GRADY, Frank Joseph, 1941-
LIGHT INDUCED FREE RADICALS FROM RETINAL,
RETINOL AND RHODOPSIN.

The City University of New York, Ph.D., 1970
Physiology

LIGHT INDUCED FREE RADICALS
FROM
RETINAL, RETINOL AND RHODOPSIN

by

Frank J.oseph Grady

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1970

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

March 6, 1970
date

Irving L. Schwartz
Chairman of Examining Committee

March 6, 1970
date

Irving L. Schwartz
Executive Officer

Irving L. Schwartz
Donald C Borg
Leonard Ornstein
Supervisory Committee

The City University of New York

ABSTRACT

Light-induced electron paramagnetic resonance (EPR) signals that disappeared upon melting were elicited from trapped paramagnetic photo-products 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°C. At about 1mM concentration of the three retinals and from rhodopsin, EPR singlet spectra were obtained at $g \cong 2.004$, with line widths of about 23 gauss and with some suggestion of further unresolved spectral detail. In general, the EPR signal was greater with increasing illumination and with decreasing polarity of the solvent. At higher concentrations an EPR photosignal also was obtained from retinol, evincing an overall, 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 fluourescence 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_.)

ACKNOWLEDGMENT

I would like to express my profound gratitude to Dr. Donald C. Borg who introduced me to electron paramagnetic resonance, and whose generosity with time and knowledge and abilities as teacher and scientist made learning and working with him a most pleasurable personal as well as intellectual experience.

I would also like to express my profound gratitude to Dr. Irving L. Schwartz whose imagination, critical insight and guidance kept me learning and working in the laboratory many extra nights, and whose Socratic prodding and encouragement was the moving force behind this thesis.

A debt of gratitude is also owed to Dr. Leonard Ornstein and Dr. Richard Bersohn for many helpful discussions and valuable suggestions.

In addition, I would like to thank Dr. Simon Freed who taught me to look to low temperature for solutions to chemical problems, and Mr. John Elmore for help in running the early samples. Last, but not least, I would like to thank Dr. Roderich Walter for his encouragement, interest, and for reading the manuscript.

TABLE OF CONTENTS

PAGE

RESULTS

DISCUSSION

B. Implications for Vision

LIST OF FIGURES

INTRODUCTION

We see light when its wavelength falls into the "visible" range of the electromagnetic spectrum, namely approximately between 4000Å and 7000Å. (1Å = 10^{-8}cm). This light is only "visible", however, because photons with energies in this range are able to effect a chemical change in the visual pigments in our eyes. After this chemical change (which will be described in detail in the next section) has occurred in only a very few molecules of visual pigment, we perceive the sensation of light[1].

The chemical changes which occur in a molecule of visual pigment when it absorbs a photon have been studied in great detail, but the connection between these chemical events and the depolarization of the photoreceptor cell (the first nervous discharge in the visual process) has never been precisely defined.

Hubbard and Kropf (1958, 1959) have speculated that the nervous impulse is triggered at a point in the rhodopsin cycle between lumirhodopsin and metarhodopsin, stating that the steps after metarhodopsin are too slow, and probably do not occur in cephalodods, but no cause and effect relationship has been elucidated. In fact, whereas the sensitivity of cells to electrical stimuli and

---

[1] Hecht, S., Schlaer, S. and Pirenne, M.H. "Energy, quanta, and vision," (1941-2), J. Gen. Physiol., 25, 819-840; Wald, G., Brown, P.K. and Gibbons, I.R. "The problem of visual excitation," (1963), J. Opt. Soc. Amer., 53, 20-35.

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[2]. Briefly, the first step, the conversion of rhodopsin to pre-lumirhodopsin, was described by Yoshizawa and Wald (1963) from studies based on material irradiated at liquid nitrogen temperature (-196°C); and it is currently believed that this first step represents the isomerization of the retinal present as 11-cis (neo-b) in rhodopsin to the all-trans from in pre-lumirhodopsin. 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[3]. 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. It would also demonstrate the first highly reactive chemical species to be found among the products

---

[2] Wald, G. "The photoreceptor process in vision," (1955), Am. J. Ophth., 40, 18-41; Hubbard, R., Bownds, D. and Yoshizawa, T. "The chemistry of visual photoreception," (1965), Cold Spring Harbor Symposia in Quant. Biol., 30, 301-315; Hubbard, R. and Kropf, A. "Molecular isomers of vision," (1967), Scient. Amer., 216 (6), 64-76.

[3] Hubbard, R. and Kropf, A. "The action of light in rhodopsin," (1958), Proc. National Acad. of Science, 81, 388-398; Hubbard, R. and Kropf; op. cit., (1967), 64-67; Wald, G., "Introductory Lecture," (1965), Recent Progress in Photobiology, (C.J. Bowen, Academic Press, New York), 133-144.

of the visual cycle-hence a first clue as to how the visual cycle triggers further chemical reactions and ultimately induces membrane depolarization.

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[4].

It was to elucidate the mechanism of the critical isomerization of retinal that the current research was undertaken. The finding that a free radical is easily induced in retinal after illumination with visible light, and can be induced in rhodopsin after similar treatment has several important implications with regard to the chemistry of vision, the origin of the nervous impulse in vision, and the origin of the early receptor potential of the electroretinogram, all of which will be considered in the discussion section. It also provides a

---

[4] Brindley, G.S. and Gardner-Medwin, A.R. "Evidence that the early receptor potential' of the retina does not depend on changes in the permeability of membranes to specific ions," (1965), J. Physiol., 180, 1P; Brindley, G.S. and Gardner-Medwin, S.R., "Origin of the early receptor potential of retina," (1966), J. Physiol. 182, 185-194; Brown, K.T. and Murakami, M. "A new receptor potential of the monkey retina with no detectible latency," (1965), Nature, 201, 626-628; Cone, R.A. "Early receptor potential of the vertebrate retina," (1964), Nature, 204, 736-739; Cone, R.A., "Early receptor potential: Photoreversible charge displacement in rhodopsin", Science , 155, 1128-1130; Hagins, W.A. and McGaughy, R.E. "Molecular and thermal origins of fast photoelectric effects in the quid retine," (1967) Science, 157, 813-816; Pak, W.L. and Ebrey, T.G. "Visual receptor potential observed at sub-zero temperature," (1965) Nature, 205, 484-486; Pak, W.L. and Cone, R.A. "Isolation and identification of the initial peak of the early receptor potential," (1964), Nature, 204, 836-838.

conciliation between the theories of Rosenberg[5] (1966; Rosenberg and Harder, 1967) and Wald (1965), which Rosenberg had until now believed to be mutually exclusive.

Further investigation into the electronic properties of retinal, the carotenoid prosthetic group of rhodopsin, may enable one to speculate on a possible mechanism to explain why a substance with an absorption maximum of approximately 380 nm can serve as the chromophore for substances with absorption maxima ranging from 430 to 570 nm.

Having thus defined the aims and some of the implications of this investigation, I shall now endeavor to provide some further background for the material to be presented.

The Visible Spectrum

Although the visible spectrum, as previously noted is "visible" because this is the energy range to which the eye pigments are sensitive, in a teleological sense one may conclude that this is the most useful range of the electromagnetic spectrum for earth-based animals to be able to detect. The sun is our main source of light and its emission has a spectral distribution resembling a black body of 6200°C, radiating

---

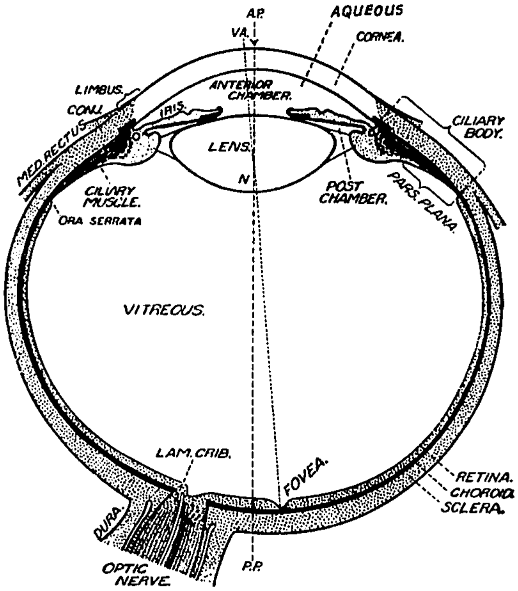
[5] Rosenberg, B. "A physical approach to the visual receptor process," (1966), Adv. in Radiation Biology, 2, 193-241; Rosenberg, B, and Harder, H.C., "Semiconduction and photoconduction activation energies of the retinals," Photochem. Photobiol, 6, 629-641, (1967). Wald, G., op. cit., (1965), 133-144.

maximally at 460 nm. (Dartnall, 1962a). The short end of this radiation never reaches the earth's surface, however, because of the earth's ozone layer which absorbs most of the light with a wavelength below 290 nm. Accordingly, 80% of the energy from the sun received at the earth's surface lies between wavelengths of 300 and 1,100 nm with a λmax of about 500 nm. (This assumes the sun to be at its zenith, because the greater the atmospheric thickness through which its rays must pass, the "cooler" the apparent temperature of the sun, due to the relatively greater scatter of shorter wavelengths.)

In addition to the availability of photons in the 300 to 1100 nm band, several other considerations also point up the advantages of this range of energies. First, quanta of higher energies, e.g. those in the far ultraviolet or x-rays have destructive effects on living tissue, while quanta of the far infrared and beyond have such low energy that the most they can do is affect molecular vibrations and rotations. Hence, the middle region between these extremes seems ideal--both because of its availability at the earth's surface and because it has enough energy to pluck some electrons from their orbits, but not so much as to cause tissue damage.

The Structure of the Verterbrate Eye

The simplest way to approach the optics of the eye is by analogy to a camera. The cornea and the lens are analogous to the



The Human Eye

Fig. 1

lenses of a camera, the cornea being like a front-mounted telephoto, and the crystalline lens therefore being the analog of the camera's own lens. (See Figure 1.) As might be expected from this analogy, the cornea physiologically has more diopteric strength (approximately 42 diopters) than the lens, the refractive power of which is variable, depending upon the state of accommodation and the age of the person; but when accommodation is relaxed, the lens has approximately 19 diopters of refractive power[6]. The reason for the greater refractive power of the cornea is at once apparent when we recall that refraction of light is dependent upon both the radius of curvature and the difference in refractive indices between the media which the light is leaving and that which it is entering. The greater refractive power of the cornea, therfore, is due to the fact that it is the first tissue surface that light encounters, and the difference of refractive index between cornea and air (1.376 and 1.000 respectively) is greater than any subsequently encountered.

