (——■) all-*trans*-retinal in acetonitrile; (——▽) 9-*cis*-retinal in heptane; (——□) 13-*cis*-retinal in heptane.

0.05 M Tris buffer (pH 7.2) and passed ten times through a no. 15 needle (flattened at the end), after which the homogenate was layered under buffer. This was then spun at 8000 rpm in a Beckman–Spinco Model L ultracentrifuge for 10 min, and the rod outer segments at the interface were collected. These were then harvested by diluting 3:1 with buffer and recentrifuging at 14,000 rpm for 20 min. The rod outer segment pellet was suspended in 4% alum ($KAlSO_4 \cdot 12\ H_2O$) and left overnight at 4°, recentrifuged, and washed twice with buffer. Lastly, it was extracted with 2% aqueous digitonin solution and centrifuged to remove the rhodopsin, the final solution being about 9×10^{-6} M (based on ϵ 40,600 for cattle rhodopsin (Wald and Brown, 1953)).

The above preparations were immersed in liquid nitrogen, as described, and were then placed within a Varian V4531 microwave reflection cavity and were examined at −196° by means of X band (9.5 GHz, 3-cm wavelength) electron paramagnetic resonance after irradiation with varying amounts of visible light. A Varian V4502 electron paramagnetic resonance spectrometer operating with 100-kHz modulation frequency and equipped with Fieldial® magnetic field control was used.

The light source consisted of a projector with a 500-W incandescent bulb and glass lensing system, by which the sample could be irradiated. Samples were contained in thin quartz tubes of 3-mm i.d., which frequently burst when aqueous solutions were quickly frozen to −196°. In order to prevent this cracking, it was necessary to precool aqueous samples rapidly in Dry Ice–

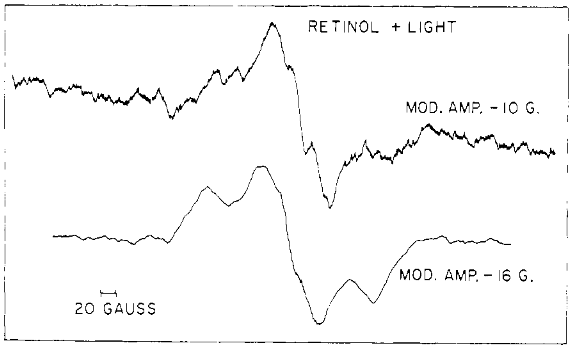

FIGURE 4: Epr spectra of 0.1 M vitamin A in acetone at −196° after irradiation. Upper: 10-gauss modulation amplitude and 37 mW of power reveal detailed spectral structure. Lower: overmodulation suppresses detail but emphasizes a basic 1:2:1 envelope with more symmetry apparent.

acetone mixture before placing them in liquid nitrogen. Fluorescence spectra were obtained with an Aminco–Bowman spectrofluorometer.

Results

On illumination with visible light, retinal in rigid solution or in the crystalline state at −196° forms paramagnetic species characterized by a single broad (approximately 23 gauss, peak to peak) electron paramagnetic resonance signal with some suggestion of further structure (Figure 2) that was not further resolvable under the conditions employed. This singlet spectrum is not accompanied by a half-field epr signal characteristic of the forbidden $\Delta m = 2$ transition of the triplet state (van der Waals and de Groot, 1959; de Groot and van der Waals, 1960; Lhoste *et al.*, 1966); and it appears after only 1-sec illumination, increasing in intensity with increasing time of illumination (Figure 3). It is apparent from Figure 2 that the 9-*cis* and 13-*cis* isomers on illumination produce radicals similar in nature to those produced from the all-*trans* isomer. The effect of solvent is also significant (Figure 3), with the signal strongest at −196° in carbon tetrachloride, slightly smaller in heptane, and then decreasing in the order acetone, acetonitrile, 2% aqueous digitonin (in which it is barely detectable); and no signal was detected from frozen ethanolic solutions. In general, then, the lower the dielectric constant of the liquid solvent, the better the yield of free radical in the corresponding frozen samples. An exception was diethyl ether, in which no signal could be detected. The fact that the aqueous digitonin solution appears less polar than ethanol, according to the criterion of electron paramagnetic resonance signal intensity, will be dealt with in the discussion. Also, carbon tetrachloride may be a special case since only in this solvent did we note any visible change, *viz.*, the irradiated surface blackened (reversible on annealing), and Walling (1957) has shown that carbon tetrachloride can interact with free radicals, perhaps stabilizing them (Smaller, 1960). In all cases, irradiation of solvent alone under identical conditions yielded no detectable signal, nor did the dissolved substances demonstrate any signal prior to irradiation.