Light entering the eye passes in sequence through the cornea, the aqueous humor, the lens and the vitreous humor to the retina. The iris, adjusting itself to the amount of light, is the eye's diaphragm. Having already compared the cornea and lens to camera lenses, the aqueous and vitreous humors which have lower refractive indices, may be likened to the air between these lenses and between

---

[6] Syndacker, D. and Newell, F. Refraction, (1952), Am. Acad. Ophth. and Oto., 71p.

the second lens and the film respectively--the retina being analogous to the film.

Since the visual pigments in which we are interested are found in the retina, the anatomy of this tissue paper thin structure is of interest to us. An excellent detailed discussion of retinal anatomy may be found in Wolff (1961), and we shall here provide a physiologically oriented (but of necessity brief) sketch of retinal anatomy.

The retina is classically divided into ten layers for descriptive purposes (Figure 2).

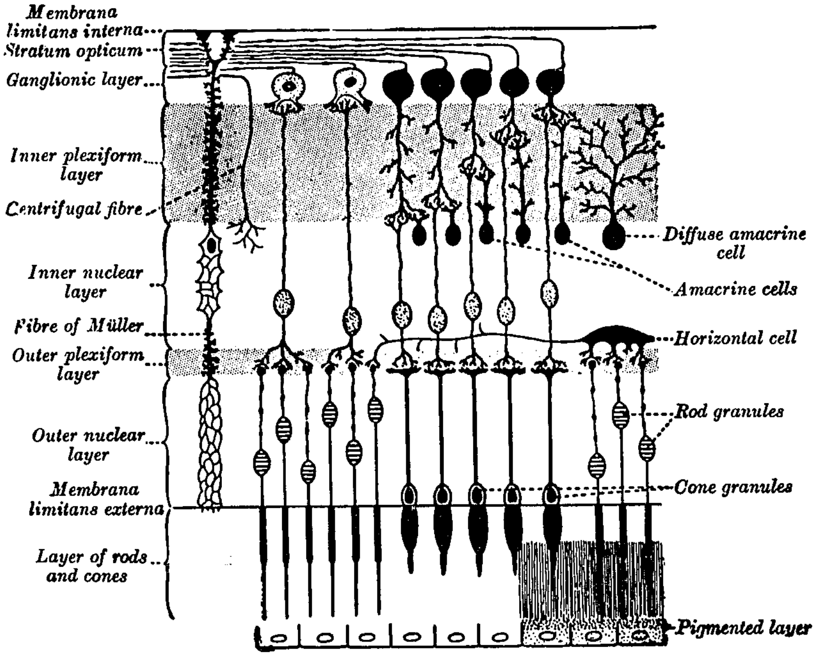


FIGURE 2

Cross section of the human retina

The outermost layer consists of the pigment epithelium. This is a one cell thick layer of relatively flat and roughly hexagonal epithelial cells derived from neuroectoderm and filled with small pigment granules. This layer may well have a function in the visual process, as will be discussed later.

Immediately in front of and in close apposition to the cells of the pigment epithelium are the visual cells, the rods and cones. These form the second layer, and it is these cells which contain the visual pigment and constitute the true sensitive part of the retina. The rest of the retina, the optic nerve, and the areas of the brain concerned with vision serve for transmission and analysis of the impulses generated in this layer.

There are approximately seven million cones in the human eye and approximately one hundred twenty-five million rods. The cones are concerned mainly with detail and color vision under conditions of bright illumination, while the rods are primarily concerned with vision under conditions of dim illumination. Both are divided into an inner and outer segment by a constriction. The inner segment is a highly metabolic structure with many mitochondria, while the outer segment serves for photoreception. It consists of several thousand discs regularly spaced and made up of one or two membranes each[7]. Each membrane is 40 to 160Å thick, depending on the species of animal[8], as shown by electron microscopic studies. Wolken (1956) showed that the double membrane discs in cattle rod outer segments consist of alternate layers of protein and lipid. Within these

---

[7] Sjöstrand, F.S. "An electron microscope study of the retinal rods of the guinea pig's eye." (1949), J. Cell. Comp. Physiol., 33, 383-404.

[8] Sjöstrand, F.S. "The ultrastructure of the outer segments of rods and cones of the eye as revealed by the electron microscope", J. Cell Comp. Physiol, 42, 25-44; De Robertis, E. "Electron microscope observations on the submicroscopic organization of the retinal rods," J. Biophys. Bioch. Cytol., 2, 319-331.

membranes, the planes containing the isoprenoid side chains of the retinal prosthetic groups (Figure 3) of visual pigment are all oriented perpendicular to the long axis of the outer segment, i.e. parallel to the membranes[9].

As one progresses inward toward the center of the eye from the layer of rods and cones, one crosses the external limiting membrane (the third layer), a fenestrated membrane though which the processes of the rods and cones pass, to the next layer--the outer nuclear layer (fourth layer) so-called because it contains the nucleus of the rod and cone cells. From this level, the "axons" of the rods and cones pass into the next (fifth) layer, the outer plexiform or molecular layer, where they synapse with the dendrites of the bipolar cells. The processes of the horizontal cells are also found in this layer.

The bipolar cells' nuclei together with the nuclei of the horizontal cells and the amacrine cells comprise the sixth layer. Both the horizontal cells and the amacrine cells are probably associational in function[10]. The axons of the bipolar cells arborise with the dendrites of the ganglion cells in the next (seventh) layer, the inner plexiform (molecular) layer. The distal processes of the amacrine cells are also found here.

---

[9] Schmidt, W.J. "Polarizationoptische analyse eines eiweiss-lipoid-systmes erlautert am Aussengleid der Sehzellen," (1938), Kolloid-Z., 85, 137-148; Denton, E.J. "A method of easily observing the dichroism of the visual rods," (1954), J. Physiol., 124, 16P-17P; Brown, P.K. and Wald, G. "Visual pigments in human and monkey retina," (1963), Nature, 200, 37-43.

[10] Wolff, E. Anatomy of the Eye and Orbit, 5th ed., (revised by R.J. Last), (1961), H.K. Lewis and Co., Ltd., London. Michael, Chas. R., "Retinal Processing of Visual Images" Sci. Amer. (1969) 220 (5), 104-114.

The eighth layer is made up of neuroglia and ganglion cells, the axons of which comprise the optic nerve and arborize on the geniculate body within the brain. Anterior to the ganglion cell layer is the nerve fiber layer (ninth layer), or stratum opticum, which contains the retinal vessels and neuroglia in addition to the processes from the ganglion cells. The tenth or inner-most layer is the membrane between the vitreous and the retina, the inner limiting membrane.

In summary, the vertebrate retina (despite its great efficiency) is pointed backwards (Figure 2), such that light must pass through all of its layers before reaching the photoreceptors which are pointed away from the light. If light succeeds in exciting a visual cell, the impulse is propagated to the bipolar cells, and in turn to the ganglion cells, from which the impulse is carried into the optic nerve, the lateral geniculate bodies, and thence to the visual cortex where it is further processed.

Photochemistry of Vision

A. Nature of the Chromophore

As Wald noted in 1935, the occurrence of vitamin A in the eye tissues, ". . . is particularly significant because this vitamin is functionally associated with vision. Animals deprived of vitamin A become abnormally insensitive to dim light (night blindness) due to failure to synthesize visual purple.[11]

The carotenoid nature of visual purple was first suggested in 1877 by Franz Boll in the paper which announced its discovery. Like many other facts in science, this belief was questioned many years after its postulation; Haurwitz (1933) and Von Euler and Adler (1934) were unable to demonstrate carotenoids in dessicated visual purple. The carotenoid nature of visual purple was reestablished when Wald (1935-36) showed vitamin A to be a product of visual purple decomposition (extractible from bleached retinas with chloroform), and concluded, therefore, that visual purple must in turn be derived from a carotenoid. He called the carotenoid which he noted to be liberated from visual purple prior to the appearance of vitamin A, "retinene".

From similarities in optical absorption spectra, R. A. Morton (1944) thought it likely that retinene was vitamin A aldehyde[12]. Morton and Goodwin (1944) first synthesized retinal by oxidizing

---

[11] Wald, G. *op*. *cit*., (1933), 316-317; Wald, G. *op*. *cit*., (1934-1935), 905-915; Fredericia, L. S. and Holm, E. "Experimental contribution to the study of the relation between night blindness and malnutrition. Influence of deficiency of fat-soluble A-vitamin in the diet on the visual purple in the eyes of rats," (1925), *Am*. *J*. *Physiol*., *73*, 63-78; Tansley, K. "The regeneration of visual purple; its relation to dark adaptation and night blindness," (1931), *J*. *Physiol*., *71*, 442-458; Wald, G., *op*. *cit*., (1935-1936), 351-369.

[12] By recently adopted international convention (Commission on Nomenclature in Biological Chemistry, 1960), vitamin A and its derivatives were renamed as follows: vitamin A_1 alcohol is now known as retin*ol*; the aldehyde, formerly called vitamin A_1 aldehyde, retinaldehyde, or retinene$_1$, is now called retin*al*, and vitamin A acid is known as retinoic acid. The corresponding derivatives of vitamin A_2 are now known as dehydroretinal, dehydroretinal, and dehydroretinoic acid.

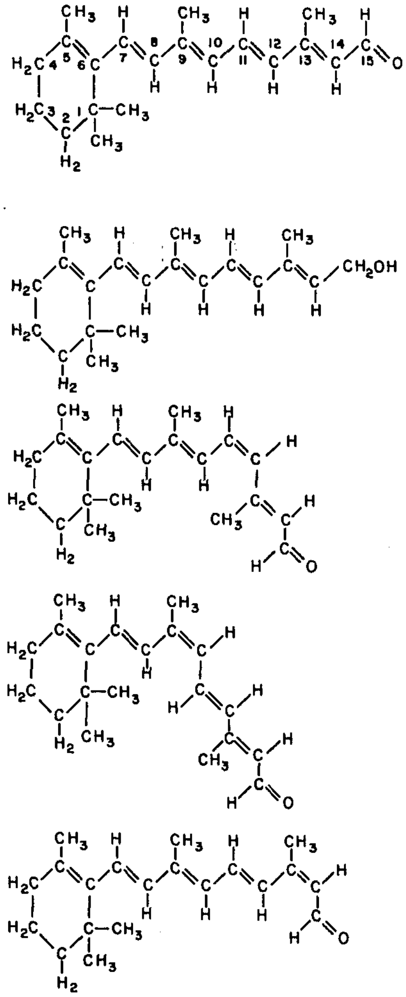
retinol with potassium permanganate, but their yields were small and inconstant. The identity of "retinene" with vitamin A aldehyde (retinal) was firmly established, however, in 1948 when this same group[13] was able to oxidize vitamin A (retinol) to retinal in good yield with MnO_2, allowing the reaction mixture to stand six to ten days. Not only was the absorption spectrum identical to that of "retinene" but the product gave the same color reaction on adding antimony trichloride (Carr-Price reaction) as "retinene". Having established the identity of the newly synthesized compound, with the substance obtained from the eye, this compound was synthesized in larger amounts and its expected aldehyde nature confirmed.

B. Cis-Trans Isomers of Retinal

Retinal and retinol have four double bonds (as may be noted from Figure 3) in the isoprenoid side chain, and therefore sixteen cis-trans isomers should theoretically be possible. According to Pauling (1939, 1949), however, only the bonds that carry methyl side chains (i.e. bonds 9- and 13-) should be able to form cis configurations, because steric hindrance from the methyl groups would impair planarity by "twisting" the molecule. This would "inhibit resonance" and thereby decrease stability. As may be noted from Figure 3, in 11-cis retinal the methyl on carbon number

---

[13] Ball, S., Goodwin, T.W. and Morton, R.A. "Studies on vitamin A, 5: Preparation of retinene - Vitamin A aldehyde," Bioch. J., 42, 516-523; Ball, S., Goodwin, T.W. and Morton, R.A. "Retinene$_1$-Vitamin A aldehyde," Biochem. J., 40, proc. 59.



ALL-TRANS RETINAL

ALL-TRANS RETINOL
(VITAMIN A)

11-cis (neo-b) RETINAL

9-cis (iso-a) RETINAL

13-cis (neo-a) RETINAL

FIG.3 GEOMETRICAL ISOMERS OF RETINAL AND RETINOL
(VITAMIN A)

FIGURE 3

13 is in the same region as the hydrogen on carbon number 10. The large size of the methyl group forces the molecule to twist such that, in the diagram, it must be either above or below the plane of the paper (in which the hydrogen atom lies), thus twisting the molecule as well as bending it. The linkage is therefore called a "hindered" cis linkage. On the other hand, in a nonhindered linkage such as is found in the 9-cis isomer, the two groups placed in apposition due to the cis linkage are both hydrogen atoms, and hence due to their smaller size, the molecule is not forced to twist out of its planar configuration as in the case of the hindered 11-cis isomer.

In the strictest sense, Pauling's theory allows only those double bonds with adjacent methyl groups to form cis configurations, therefore there should be only four possible isomers: all-trans, 9-cis, 13-cis and 9,13-dicis. These four isomers have been synthesized (Robeson et al., 1955a, 1955b), but, as might have been suspected from my previous discussion of the 11-cis isomer, the "disallowed" 11-cis and 11,13-dicis retinal have also been prepared[14].

Synthesis of the "disallowed" 7-cis compounds, however, has been attempted (Wald, Hubbard, Brown, and Oroshnik, 1955, as cited by Pitt and Morton, 1960) but not successfully accomplished, and the 7-8 acetylene, which on reduction would yield a cis linkage,

---

[14] Oroshnik, W. "The synthesis and configuration of neo-b vitamin A and neo-b retinene," (1956), J. Am. Chem. Soc., 78, 2651.

does not add hydrogen[15]. Since the 11-cis form is, after all, allowed, two further isomers may (as Dartnall, 1962b points out) be possible, namely the 9,11-dicis, and the 9,11,13-tricis, but so far these have not yet been synthesized.

Due to thermodynamic considerations, the planar all-trans retinal is most stable, followed by the unhindered cis isomers: 9-cis, 13-cis, and 9,13-dicis. Considerably less stable are the hindered and twisted 11-cis and 11,13-dicis forms, since the more planar the isomer the more stable[16].

C. Spectra of Retinal Isomers

Fortunately, the various isomers of retinal can be distinguished to some extent by their absorption spectra[17]. This is useful both for identifying various isomers and for following isomerization reactions.

Zechmeister has shown that a single cis linkage tends to shift the absorption maximum 4 to 6 nm toward shorter wavelengths,

---

[15] Oroshnik, W., Brown, P.K., Hubbard, R. and Wald, G., "Hindered cis isomers of vitamin A and retinene; the structure of the neo-b isomer," Proc. Nat. Acad. Sci., 42, 578-580; Hubbard, R., et al. op. cit., (1965), 301-315.

[16] Zechmeister, L., "Cis-trans isomerization and stereochemistry of carotenoids and diphenylpolyenes," (1944), Chem. Revs., 34, 267.

[17] Hubbard, R., Gregerman, R. Wald, G. "Geometrical isomers of retinene," (1953), J. Gen. Physiol., 36, 415-429; Wald, G., et al., op. cit., (1955), 438-451; Robeson, C.D., Blum, W.P., Dieterle, J.M. Cawley, J.D., and Baxter, J.G. "Chemistry of vitamin A, XXV: Geometrical isomers of vitamin A aldehyde and an isomer of its alpha ionone analog," J. Am. Chem. Soc., 77, 4111-4119; Hubbard, R. op. cit., (1956), 935-962; Brown, P.K. and Wald, G. "The neo-b isomer of vitamin A and retinene," (1956), J. Biol. Chem., 222, 865-877.

two cis linkages shifting it about twice as much (Zechmeister, 1944). Thus the λ max for all-trans retinal at room temperature in ethanol is 381 nm while that for 11-cis is 376.5 nm (Dartnall, 1962b). Carotenoids may also have a peak in the region 255 to 260 nm which Zechmeister calls a cis peak. Its presence indicates a cis linkage, and its height is proportional to the degree of bending of the molecule. Thus, the 11-cis and 9-cis isomers are the most bent, and therefore these have the highest cis peaks[18], that of the 11-cis being slightly larger[19]. It is interesting to note, too, that only these two isomers will spontaneously combine with opsin, because they both have about the same amount of bending, and therefore presumably fit the molecule. The combination of opsin with 11-cis retinal is now known to be rhodopsin, and that with 9-cis retinal is known as isorhodopsin. It took a great many years of hard work to come to these conclusions, and I shall try to tell part of the story in the following sections, after further examining the spectra and other interesting properties of retinal.

The presence of a cis linkage not only shifts the absorption maximum, but also results in a decrease of the molar extinction coefficient[20]; for example ϵ max (in liters/ mole-cm.) of 11-cis

---

[18] Hubbard, R. *et al.* *op.* *cit.* (1952), 269-315; Hubbard, R., *et al.* *op.* *cit.*, (1953), 415-429; Hubbard, R., *op.* *cit.*, (1956a), 4662-4667.

[19] Hubbard, R. *et al.*, *op.* *cit.*, (1952), 269-315.

[20] Hubbard, R., *op.* *cit.*, (1956a), 4662-4667; Pitt, G.A.J. and Morton, R.A., "Cis-trans isomers of retinene in visual processes," (1960), *Biochem.* *Soc.* *Symposia* (Cambridge), No. 19, 67-89.

retinal at room temperature is 24,900 (in ethanol), while that of all-trans retinal under the same conditions is 43,400 (Dartnall, 1962b).

The main bands of the unhindered isomers of vitamin A show some fine structure which is most pronounced in 9-cis. This is completely lacking in the spectrum of the 11-cis isomer, another aspect of the "degradation" of absorption spectrum also associated with a hindered cis linkage[21].

Since this study was done at low temperature, the changes in the absorption spectrum which occur on cooling are of interest. Jurkowitz and others in Wald's lab[22] showed that there is a significant difference in behavior between the hindered and the non-hindered isomers of retinal as regards their absorption spectrum at low temperatures. The absorption of all-trans retinal in ether-isopentane-ethanol (5:5:2) at room temperature is maximal at 373 nm (λ max) with a molar extinction coefficient of 47,600 (note that these values are slightly different from those cited above, referring to ethanol as solvent); upon cooling to -185°C, the λ max moves about 14 nm toward longer wavelengths to 387 nm, and the molar extinction coefficient at the absorption maximum increases about ten percent, as is characteristic of

---

[21] Hubbard, R. "Geometrical isomerization of vitamin A, retinene, and retinene oxime," (1956a), <u>J. Am. Chem. Soc.</u>, <u>78</u>, 4662-4667.

[22] Jurkowitz, L., Loeb, J.N., Brown, P.K. and Wald, G., "Photochemical and stereochemical properties of carotenoids at low temperatures - photochemical behavior of retinene," (1959), <u>Nature</u>, <u>184</u>, 614-624.

polyenes brought to low temperatures[23]. If, however, a solution of 11-cis retinal is similarly cooled, its λ max and ϵ max which at room temperature are 369 nm and 26,400 respectively, increase to 385 nm and 43,000 respectively[24]. Thus, the molar extinction of the all-trans isomer increases about ten percent while that of the 11-cis isomer increases sixty-two percent on cooling, changes that are completely reversed on warming. The authors interpret their data as showing that cooling results in contraction of the Van der Waals radii of the methyl groups, thereby allowing rotation about the hindered linkage, in effect relieving the steric hindrance and allowing the molecule to approach planarity more closely.

D. Isomerization of Retinal

Retinal is isomerized by simple exposure to light[25]. Beginning with any single geometrical isomer, light exposure leads to the same steady state mixture of all possible isomers[26]. The all-trans predominates in this mixture with lesser amounts

---

[23] Hausser, K.W., Kuhn, R. and Seitz, G., "Lichtababsorption und Doppelbindung, V: Uber die Absorption von Verbindungen mit Konjugierten Kohlenstoffdoppelbindungen bie tiefer temperatur," (1935), Z. Physikal. Chemie. Abt. B, 29, 391-116; as cited by Jurkowitz, L., et al. op. cit. (1959), 614-624.

[24] Jurkowitz, L., et al. op. cit., (1959), 614-624.

[25] Hubbard, R. and Wald, G., op. cit.,(1952), 269-315.

[26] Hubbard, R. et al. op. cit., (1953), 415-429, Hubbard, R., op. cit. 1956A 4662-4667; Hubbard, R., "Retinene Isomerase", (1956), J. Gen. Physiol. 935-962.

of 11-cis and 9-cis and a very small amount of 13-cis[27]. The relative proportions depend first on whether the irradiation time is sufficient for the mixture to come to equilibrium, and the equilibrium mixture depends on the solvent[28], the compounds with which the retinal is combined, and perhaps the pH[29]. For example, Brown and Wald (1956) have shown that a greater proportion of cis isomers results if the irradiation is carried out in polar solvents, and Hubbard, Bownds and Yoshizawa (1965) state that while irradiation of retinal in solution results in ". . . a mixture of the all-trans with the three mono cis isomers", similar treatment of rhodopsin produces ". . . only the 11-cis, all-trans, and 9-cis chromophores", thus illustrating the importance of the environment in influencing the composition of the final mixture of retinal isomers after illumination.