A preliminary attempt at obtaining an action spectrum for the electron paramagnetic resonance signal from retinal revealed most efficient production at wavelengths corresponding approximately to the range of retinal's absorption spectrum. For this purpose, a Bausch and Lomb monochromator was placed in front of the source, and corrections were made for source emission spectrum and monochromator efficiency. Since the rhodopsin signal was so much weaker than that from the pure carotenoids, it was difficult to determine an action spectrum for it.

The slow rise of the retinal electron paramagnetic resonance signal and its failure to saturate after several hours of exposure to light suggested the possibility of a double-quantum event. This was rendered less likely by the finding that the effect on signal formation of a 25% transmission neutral density filter was exactly compensated by a four-time prolongation of light exposure, although a two-quantum transition with freezing-in of an intermediate photoproduct could not be ruled out. The slow rise in electron paramagnetic resonance signal intensity, in turn, may have reflected the low statistical likelihood of reaction *within* the samples due to severe self-absorption of light with little penetration beyond the surface. Indeed, our solutions of 10^{-4} M retinal would decrease the light intensity to 10% of its incident value within 200 μ of the surface, since $\epsilon_{-65°}$ 47,000 for all-*trans*-retinal (Hubbard and Kropf, 1959).

Retinol (vitamin A) was also induced to form a paramagnetic product on illumination, although less easily than retinal, requiring several hundred times the concentration for detection. The reasons for this, however, are at least partially evident. First, the absorption maximum of all-*trans*-retinol is approximately 335 mμ in a low-temperature glass, while that of all-*trans*-retinal is 385 mμ, and we were irradiating through glass lenses with an incandescent source, resulting in many fewer quanta at 335 mμ than at 385 mμ. Secondly, the retinol signal is about 210 gauss wide[1] (Figure 4) compared with 23 gauss for retinal, necessitating many more unpaired spins before the signal can be detected. Yet another possible contributing factor, the fluorescence of retinol, is dealt with in the discussion.

Rhodopsin in 2% digitonin solution yields upon illumination an electron paramagnetic resonance signal qualitatively similar to that of retinal (Figure 5), but it is considerably weaker and cannot be made to grow on continued irradiation. Furthermore, unlike

[1] The anisotropy of triplet-state magnetic resonance gives rise to very broad electron paramagnetic resonance signals (often undetectable) for the $\Delta m = 1$ transition, but (as was the case with the aldehydes) no $\Delta m = 2$ signal was detected from light-irradiated retinol.

the carotenoid signals which disappear immediately and completely on annealing and may be regenerated an indefinite number of times, the rhodopsin signal only diminishes on melting at about 0°, and then persists for several minutes before disappearing after the solution has been warmed to room temperature. However this signal, like that from the carotenoids, is regenerable upon reexposure to light at −196°. (A digitonin blank gave no signal on similar treatment.)

Discussion

Dawson (1962) and others (Abrahamson *et al.*, 1959; Dawson and Abrahamson, 1962; Grellman *et al.*, 1962) have shown that the triplet state(s) of retinal may be easily populated by flash photolysis, while that of vitamin A (retinol, the corresponding alcohol) shows no triplet on flash illumination. A triplet-exicted molecule may lose its energy by phosphorescence (although Dawson (1962) could detect no phosphorescence from retinal), or by nonradiative (vibrational) decay, or it may capture an electron to form a free-radical anion. This latter path is postulated because the triplet has a low-lying positive hole, and hence is relatively electron attracting. Furthermore, it is not uncommon for the triplet state to be a precursor of free-radical formation (Sogo *et al.*, 1957; Ilten and Calvin, 1965). Additionally, the finding of Mousseron-Canet *et al.* (1966) that a high yield of dimer is produced upon irradiation at 325 mμ of vitamin A in hexane is also consistent with a free-radical intermediate.