The isomerization of each bond is essentially independent of the configuration of the other double bonds in the conjugated system[30]. As a virtual corollary of this[31], Hubbard *et al.* (1965)

---

[27] Pitt, G. A. J. and Morton, R.A., "Cis-trans isomers of retinene in visual processes," (1960), *Biochem. Soc. Symposia* (Cambridge), No. *19*, 67-89.

[28] Brown, P.K. and Wald, G., "The neo-b isomer of vitamin A and retinene," (1956), *J. Biol. Chem.*, *222*, 865-877.

[29] Hubbard, R. and St. George, R. C. C., "The rhodopsin system of the squid," (1958), *J. Gen. Physiol.*, *41*, 501-528; Pitt, G. A. J. *et al.* *op. cit.* (1960), 67-89.

[30] Pinckard, J. H., Wille, B. and Zechmeister, L., "A comparative study of the three stereoisomeric 1, 4, diphenylbutadienes," (1948), *J. Am. Chem. Soc.*, *70*, 1938-1944; Magoon, E. F. and Zechmeister, L., "On the cis forms of some biphenylene derivatives of butadiene and hexatriene," (1955), *J. Am. Chem. Soc.*, *77*, 5642-5646; Hubbard, R., *op. cit.*, (1956a), 4662-4667.

[31] Hubbard, R. *et al.* *op. cit.*, (1965), 301-315.

noted that the conjugated system cannot be considered to have a single excited state, but each linkage must have its own excited state. Carrying this one step further, since the cis isomers absorb at shorter wavelengths than the trans, and are also less stable, one must conclude that the cis and trans isomers of the same bonds have different excited states, since if they shared a common excited state, the cis isomer whose ground state presumably has more energy, would require less energy to reach the excited state and hence should absorb at longer wavelengths[32].

In the light of the above, the fact that all linkages do not isomerize at the same rate is not surprising. Thus, the 11- and 13-cis linkages are rapidly isomerized, while the 9-cis linkage is only slowly isomerized[33], the 9-cis isomer requiring about five times as much time of light exposure as the all-trans or the other cis isomers[34] to reach the equilibrium mixture.

As may be noted from Figure 3, the side chain and the two methyl groups on the beta-ionone ring may be either cis or trans to each other, depending on the rotary position of the ring. Pullman and Pullman (1961) have postulated that perhaps the side chain and methyl groups are cis to each other (s-cis) in the 9-cis species and trans in the others, thus accounting for the difficulty

---

[32] Ibid.

[33] Hubbard, R. et al, op. cit., (1953), 415-429; Hubbard, R., op. cit. (1956a), 4662-4667; Pitt, G. A. J. et al. op. cit., (1960), 67-89.

[34] Hubbard, R. et al. op. cit., (1953), 415-429.

in isomerizing the 9-cis isomer, as this would necessitate two separate configurational changes. To date, this postulation has neither been proved nor disproved.

Since it was noted earlier that the λ max of the all-trans isomer is at longer wavelengths and that this isomer has a higher molar extinction coefficient, it follows that when a mixture of all-trans retinal is irradiated, its λ max goes to lower wavelengths, and its molar extinction decreases; similarly, when a cis isomer is irradiated, its λ max shifts to longer wavelengths, and its molar extinction coefficient increases[35].

Iodine is an excellent catalyst for the isomerization of carotenoids (Zechmeister, 1944), but as noted earlier, light alone is sufficient for retinal. On the other hand, retinol requires iodine to isomerize in visible light[36], although even when both are in the presence of iodine, retinal isomerizes faster than retinol, presumably due to the resonance involved in the terminal carbonyl group of retinal[37]. Hubbard and Wald (1952) feel that iodine makes two kinds of contributions to isomerization of retinol, the first being to capture the energy of visible light for the reaction (since retinol absorbs in the near ultraviolet), and the other is postulated to be a momentary addition across the

---

[35] Hubbard, R. *et al. op. cit.*, (1953), 415-429; Brown, P.K., and Wald, G. *op. cit.*, (1956), 865-877; Wald, G., "Photochemical Aspect of visual excitation," (1958), *Exptl. Cell, Res. Suppl.*, *5*, 389-410.

[36] Brown, P. K. *et al. op. cit.* (1956), 865-877.

[37] Hubbard, R., *op cit.*, (1956a), 4662-4667.

double bond converting it to a single bond for an instant, thus allowing free rotation, with subsequent dissociation of the iodine. In a later paper dealing with retinal, Hubbard (1966) seriously questions this latter mechanism, because iodine does not change the Arrhenius activation energy as would be expected if it formed a frank addition product, and her conclusions will be cited in the section on possible mechanisms of isomerization.

Another point of interest regarding the isomerization of pure carotenoids is that this reaction can go on at liquid nitrogen temperature (ca. 77° K). Jurkowitz et al. (1959) noted that crystalline retinal was not isomerized by light, and they investigated whether low temperature or a rigid solvent would also impair isomerization because of the necessity for rotation. They concluded that "...neither low temperature nor a rigid solvent inhibits geometrical isomerization of this molecule, and the process appears to go about as well in these circumstances as at room temperature. Apparently the rigid solvent leaves the molecule sufficient elbow room to allow free play for the rotations involved in geometrical isomerization[38]".

The importance of isomerization in vision was first suspected by Hubbard and Wald (1951, 1952, 1952a, 1962b) when they noted that bleached (i.e. light exposed) rhodopsin could be regenerated in the presence of DPN (NAD) and alcohol dehydrogenase by addition of fish liver oil concentrate as the source of vitamin A, but not if crystalline (all-trans) vitamin A was employed[39].

---

[38] Jurkowitz, L. et al. op. cit. (1959), 616. See Discussion, p. 106.

[39] Hubbard, R. and Wald, G., op. cit. (1952), 269-315.

However, after exposing the crystalline retinol to light in the presence of iodine, a process known to isomerize carotenoids, (Zechmeister, 1944), this proved as good a precursor of rhodopsin as fish liver oil. Thus, it became apparent that the active species which reacted to reform the visual pigment was a particular isomer of retinol other than the all-trans form. It was already suspected from examination of the absorption spectrum of rhodopsin that the active isomer had a cis linkage. In the light of these findings, the earlier experiments of Chase (1937) and Chase and Smith (1939) became somewhat more understandable, too. These authors had found that when blue (isomerizing) light was employed to bleach rhodopsin, there was partial regeneration, but no regeneration occurred if yellow (non-isomerizing) light was used for the bleaching. The importance of isomerization being thus discovered, the search was on to find the active isomer or isomers.

As noted earlier, sixteen stereoisomers of retinal or retinol are theoretically possible, but to date only six have been prepared: 9-cis, 11-cis, 9,11-dicis, 11,13-dicis, and all-trans. Before the chemical nature of each of the isomers was known, they were designated by common names--thus, the isomer released from rhodopsin on bleaching was known to be a cis isomer and was called the neo-b isomer. The other isomers were designated neo-a (13-cis), iso-a (9-cis), iso-b (9,13-dicis), and neo-c (11,13-dicis). The neo-b isomer was first thought to be 9-cis (Hubbard and Wald, 1952; Hubbard, Gregerman and Wald, 1953) because at this time it was still thought that hindered isomers could not exist. Subsequently, when an isomer thought to be 11-cis (which was actually 11,13-dicis) proved not to be identical with the neo-b form, 7-cis was settled upon by exclusion[40]. The following year, an isomer identical to that obtained from the eye was synthesized by Oroshnik who was working with Wald's group. This proved to be 11-cis retinal[41].

Although this research is concerned mainly with rhodopsin, the pigment of the vertebrate rod, Hubbard and Kropf (1958) have pointed out that stereoisomerization of retinal, "...seems to be a general situation that involves all the known pigments--rhodopsin (vertebrate rod pigment), iodopsin (vertebrate cone pigment), porphyropsin (fresh water fish rod pigment) and cyanopsin (fresh water fish cone pigment) --and the corresponding isopigments[42].

E. Structure of Rhodopsin

As early as 1935 Wald concluded that rhodopsin or visual purple, as it was known then, consisted of "retinene" (retinal) bound to protein[43]. He arrived at this conclusion since it did not diffuse through a semi-permeable membrane (Ewald and Kühne, 1878), and it could be salted out with magnesium or ammonium sulphate (Kühne, 1895).

---

[40] Wald, G., *et al. op. cit.*, (1955), 438-451.

[41] Oroshnik, W., et al. *op. cit.* (1956); Oroshnik, W., op. cit. (1956).

[42] Wald, G., Brown, P.K. and Smith, P.H. "Cyanopsin, a new cone pigment of vision, (1953), *Science*, *118*, 505-508; Wald, G., Brown, P.K. and Smith, P.H., "Iodopsin", (1954-1955), *J. Gen. Physiol.*, *38*, 623-681; Wald, G., "The photoreceptor process in vision", (1955), *Am. J. Ophth.*, *40*, 18-41.

[43] Wald, G., "Carotenoid and the visual cycle,", (1935-1936), *J. Gen. Physiol.*, *19*, 351-369.

Furthermore, combinations of carotenoids with protein were known to exist, e.g. in serum, carotene is bound to albumin[44]; and pigments of several crustaceans were known to be of this type[45], and they behaved exactly as did visual purple with regard to salting out and extraction of carotenoid.

Yet another protein-like characteristic was demonstrated by Broda and Victor in 1940[46] when they demonstrated that the electrophoretic mobility of frog rhodopsin varied with pH, and that it had an isoelectric point of 4.47. The characteristic 280 nm absorption band of the aromatic amino acids was also shown to be part of the rhodopsin spectrum[47], and Lythgoe and Quiliam (1938) had shown that rhodopsin was destroyed by heat, yet another characteristic of protein.

The large molecular weight of rhodopsin is also due to its protein moiety. Hubbard (1954) calculated the molecular weight of rhodopsin to be approximately 40,000. This calculation took advantage of the fact that digitonin contains no nitrogen. Thus, if a Kjeldahl nitrogen analysis is performed on a dried

---

[44] Palmer, L.S. and Eccles, C.H., "Carotin - the principal natural yellow pigment of milk fat: Its relations to plant carotin and the carotin of body fat, corpus luteum and blood serum. III, the yellow lipochrome of blood serum," (1914), J. Biol. Chem., 17, 223-237.

[45] Wald, G., "Carotenoid and the visual cycle", (1935-1936), J. Gen. Physiol., 19, 351-369.

[46] Abrahamson, E.W. and Ostroy, S.E., "The photochemical and micromolecular aspects of vision," (1967), Progress in Biophysics and Molecular Biology, 179-215.

[47] Collins, F.D., Love, R.M. and Morton, R.A., "Studies in rhodopsin,4: Preparation of rhodopsin," (1952), Bioch. J., 51, 292-298.

sample of digitonin solution of rhodopsin, and one assumes that rhodopsin, like most proteins, contains about 15% nitrogen by weight, one can calculate the fraction of the weight contributed by rhodopsin. This comes to approximately 14%. Since the molecular weight of the digitonin-rhodopsin micelle or particle is 260,000 to 290,000, as demonstrated by Hubbard (1954) and earlier by Hecht and Pickels (1938), it contains 36,000 to 41,000 grams of rhodopsin per mole of micelles. Thus, assuming one molecule per micelle, (as had been shown by Hubbard (1954)) the maximum molecular weight of rhodopsin is approximately 40,000, from which it can be calculated that an average micelle contains roughly 200 molecules of digitonin to one molecule of rhodopsin.

Krinsky (1958) estimated the molecular weight of rhodopsin to be approximately 32,000 and further noted that more phospholipid could be extracted after bleaching, suggesting that lipid may be associated with the chromophore--a finding very significant in the light of our investigation and the recent work of Poincelot _et al_. (1969). Yet another estimate of the molecular weight of rhodopsin is offered by Shields _et al_. (1967) who believe the molecular weight to be approximately 28,600. His group has also recently estimated the amino acid composition of bleached rhodopsin as follows: $\mathrm{Asp}_{18}\text{-}\mathrm{Thr}_{20}\text{-}\mathrm{Ser}_{17}\text{-}\mathrm{Glu}_{21}\text{-}\mathrm{Pro}_{19}\text{-}\mathrm{Gly}_{19}\text{-}\mathrm{Ala}_{22}\text{-}\mathrm{Cys}_{6}\text{-}\mathrm{Val}_{20}\text{-}\mathrm{Met}_{8}\text{-}\mathrm{Ile}_{14}\text{-}\mathrm{Leu}_{22}\text{-}\mathrm{Tyr}_{11}\text{-}\mathrm{Phe}_{21}\text{-}\mathrm{Lys}_{13}\text{-}\mathrm{His}_{6}\text{-}\mathrm{Arg}_{10}$[48].

---

[48] Shields, J.E., Dinovo, E.C., Henriksen, R.A., Kimbel, R. L., Jr., and Millar, P.G., "The purification and amino acid composition of bovine rhodopsin," (1967), _Biochim_. _Biophys_. _Acta_, _147_, 238-251.

In a very recent report, Shichi et al. (1969) after extracting rhodopsin with the detergent emulphogene BC-720 found the molecular weight to be about 28,000 which is not far from Shields figure of 28,600.

Although the light sensitive reaction is based on the photoisomerization of retinal, the nature of the protein is very important, since the characteristics of the protein determine whether the pigment is rhodopsin or one of the iodopsins[49]. Wald has coined the terms scotopsin and photopsin for the proteins (opsins) involved in forming the rod (rhodopsin) and cone (iodopsin) pigments respectively.

Iodopsin, the cone pigment which was originally thought to be one pigment was recently shown to be at least three different pgiments with their λ max values approximately 440 nm (cyanolabe), 540 nm (chlorolabe), and 565-580 nm (erythrolabe)[50]. Despite the existence of these pigments and also of pigments analogous to rhodopsin and iodopsin but based on dehydroretinal in some fresh water fish[51], I will, as noted above, deal principally with the photochemistry of rhodopsin.

---

[49] Wald, G., Brown, P.K. and Smith, P.H., "Iodopsin", (1952), Fed. Proc., 11, 34; Wald, G., et al. op. cit., (1954-1955), 623-681; Dartnall, H.J.A., "The chemical structure and photochemistry of the visual pigment," (1926b) Chapter 19 in The Eye, 427-471.

[50] Thompson, L.C., "The effect of change of brightness level upon the foveal luminosity curve measured with small fields," (1947), J. Physiol. 106, 368-377; Thompson, L.C. (1951), 114-132; Hsia, Y. and Graham, Ch.H., "Spectral sensitivity of the cones in the dark adapted human eye," (1952), Proc. Nat. Acad. Sci. 38, 80-85; Brown, P.K. and Wald,G., "Visual pigments in human and monkey retina," (1963), Nature, 200, 37-43.

[51] Wald, G., Brown, P.K. and Smith, P.H., "Cyanopsin, new cone pigment of vision", (1953), Science, 118, 505-508.

The mechanism of the initial step, namely the action of light on the pigment, is probably the same in all of these pigments[52], since all have the 11-cis configuration of retinal as their chromophore[53]. That retinal is involved in both rod and cone vision is in agreement with the clinical fact that vitamin A deficiency leads to degeneration of both rod and cone vision and also with the observation that addition of 11-cis retinal in vitro to bleached rod or cone pigment results in 50% regeneration[54].

F. The Retinal-Protein Bond

Since the aldehyde group is the most reactive group which retinal possesses and since neo-b (11-cis) vitamin A will not bind to opsin in the absence of alcohol dyhydrogenase and NAD[55], it was readily accepted that the aldehyde end of the retinal was the main linkage site to the protein. There were, however, until recent years, two competing theories regarding the site on the protein to which the aldehyde was bound. Morton and his colleagues at Liverpool favored

---

[52] Ibid

[53] Wald, G., et al. op. cit., (1954-1955), 623-681; Dartnall, H.J.A., op. cit., (1962b), 427-471.

[54] Brown, P.K. and Wald, G., "Visual pigments in human and monkey retina", (1963), Nature, 200, 37-43.

[55] Hubbard, R. and Wald, G., op. cit., (1952), 269-315.

a Schiff base linkage through an amino nitrogen called retinal-N-opsin[56], while Wald and Brown (1950, 1952) favored a linkage through a sulfhydryl group and considered rhodopsin to be retinal-S-opsin. The evidence for both was strong, Morton and his colleagues having demonstrated that retinal and primary amines would form compounds if reacted under alkaline conditions which behaved like Lythgoe's indicator yellow (Lythgoe, 1937), which was recovered after bleaching visual pigments and has a λ max of 365 nm in alkali and 400 nm in acid. Granting the presence of indicator yellow, Wald (1938) felt that it was just an artifact resulting from recombination of retinal and protein after they had been cleaved (Wald, 1949). Dartnall (1948) and Bliss (1948) felt that indicator yellow was a true intermediate and did not result from recombination. This was shown to be the case by Collins and Morton (1950) who found that immediately after bleaching there was no free retinal, and if one bleached in acid, indicator yellow could be detected. Recalling that the recombination of retinal with protein only occurs in alkali (although once formed the product behaves as an indicator), the finding of indicator yellow when rhodopsin is bleached in acidic sholution shows it to be a true intermediate. Thus Collins and Morton (1950) concluded that, "The C-N link of

---

[56] Ball, S., Goodwin, T.W. and Morton, R.A., "Studies in Vitamin A 5: Preparation of retinene-vitamin A aldehyde," (1948), *Biochem. J.*, *42*, 516-523; Pitt, G.A.J. and Collins, F.D., Morton, R.A. and Stok, P., "Studies on rhodopsin, 8: Retinylidene methylamine and indicator yellow analog," (1955), *Bioch. J.*, *59*, 122-128; Morton, R.A. and Pitt, G.A.J. "Studies on rhodopsin, 9: pH and the hydrolysis of indicator yellow," (1955), *Bioch. J.*, *59*, 128-134.

indicator yellow must also occur in rhodopsin," and this was again confirmed by further work of Collins (1953).

Wald and Brown's evidence for retinal-S-opsin was that regeneration of rhodopsin was inhibited by substances that react with SH groups (e.g. para-chloromercuribenzoate, PCMB), and that SH groups do not appreciably ionize below pH 8; it was felt that optimal regeneration occurred under conditions which were favorable to condensation with SH groups and not so favorable for condensation with NH_2 groups.

To reconcile the two theories Collins et al. (1954) proposed that freshly synthesized retinal-S-opsin rearranges immediately after synthesis to retinal-N-opsin, but Dartnall (1957) rejected the theory of retinal-S-opsin altogether, pointing out that Wald and Brown had not shown that the aldehyde group partakes in the reaction with sulfhydryl groups. He also concluded that if the aldehyde group were the only adherent point, all isomers would be equally well bound, but as we know only 9-cis and 11-cis retinal will spontaneously combine with opsin[57], and when these are isomerized to the all-trans configuration, they are split off, presumably because the 9-cis and 11-cis (which we noted earlier were bent more than the other isomers, as shown by the height of their cis peaks) "fit" opsin better. Presumably, the SH groups of the protein also form hydrogen bonds with the

---

[57] Pitt, G.A.J., et al. op. cit., (1960), 67-89; Hubbard, R. op. cit., (1956a), 4662-4667.

conjugated backbone of the retinal.

Even Wald's group (Hubbard, 1958) have now come to accept the retinal-N-opsin structure, while Morton's group acknowledges, "...at least three linkages joining retinene to the protein."[58]

Having agreed that there was a linkage through nitrogen, further investigation showed it to be of the Schiff base type to an amino group on opsin[59]. Since no terminal groups were found by Albrecht (1957), and Bownds (1967) found that after reduction of metarhodopsin II with sodium borohydride, the retinol remains attached to lysine after the protein has been hydrolyzed, it is probable that the retinal is bound to an epsilon amino group of lysine. He also isolated and identified some small peptides which were still attached to the retinyl group after only partial hydrolysis with pronase. The largest of these was phe_3-ala_3-ile-pro-thr-ϵ N retinyl lysine.

He had to use metarhodopsin II instead of rhodopsin because sodium borohydride cannot reduce the carbon-nitrogen linkage in rhodopsin. The reason for this is not clear. Bownds (1967) has suggested that the protein is not as "open" when it is present as rhodopsin with 11-cis retinal. It is well known, for example, that

---

[58] Morton, R.A. and Pitt, G.A.J. "Visual Pigments," (1957), Fortschr. Chem. Org. Naturst., 14, 244-316.

[59] Hubbard, R., "On the chromophores of the visual pigments" (1958) in Visual Problems of Colour, Nation Physical Lab. Symp. #8. H.M. Stationery Office, London, 153-169; Matthews, R., Hubbard R., Brown, P.K. and Wald, G., "Tautomeric forms of metarhodopsin," (1963-1964), J. Gen. Physiol., 47, 215-240.

rhodopsin is stable over a wider range of pH[60] and up to higher temperature (Hubbard, 1958a) than its protein segment (opsin) without the carotenoid. This also is thought to be a result of the protein existing in a more tightly coiled state in rhodopsin. Thus, the analogy drawn a great many years ago by Wald (1935-6), Mirsky (1936) and Wald and Brown (1952) between the bleaching of rhodopsin and protein denaturation still appears to have merit , and will be dealt with in the next section.