However, in our experiments retinals and retinol alone frozen at −196° were induced to form their characteristic free radicals by irradiation with visible light in the absence of added redox agents. This is reasonable if it is assumed that one molecule of carotenoid serves as the donor of an electron to a second molecule, which may be in the triplet-excited state to act as an electron acceptor. Pullman (1960) has calculated resonance energies, bond orders, and electronic charges for these compounds and concludes that they can be both good donors and acceptors of electrons. We believe this mechanism of radical anion formation from the triplet state to be the most likely explanation of the retinal and rhodopsin paramagnetic resonance, and electron paramagnetic resonance investigation of the characteristics of charge-transfer complexes of retinal and retinol (in preparation) further supports the conclusion that a free-radical anion is the species whose spectrum we have observed in this work.

Dawson (1962) and Grellman *et al.* (1962) have pointed out that the relative percentage of triplet yield is best in nonpolar solvents and decreases as the polarity of the solvent increases. Similarly, Hubbard (1966) also has concluded from thermodynamic measurements that the possible importance of a triplet mechanism of 11-*cis*-retinal isomerization depends upon solvent polarity and dielectric properties. This relation to solvent holds also, in a general way, for free-radical production, no radical at all being observable in ethanol solutions, whose static dielectric constant in solution is higher than those of all the other solvents, save acetonitrile (Handbook of Chemistry and Physics, 1964). However, the relationship of liquid-state polarities to the dielectric environment in the frozen solutions may be complex.

That retinal in 2% aqueous digitonin behaves as if it were in a somewhat nonpolar environment was shown both by our finding that we could still detect a signal in this medium (although barely) and the study of Hubbard *et al.* (1965) of the isomerization of 11-*cis*- to all-*trans*-retinal in which they conclude that ". . . . the changes in configuration . . . . which accompany the isomerization . . . . from the *cis* to the *trans* configuration are favored by such nonpolar environments as *n*-heptane. The fact that aqueous digitonin behaves like heptane in this regard suggests that retinal fits itself into the digitonin micelle in such a way as to achieve a nonpolar environment. A similar relationship may well obtain between the retinal and opsin . . . ."

There is some evidence for retinal's having an even more nonpolar environment in rhodopsin than in digitonin. While the retinal electron paramagnetic resonance signal was just barely detectable in 2% digitonin, it was beyond a doubt present in 2% digitonin solutions of rhodopsin, although considerably smaller than in solutions of retinal in nonpolar solvents such as heptane. In fact, Grellman was able to detect the retinal triplet in nonpolar solvents with ease (up to 28% yield), but could not detect it in 1% aqueous digitonin solutions (Grellman *et al.*, 1962).

Rosenberg (1958, 1966) and his associates (1961) have shown β-carotene and dried rod outer segments to be photoconductive, and they believe the triplet state to be an intermediate in producing the photoconductivity. Perhaps, in fact, the triplets are precursors of radical anions and positive holes, the latter serving, according to Rosenberg, as the mobile charge carriers; because it is known that β-carotene will form free radicals on irradiation with visible light (Smaller, 1960).

A possible mechanism, therefore, for the isomerization of *cis* to *trans* might be the following:[2]

$$\begin{array}{ll} \mathrm{C{=}CC{=}CC{=}C} & \text{(a: } cis\text{)} \\ \quad\big\downarrow h\nu & \\ \mathrm{\dot{C}C{=}CC{=}C\dot{C}} & \text{(b: one of several possible contributions to the triplet state)} \\ \quad\big\downarrow \Delta & \\ \mathrm{\dot{C}C{=}C\vartheta C{=}C\dot{C}} & \text{(c: 90° twist)} \\ \quad\big\downarrow & \\ \mathrm{C{=}CC{=}CC{=}C} & \text{(d: } trans\text{)} \end{array}$$

The light-excited species which actually isomerizes may thus be a triplet, which may or may not trap an extra electron to form the radical anion, since, as we have noted, promotion of an electron to a triplet

[2] Modified from a suggestion by a referee and very similar to the mechanism proposed by Hubbard and Kropf (1967) for *cis–trans* isomerization of excited states.

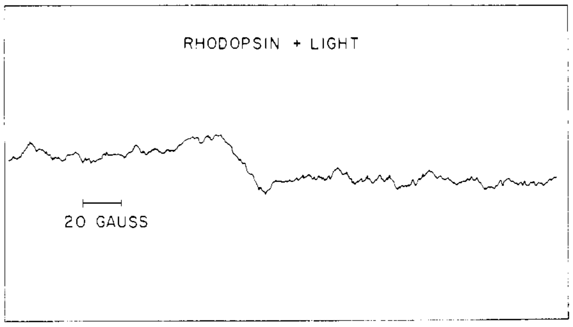

FIGURE 5: Epr spectrum of rhodopsin irradiated in 2% aqueous digitonin at $-196°$. Modulation amplitide, 16 gauss; power, 37 mW. g = 2.004 ± 0.001.