The differential stability of opsin and rhodopsin may be of medical significance as well, for deprivation of vitamin A results in degeneration of visual receptor cells, perhaps because the opsin is less stable. Rhodopsin thus appears necessary for the integrity of these cells. This was nicely shown by Dowling and Gibbons (1961) when they deprived rats of vitamin A to watch the visual changes, but as vitamin A deprivation results in systemic symptoms as well, they maintained their animals on retinoic acid which can be used instead or retinol as a precursor in all tissues other than the retina. After prolonged retinol deprivation, irreversible changes occurred in the outer segments of both rods and cones.

The retinal to protein linkage through nitrogen, as indicated above, has become widely accepted. However, a very recent paper by Poincelot _et_ _al_. (1969) suggests that in the native state of rhodopsin the 11-cis retinal may be bound to a lipid phosphatidyl ethanolamine

---

[60] Radding, C.M. and Wald, G., "Acid-base properties of rhodopsin and opsin," (1956) _J_. _Gen_. _Physiol_., _39_, 909-922.

moiety of rhodopsin, and that it may migrate to the protein in the course of bleaching. This very interesting possibility still awaits further confirmation.

G. Wald's Visual Cycle

When it strikes the visual pigments, light effects a series of chemical changes, some of which are even observable at room temperature. Because of what has been shown to be the complex nature of the changes involved, the early work is somewhat confusing, and the review by Dartnall (1957) tries to summarize it in as orderly a fashion as is practicable. The means by which most of the currently known intermediates were isolated, namely examination of preparations at low temperatures at which labile species decay less rapidly and hence persist long enough to be detected, was first successfully employed by Lythgoe (1937). He divided a solution of frog visual pigment into two aliquots, maintaining one at room temperature and placing the other in ice. He then exposed both to light and noted that the solution at ambient temperature turned yellow while the cold solution turned orange and only slowly faded to yellow if kept cold, although it rapidly faded if warmed. As Broda and Goodeve (1941) later showed, bleaching is arrested at the transient orange stage indefinitely if the solution is kept sufficiently cold ($-70^{\circ}C$). Nevertheless, Lythgoe (1937) has discovered "transient orange" to be an intermediate between "visual purple" and "indicator yellow" (with a λ max of 440 nm in acid solution and "below 400" in alkali (Lythgoe, 1937). Wald (1937) had also noted the red-to-orange to yellow transformation of visual purple when it was exposed to light. The following year Lythgoe and Quilliam (1938a) obtained the spectrum of "transient orange" at 3° C and also demonstrated that it was a precursor of "indicator yellow".

Some years later, Bliss (1948) showed that indicator yellow in acid solution further decomposed into retinal and protein[61], and that the retinal further became vitamin A in fresh preparations if the pH was between 5.5 and 8. He thus concluded this last reaction was enzymatic because of its pH optimum and requirement for fresh preparations. [The identification of retinene as vitamin A aldehyde (shown by manganese dioxide oxidation of vitamin A by Ball et al., 1946, 1948) and its subsequent renaming to retinal has already been discussed.]

The changes which occur during and after the photolysis of rhodopsin have been exhaustively studied and reviewed[62], but there are still many aspects which are not settled, especially those regarding whether or not certain substances are actually intermediates or whether they are merely side products off the main reaction pathway. Accordingly, in describing the reactions of rhodopsin in the visual cycle, one must make a choice as to whose

---

[61] The nature of retinal was not known at that time. It was, in fact, called retinene, and identified since its λ max of 385 nm was different from that of acid indicator yellow, which was 440 nm.

[62] Dartnall, H.J.A., op. cit., (1962b), 427-471; Abrahamson, E.W. and Ostroy, S.E. "The photochemical and micromolecular aspects of vision," (1967), Progress in Biophys. and Molecular Biology, 179-215; Matthews, R., et al., op. cit., (1963-1964), 215-240.

scheme to follow. I have chosen that of the Harvard group: Wald, Hubbard, Brown and their colleagues, from whose laboratories so much of the early and current contributions have come. I have also, however, endeavored to point out several of the places in the cycle where other investigators would make revision and to correlate this scheme with the partial scheme of Lythgoe and Quilliam (1938a):-

$$\begin{tikzcd}
\text{Rhodopsin (498 nm)} \arrow[r] & \text{Transient orange (465 nm)} \arrow[r] & \text{Acid indicator yellow (440 nm)} \arrow[r] \arrow[d, shift right] & \text{Trans retinal (387 nm)} \\
& & \text{Alkaline indicator yellow (365 nm)} \arrow[u, shift right] &
\end{tikzcd}$$

"Under physiological conditions, the only thing that light does in vision is to isomerize the visual pigment chromophore from the 11-cis to the all-trans configuration. This corresponds to the conversion of rhodopsin to prelumirhodopsin. All the subsequent steps are thermal reactions that occur equally well in light or in darkness"[63]. The first product (which has a λ max of 543 nm at -193°C), called pre-lumirhodopsin by Yoshizawa and his colleagues (Yoshizawa and Kito, 1958; Yoshizawa, Kito and Ishigami, 1960: Kito _et_ _al_., 1961) at Osaka University, to occur between rhodopsin and the previously known intermediate lumirhodopsin. It is believed (Yoshizawa and Wald, 1963) that this represents all-trans retinal in combination with opsin as the protein exists in rhodopsin. As is the case for retinal itself (Jurkowitz _et al_., 1959), the isomerization of rhodopsin (11-cis) to pre-lumirhodopsin (all-trans)

---

[63] Hubbard, R., _et_ _al_., _op_. _cit_., (1965), 301-315.

can go on in a rigid solvent (Yoshizawa and Wald, 1963) to some extent.

While it has been assumed that the major function of light is to isomerize the retinal (Hubbard, Bownds and Yoshizawa, 1965) with little or no change in the conformation of the protein, opsin, Yoshizawa and Wald (1963) have suggested that some of the energy of the absorbed photon may cause small local changes in the protein configuration to occur in the succeeding steps, with the unwinding of the protein, as Hubbard and Kropf (1959) and Erhardt and Ostroy (1966) picture it.

Since the protein has not changed significantly, and since light effects the isomerization of the chromophore, rhodopsin (11-cis), pre-lumirhodopsin (all-trans) and isorhodopsin (9-cis) are all intercovertible by light. Since their absorption maxima are different, illuminating with light at the absorption maximum of any one converts it into a mixture of the other two (Yoshizawa and Wald, 1963).

While pre-lumirhodopsin is considered by some to be a physiological intermediate, the role, if any, of isorhodopsin has been debated. As mentioned earlier, the 9-cis isomer of retinal is very bent, being second only to 11-cis retinal in this characteristic. Thus, it may fit the opsin almost as well as 11-cis. The λ max of isorhodopsin is 487 nm instead of the 498 nm which is characterisitic of cattle rhodopsin. Most authorities believe, however, that isorhodopsin is only an artifact, having no physiological role, and that it does not exist *in vivo* under normal conditions[64].

On warming a solution of pre-lumirhodopsin above -140° C, it undergoes a thermal decay to lumirhodopsin (λ max 497 nm at -50° C), first observed by Broda and Goodeve (1941) and named by Wald, Durell and St. George (1950). In this study, those authors cooled a rhodopsin solution to -45° C and measured the spectrum after first taking a spectrum at 23° C. They then exposed it to light, maintaining the temperature at -45° C and obtained the spectrum of lumirhodopsin. The solution was then warmed to -15° C and recooled, at which time the spectrum was once again taken. This proved to be the spectrum of the intermediate, metarhodopsin (λ max 478 nm), because lumirhodopsin decomposes rapidly when warmed above -40° C (See Figure 4). Because of subsequently finding even more intermediates, the above metarhodopsin was subsequently renamed metarhodopsin I[65].

Although most investigators agree that pre-lumirhodopsin, lumirhodopsin and metarhodopsin I are in the main line of reactions, there are several theories regarding the nature of these substances. (See footnote)[66] Briefly, Hubbard and the Harvard

---

[64] Hubbard, R. and Kropf, A., op. cit., (1958), 130-139; Dowling, J.E., and Hubbard, R., "Effects of brilliant flashes on light and dark adaptation," (1963), Nature, 199, 972-975; Yoshizawa, T. and Wald, G. "Transformation of squid rhodopsin at low temperatures," (1964), Nature, 201, 340-345.

[65] Matthews, R., et al., op. cit., (1963-1964), 215-240.

[66] Abrahamson and Ostroy (1967) suggest that pre-lumirhodopsin may not be the primary intermediate at room temperature because it is thought to be non-planar, twisted species, and the torsional potential curve is probably temperature dependent. Thus, in contrast to the Harvard group, they conclude that it is probably not a physiological intermediate, although they also agree that isomerization of retinal is the first step in the visual process.

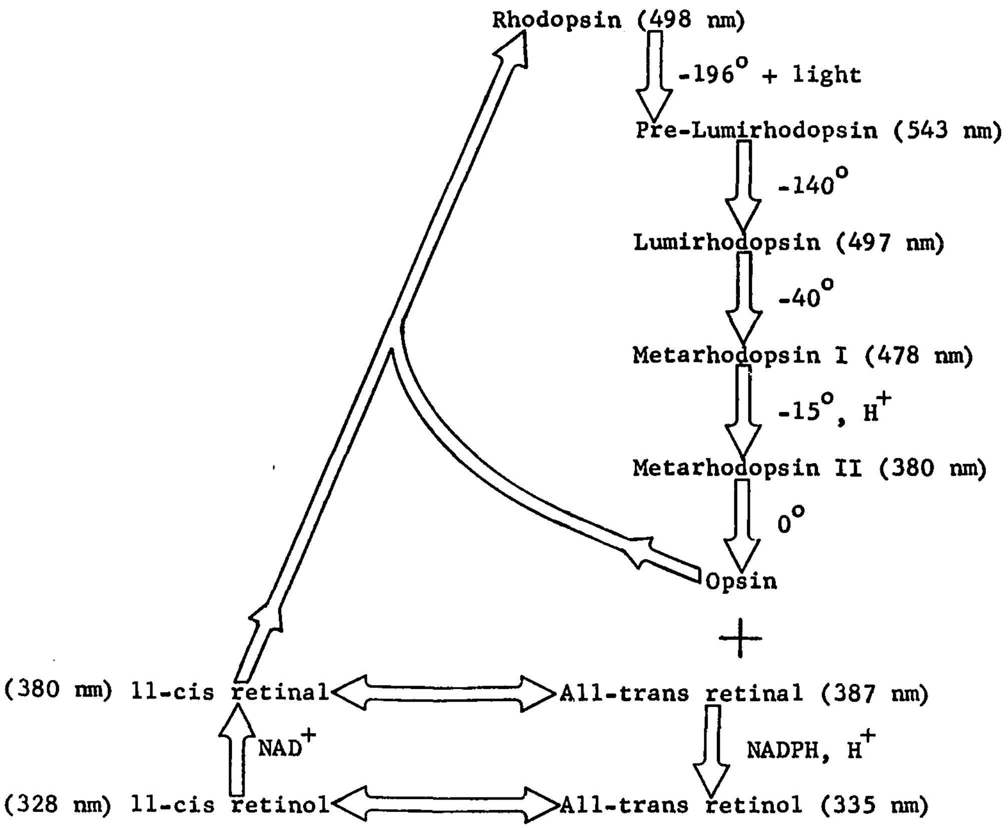


FIGURE 4

Photolysis of rhodopsin, scheme
of
Matthews, Hubbard, Brown and Wald

School[67] believe that lumirhodopsin consists of opsin in combination with all-trans retinal, the inital light reaction having isomerized the labile 11-cis configuration to predominantly all-trans retinal. Hagins (1957), on the other hand, postulated that lumirhodopsin is a complex mixture of stereoisomeric lumirhodopsins rather than mostly all-trans. (He spoke of lumirhodopsin and not pre-lumirhodopsin, since pre-lumirhodopsin had not been discovered at that time.)

Wulff *et al*. (1958) have shown from flash photolysis experiments at room temperature on cattle rhodopsin that the earliest detectable species after exposure has a λ max of 486 nm and consists of several fractions, one of which decomposes to indicator yellow in 100 microseconds, a second in 11 milliseconds, a third in 100 milliseconds, and a fourth in about an hour, leaving a fifth stable fraction which Wulff considers to be a mixture of rhodopsin (11-cis) and isorhodopsin (9-cis). The four unstable products, he feels, represent the other possible isomers of retinal (all-trans, 13-cis, 11,13-dicis, 9,13-dicis) with opsin, thus implying that the product of the light flash is a mixture of isomers, as proposed by Hagins (1957). Wulff, nevertheless, acknowledges that it is still possible that (as the Harvard group postulates) the *initial* effect of light is to produce the all-trans species, from which the other isomers are produced by further

---

[67] Hubbard, R., "Bleaching of Rhodopsin by light and by heat", (1958b), *Nature*, *181*, 1126; Hubbard, R. and Kropf, A., *op*. *cit*., (1958), 130-139; Hubbard, R., *et*. *al*.; Hubbard, R. Brown, P.K. and Kropf, A., "Action of light on visual pigments, vetebrate lumi and metarhodopsins", (1959), *Nature* *183*, 442-446.

isomerization due to the bright (65 joules) light flash during the 20 microseconds of exposure. In summary, then, this study, while interesting, is compatible with both the theory of Hubbard and her co-workers and that of Hagins. The previously noted demonstration of Yoshizawa and Wald (1963) that rhodopsin and isorhodopsin are inter-convertible at liquid nitrogen temperature only through pre-lumirhodopsin is, however, consistent with pre-lumirhodopsin being, as Wald believes, predominantly all-trans and not a mixture.

Although Wald and his associates initally thought that metarhodopsin decayed directly into all-trans retinal and opsin[68], as demonstrated by the scheme shown in Figure 4, they expanded their cycle in 1963 to include a pigment absorbing at 380 nm, metarhodopsin II. This pigment is thought to have picked up a hydrogen ion from the surrounding medium and is called acid metarhodopsin, the hydrogen being bound by a group with pK 6.6, as will be discussed shortly. In the course of bleaching, the protein unwinds, resulting in a change in isoelectric point (Radding and Wald, 1956) and in exposure of two to three sulfhydryl groups (Wald and Brown, 1952) or even four[69] and one acid binding group with a pK of about 6.6[70]. Radding and Wald propose that this

---

[68] Wald, G., Durell, J. and St. George, R.C.C., "The light reaction in the bleaching of rhodopsin", Science 111, 179-181; Kropf, A. and Hubbard, R., "The mechanism of bleaching rhodopsin," (1958), Ann.N.Y. Acad. Sci., 74, 266-280.

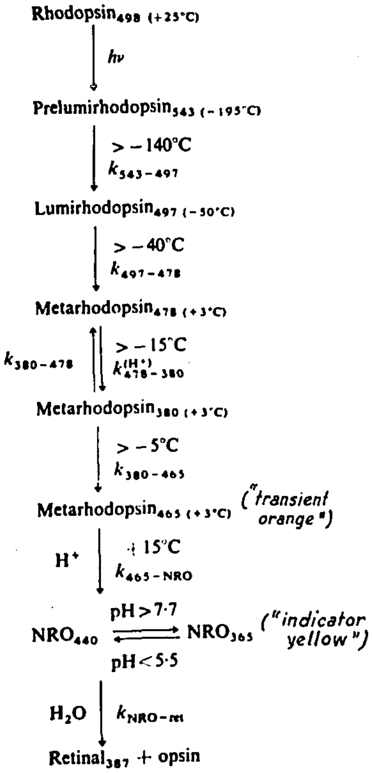
[69] Ostroy, S., Rudney, H. and Abrahamson, E.W., "Sulfhydryl groups of rhodopsin," (1966a), Biochim. Biophys. Acta, 126, 409-412.

[70] Radding, C.M. et al., op. cit., (1956), 909-922.

may be due to the exposure of a histidine residue, since this pK is close to that of the imidazole group of histidine. The exposure, during bleaching, of groups on the protein which previously had not been titrable is also consistent with the previously mentioned analogy between bleaching and partial denaturation (e.g. Mirsky, 1936). Recently, Wald (1964a, as cited by Rosenberg, 1966) found that the SH groups were not yet detectable at the metarhodopsin stages, therefore concluding that the exposure of sulfhydryl groups "...cannot play a part in visual excitation." Accordingly, they will not be considered in the later discussion of visual excitation.

Matthews _et_ _al_. (1963) (Figure 4) believe that metarhodopsin II decays directly to all-trans retinal and opsin, with a product having a λ max of 465 lying on a side pathway. In contrast, Ostroy _et_ _al_., (1966) include metarhodopsin 465 in the main pathway--their chief reason for so doing is that it corresponds most closely to the "transient orange" of Lythgoe and Quilliam (1938a). These authors also include on the main pathway N-retinilidene opsin, which corresponds to Lythgoe and Quilliam's "indicator yellow." (See Figure 5) This view is shared by most workers outside of Harvard, viz. Collins (1953), Morton and Pitt (1955), Bridges (1962), and Dartnall (1957).

In summary, then, Lythgoe and Quilliam's two intermediates, "Transient orange" and "indicator yellow" correspond most closely with the two last intermediates in the cycle before cleavage of retinal from opsin occurs: metarhodopsin$_{465}$ and N-retinilidene



Photolysis of Rhodopsin, Scheme of Ostroy et al.

FIG. 5

opsin respectively. The N-retinilidene opsin then decomposes to opsin and all-trans retinal.

Looking at the above series of changes from the point of the tertiary configuration of the protein, it has already been noted that the conversion of rhodopsin to prelumirhodopsin results in very little, if any, change in protein configuration[71]. However, as Williams (1964) points out, the all-trans isomer does not fit well with opsin, apparently rendering the protein structure unstable and resulting in eventual "denaturation". From prelumirhodopsin on, each intermediate is believed to contain a protein whose structure is slightly more opened. Hubbard *et al.* (1965) feel that the transition from prelumirhodopsin to lumirhodopsin probably involves only minor configurational changes, since it is assumed that major configurational changes of proteins are accompanied by large positive heats and entropies of activation[72], and the heat and entropy of activation for the reaction of pre-lumirhodopsin going to lumirhodopsin are only 10 kilocalories per mole and 0 entropy units, respectively[73]. On the other hand, the change from lumirhodopsin to metarhodopsin I

---

[71] Yoshizawa, T. and Wald, G., "Pre-lumirhodopsin and the bleaching of visual pigments", (1963), *Nature*, *197*, 1279-1286; Erhardt, F., Ostroy, S.E. and Abrahamson, E.W., "Protein configuration changes in the photolysis of rhodopsin, I: The thermal decay of cattle lumirhodopsin *in vitro*," (1966), *Bioch. Biophys. Acta*, *112*, 256-264.

[72] Stearn, A.E., "Kinetics of biological reactions with special reference to enzymic processes,", (1949), 25-74; Lumry, P. and Eyring, H., "Conformation changes of proteins,", (1954), *J. Phys. Chem*, *58*, 110-120.

[73] Grellman, K.H., Livingston, R. and Pratt, D., "A flash photolytic investigation of rhodopsin at low temperatures," (1962), *Nature*, *193*, 1258-1260; Pratt, D.C., Livingston, R. and Grellmann, K.H., "Flash photolysis of rod particle suspensions," (1964), *Photochem. and Photobiol.*, *3*, 121-127.

is characterized by a 60 kcal heat of activation and an entropy change of 160 e.u.[74]. These authors, accordingly, feel that most of the unwinding occurs at this step, pointing out that the heat and entropy of activation of this reaction are about as large as those measured for the thermal denaturation of opsin (Hubbard, 1958a). Indeed, the fact that opsin is much more susceptible to denaturation than rhodopsin, both by heat (Hubbard, 1958a) and by exposure to extremes of pH (Radding and Wald, 1956), is taken as further evidence that the 11-cis retinal somehow holds the protein together and in a closed configuration in rhodopsin.

Most of these protein configurational changes cannot occur at very low temperatures, and Hubbard and Kropf (1959) point out that when the cis chromophore is isomerized to the all-trans in rigid solvent, "...the configuration of the protein, although unstable, is unable to rearrange until the temperature is increased sufficiently to render the system more fluid."[75] Hubbard *et al.* (1959) have also pointed out in support of the fact that low temperature blocks the rearrangement of opsin, that rhodopsin and lumirhodopsin are readily intercovertible by light at -65°C, as are rhodopsin and metarhodopsin at -20°C, but irradiation of metarhodopsin at -65°C does not yield rhodopsin. This also implies that there is more internal rearrangement between lumirhodopsin and metarhodopsin than between rhodopsin and lumirhodopsin.

---

[74] Hubbard, R., *et al.*, *op. cit.*, (1965), 301-315.

[75] Hubbard, R., *et. al.*, *op. cit.*, (1959), 442-446.