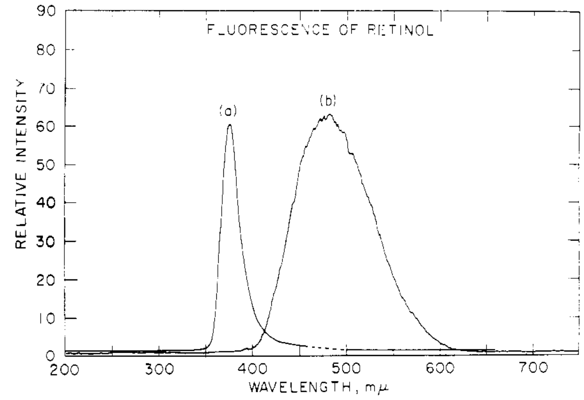

FIGURE 6: Fluorescence of 10^{-3} vitamin A in acetone at 23°. (a) Excitation spectrum, with emission measured at 480 mμ. (b) Emission spectrum, with excitation at 375 mμ.

orbital leaves a low-lying hole. If, as Morton, Pitt, Hubbard, and Kropf believe (Abrahamson and Ostroy, 1967), the primary binding of retinal to protein is *via* a protonated Schiff base, the positive charge on the nitrogen, in addition to the low-lying hole, would make electron capture a likely phenomenon should the above triplet (b) be generated. Nevertheless, because of the much higher quantum yield of vision (*ca.* 0.5–0.6) than of triplet excitation, Abrahamson and Ostroy (1967) conclude that participation of the triplet state is unlikely. In that case the isomerizing intermediate might be the free radical itself, formed from the excited singlet precursor according to the more common photochemical mechanism, an interpretation fully as consistent with our own data as is the triplet pathway we conjectured in the earlier discussion.

There is evidence that each isomer of retinal has its own excited state, and that there is no common excited state for these isomers (Hubbard, 1956). Furthermore, although the electron paramagnetic resonance spectra of the light-produced free radicals of 9-*cis*-, 13-*cis*-, and all-*trans*-retinal appear almost identical at low temperature, the width and slight asymmetry of the spectra suggest the presence of hyperfine structure which is not resolved in the frozen samples. Accordingly, the electron paramagnetic resonance data are not inconsistent with there being similar but different excited states for the different isomers, and these may give rise to either similar or different free-radical products.

In contrast to the above similarities, however, the signal obtained from vitamin A (retinol) is altogether different from that obtained from the retinals and rhodopsin. These latter signals are relatively narrow (*ca.* 23 gauss, exactly the expected signal width from a free radical whose unpaired electron interacts with the majority of the protons), and this is consistent with considerable delocalization of the unpaired electron over these protons and the magnetically inert carbon atoms along the conjugated backbone of the molecule. On the other hand, the broad spectrum (*ca.* 210 gauss) from the retinol radical represents strong interaction with a few magnetic nuclei, as would result from odd electron density highly localized in the vicinity of two or three hydrogen nuclei, perhaps involving electron or hydrogen abstraction from the terminal alcoholic group. Since the retinol spectrum is obviously complex but unresolved, especially on the high-field side, we are unable at this time to identify the chemical nature of the radical giving rise to it, except to point out that it is probably different from the "broad singlet" described by Rexroad and Gordy (1956) after X-irradiation of the vitamin. However, the fact that the odd electron on the retinal free radical is more delocalized and "free" to interact with the environment than the corresponding electron on the retinol radical may be significant regarding the physiological role of the aldehyde (retinal) as the prosthetic group of rhodopsin in contrast to the alcohol (retinol). One possible consequence of the greater delocalization of the electron in retinaldehyde radicals than in those from retinol, as suggested by Dartnall (1948), is that only in the former would the electron be readily transferred .... "down the carotenoid chain to the protein base and thence *in vivo* to the retinal end organ to which in all probability the visual purple molecules are attached."

Since excitation energy localized to a part of a molecule not strongly coupled to the matrix is less apt to be dissipated by nonradiative decay, localization and relative sequestration of excitation energy by retinol is also suggested by its fluorescence (at room temperature) following near-ultraviolet light stimulation (Figure 6), whereas ultraviolet-irradiated retinal (at room temperature) discharges its excitation nonradiatively,[3] *via* triplet states or other mechanisms, including chemical pathways. Indeed, Hubbard (1956) has shown that in the presence of iodine and light all isomers of retinal

[3] Although Dawson (1962) was able to observe strong fluorescence from retinal in ethyl ether–isopentane–ethanol *glass* we were unable to demonstrate any fluorescence at room temperature in a variety of solvents.

isomerize faster than the corresponding isomers of vitamin A. Functionally, therefore, the delocalization indicated by the electron paramagnetic resonance and fluorescence data may suggest the selective advantage of retinal over retinol in the evolution of the chromophore of the visual pigment.

As noted earlier, the signal from the rhodopsin was considerably smaller than that from the carotenoid solutions, because to avoid denaturing protein, the solvent system was 2% digitonin instead of one of the nonpolar hydrocarbons that enhanced photosignal formation (Figure 3) (although no doubt the 100-fold lower concentration of carotenoid in the rhodopsin solutions also contributed to the difference). Since the lamellae of the rod outer segments contain a great deal of lipid (Wolken, 1961), rhodopsin *in vivo* is in a relatively nonpolar environment, and production of a free radical from the rhodopsin molecule may be an *in vivo* physiological response to light, either on the path of isomerization and/or as a source of an electrical impulse. In fact, Rosenberg (1966) has postulated that an electrical response, such as the photoconduction discussed above, operates in competition with Wald's (chemical) visual cycle. Nevertheless, we were unable to demonstrate an electron paramagnetic resonance signal from suspensions of rod outer segments in sucrose, presumably because of greater light transmission through the essentially transparent rhodopsin glass than through the semiopaque ice of suspended outer segments, and possibly because of greater dilution of rhodopsin in the suspension than in the concentrated extract (about 9×10^{-6} M), from which only a weak signal (Figure 5) could be obtained in any case. The inability to elicit an electron paramagnetic resonance signal from the outer segment preparations is in agreement with the findings of Pitt and Tinkham (1965) who also were unable to demonstrate a signal from freeze-dried rod outer segments irradiated at 90°K. However, we noted earlier that time-averaging techniques enabled Polis and Wyeth (1962) to confirm the presence of electron paramagnetic resonance in illuminated rod outer segments at room temperature, although no electron paramagnetic resonance spectrum, *per se*, could be obtained (B. D. Polis, personal communication).

Falk and Fatt (1966) have recently demonstrated a rapid hydrogen ion uptake of rod outer segments and rhodopsin solutions on illumination, corresponding to an uptake of one hydrogen ion per molecule of rhodopsin. This would be consistent with our supposition that the initial light-promoted chemical step is the formation of a free-radical anion, the hydrogen ion being subsequently taken up to restored electrical neutrality.

Recently there has been a great deal of interest in the early receptor potential which appears within 0.5–50 μsec of the light stimulus and has been attributed to rhodopsin because of this short latency (Brown and Murakami, 1964; Pak and Cone, 1964), its linearity with light intensity (Cone, 1964), its action spectrum (Cone, 1964), and its lack of sensitivity to anoxia (Brown and Murakami, 1964) and to the ionic solutions (Brindley and Gardner-Medwin, 1965, 1966) which destroy membrane conductivity. The R1 component of the early receptor potential has also been demonstrated at temperatures as low as −35° (Pak and Ebrey, 1965). (This was the lowest temperature examined: perhaps it exists at even lower temperatures.) Pak and Ebrey (1965) therefore rule out the steps below metarhodopsin I for its production, since they require higher temperatures. Rosenberg (1962), Cone (1967), and Hagins and McGaughy (1967) believe that the initial receptor potential[4] is due to charge separation on the rhodopsin molecule, and since our evidence leads us to hypothesize that incident light may generate charge separation on rhodopsin by the creation of radical ions, these may be the charged species responsible for the early receptor potential. Should this plausible reaction sequence deduced from observations *in vitro* actually apply to the process of visual excitation, one would have a second chemical mechanism for an important electrophysiological event, in addition to the sodium–potassium flux associated with nerve potentials.

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[4] Rosenberg actually conjectured about the electroretinogram itself, because the early receptor potential was not yet known, while Hagins and McGaughy (1967) concluded that the comparable fast photovoltage from squid retina arises from charge redistribution in excited rhodopsin molecules.

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