To recapitulate, the rhodopsin molecule absorbs a quantum of light, the chromophore is isomerized, and the rhodopsin is then converted to a series of intermediates all bearing the all-trans configuration of retinal, viz pre-lumirhodopsin, lumirhodopsin, metarhodopsin I, metarhodopsin II, perhaps metarhodopsin$_{465}$ (III) and N-retinyl opsin, this latter being split to all-trans retinal and opsin. The intermediates listed above were all isolated at low temperature, and each succeeding intermediate was identified as the sample was warmed to a higher temperature and its spectrum remeasured. As Hubbard _et al_. (1965) note (p. 301), "Although these intermediates are usually observed at low temperatures in rhodopsin solutions, there is no reason to assume that bleaching occurs differently in the retina and at physiological temperatures."[76] That these same substances are true intermediates at room temperature and not merely artifacts of the low temperatures employed has been shown for at least some of them by two techniques. First, Wald _et al_. (1950) incorporated rhodopsin into a dried gelatin film and examined it at room temperature. After taking an initial spectrum of the film (rhodopsin), it was exposed to a brief flash of light and the spectrum taken again one minute after the exposure. This spectrum was similar to that of lumirhodopsin. After one hour more in darkness, the spectrum was again recorded, and Wald called this spectrum "metarhodopsin" since it was similar to that of metarhodopsin I (metarhodopsin II

---

[76] Hubbard, R., _et_. _al_., _op_. _cit_., (1965), 301-315.

had not yet been described). The film was then soaked in buffer solution for ten minutes and redried, after which its spectrum was once again recorded. At this point it appeared to approximate that of retinal and opsin.

Flash photolytic studies of bleaching of rhodopsin in solution and of rod outer segment suspensions have shown that metarhodopsin I is detectable at temperatures above 0° C[77], and that very low temperatures are not necessary to detect prelumirhodopsin and lumirhodopsin, both of which are detectable at -25° C[78]. Hubbard _et al_. (1965) also feel that Grellman _et al_. (1962), whose work was done before the identification of prelumirhodopsin, probably observed this intermediate at temperatures of -25° C and below in their flash photolytic studies.

Once the retinal is liberated from its linkage with the opsin, it may either be isomerized to 11-cis retinal and recombined spontaneously to reform rhodopsin, or it may be reduced to vitamin A, which in turn may be isomerized and reoxidized to retinal, which may then combine with opsin. Although the isomerization of retinal is much faster than that of retinol, presumably due to resonance involving the terminal carbonyl of the aldehyde, as noted earlier (Hubbard, 1956a), most of the carotenoid goes through the retinol

---

[77] Hubbard, R., _et_. _al_., _op_. _cit_., (1965), 301-315; Ostroy, D.S., _et_. _al_., _op_. _cit_., (1966), 578-580.

[78] Pratt, D.C., Livingston, R. and Grellman, K.H. "Flash pholoysis of rod particle suspensions" (1964), _Photochem_. _and_ _Photobiol_., _3_, 121-127; Hubbard, R., _et_. _al_., _op_. _cit_., (1965), 301-315.

pathway, because reduction is more rapid than isomerization. Much of the vitamin A is esterified in the eye (Krinsky, 1955), and it is not known whether or not this is the state of the retinol in which isomerization occurs, or whether this merely serves as a trapping mechanism for the 11-cis isomer, as will be discussed shortly. It is also not known where isomerization of the alcohol occurs, but it is likely that it occurs in the pigment epithelium (Dowling, 1960). The possibility also exists that there is some exchange of stereoisomers of retinol between the retina and the blood[79].

While it is known that light can reisomerize retinal, light has only a limited opportunity to do so in the eye (and light has even less of an opportunity to isomerize vitamin A in the eye, because its absorption lies further into the ultraviolet, and the vertebrate lens does not transmit much ultraviolet light. Although I have already pointed out that metarhodopsin is interconvertable with rhodopsin at temperatures above -20° C[80], this would obviously require a second quantum of light, the first having isomerized the rhodopsin to metarhodopsin. Hence, this could only occur under conditions of extremely high light intensity. Williams (1964, 1966) has shown under conditions of high light intensity that the fraction of rhodopsin in solution which is bleached by a single flash approaches 50% asymptotically. This is so because only species which

---

[79] Hubbard, R., and Wald, G., op. cit., (1952), 269-315; Wald, G., "Photochemical aspects of visual excitation," Exptl. Cell. Res. Suppl., 5, 389-410.

[80] Yoshizawa, T., et. al., op. cit., (1963), 1279-1286; Pitt, G.A.J., et. al., op. cit., (1960), 67-89.

have received an odd number of quanta go on to bleach. At physiological light intensities, however, he implies that the number of molecules receiving a second quantum would be quite small indeed. Nevertheless, both Williams (1964) and Hubbard and Kropf (1958) feel that even at high intensities, the quantum efficiency of vision "...probably depends only on the initial photoisomerization of rhodopsin to all-trans lumirhodopsin and should not be affected by the photochemical back reactions."[81] (This was written by Hubbard and Kropf before pre-lumirhodopsin was discovered, and would now probably be revised to substitute pre-lumirhodopsin for lumirhodopsin.)

Having ruled out light as unlikely, the probable mechanism for isomerization of all-trans retinal to 11-cis retinal is by means of enzymic catalysis. The enzyme, retinene isomerase, was first isolated by Hubbard (1955, 1956) from cattle and frog retinae. It specifically catalyzes the reaction between all-trans and 11-cis retinal and will neither act on nor produce other isomers. A difficulty lies in the fact that it does not seem to produce the 11-cis isomer fast enough to account for the rate of regeneration in the living eye. With both enzyme and light this discrepancy vanished, but Lewis (1957) has shown that the rate of regeneration of rhodopsin is the same in light as in darkness, and Rushton (1958) also feels that light does not affect the action of retinene isomerase under physiological conditions.

Although free retinal does not exist in the eye in large

---

[81] Hubbard, R., and Kropf, A., *op. cit.*, (1958), p. 138.

amounts, there are stores of 11-cis retinol (Krinsky, 1958a). Hubbard and Colman (1959) note that while the ratio of retinal isomers produced by retinene isomerase is 95% trans to 5% 11-cis, the 11-cis retinol in the retina dominates, and they therefore cite Krinsky's postulation of a trapping mechanism for it, e.g. selective esterification (Krinsky, 1958a). A reserve of 11-cis carotenoid also could help to account for the fact that rhodopsin synthesis is faster than the production of the 11-cis isomer by retinene isomerase. Rushton (1958) has shown that the demand for 11-cis retinal is such that any reserve would be rapidly exhausted, so that a mechanism for rapid reisomerization is still necessary. This is especially true when one considers cone pigment synthesis which is even faster than rhodopsin synthesis[82]. Morton and Pitt (1960) conclude that, "The most plausible assumption is that, in the eye, retinene isomerase is working without photoactivation and that *in vivo* it works more rapidly than in Hubbard's (1956a) simplified *in vitro* system."[83]

The interconversion of retinol and retinal is catalyzed by retinene reductase (alcohol dehydrogenase), which employs DPNH (NADH) and TPN (NADP) (Futterman, 1965) as coenzymes. The oxidation-reduction reaction is nonisomerizing and hence 11-cis retinol is oxidized to 11-cis retinal, which can spontaneously combine with opsin

---

[82] Wald, G., *et. al.*, *op. cit.*, (1954-1955), 623-681; Wald, G., Brown, P.K. and Kennedy, D. "The visual system of the alligator", (1957), *J. Gen. Physiol*, *40*, 703-713; Wald, G., *op. cit.*, (1958), 222-226.

[83] Hubbard, R., *op. cit.*, (1956), 935-962.

to form rhodopsin. This regeneration is spontaneous and energy yielding[84] occurs optimally around pH_6,[85] and is inhibited by p-chloromercuribenzoate and other substances which react with sulfhydryl groups[86] (Brown and Wald, 1952; Wald, 1958). The recombination is second order as expected, since it involves the comibination of two reactants[87]. With this reaction complete, the cycle has gone all around; the rhodopsin is resynthesized, again ready to undergo photolysis.

Possible Mechanisms of Isomerization

Although I have described the entire visual cycle, the step which is of primary interest to this study is the first one: the isomerization of the 11-cis chromophore to all-trans (assuming Wald's hypothesis that prelumirhodopsin is predominantly all-trans). In this step the two substituents on either side of the 11-double bond begin cis to each other, and rotation occurs such that they become trans to each other. Ordinarily, the energy barrier to rotation about a double bond is such that free rotation does not occur unless some of the double bond character is lost. This system is

---

[84] Wald, G., and Brown, P.K., *op. cit.*, (1950), 84-92.

[85] Wald, G., and Brown, *op. cit.*, (1952), 797-821; Radding, C.M. and Wald, G., "Acid-base properties of Rhodopsin and opsin," (1956), *J. Gen. Physiol.*, *39*, 909-922.

[86] Wald, G., *op. cit* (1958), 389-410, Brown and Wald (1952).

[87] Hubbard, R., *et. al.*, *op. cit.*, (1952), 269-315; Wald, G., *et. al.*, *op. cit.*, (1957), 703-713; Wald, G. and Brown, P.K. "Human rhodopsin,", *Science*, *127*, 222-226.

no exception[88].

If some energy were absorbed by a molecule of retinal, an electron might be promoted to a higher, perhaps nonbonding orbital, such as a triplet state; or it might be completely driven from the molecule, thus forming a free radical. In either case, the bond to which the electron made a bonding contribution would lose some or all of its double bond character.

It has been found that light incident on beta-carotene results in free radical production (Smaller, 1960). Since results of such a study with retinal, have never been published, the possibility that retinal on illumination might also form a free radical appeared a reasonable one, because retinal is really one-half beta-carotene with an oxidized terminal group, and rhodopsin contains this same molecule with further modification of its terminal group. Hence, free radical formation, if it occurred readily, might be the mechanism (or a mechanism) through which isomerization occurs, both *in vitro* and *in vivo*.

Several investigators became interested in the possible existence of a retinal triplet because of the long lived triplet which has been demonstrated in chlorophyll, another biological molecule whose function is to interact with light. In chlorophyll, an excited triplet with a half-life of about 10^{-3} seconds has been identified by flash

---

[88] Hubbard, R. and Kropf, A., "Molecular isomers of vision, *Scient. Amer.*, *216 (6)*, 64-76.

photolysis[89].

Flash photolysis of retinal in non hydroxylic solvents has been carried out by Abrahamson *et al*. (1959, 1960) and Grellman *et al*. (1962a), and an intermediate absorbing at 450 nm with a half-life of 10^{-5} seconds has been demonstrated. It was felt to be a triplet because of its rapid quenching with oxygen. Its extinction coefficient was determined by Dawson and Abrahamson (1962) to be 80,000, but they also noted that the quantum yield for its production was only 11%. Because the formation constant of the hydrogen-bonded complex between retinal and hydroxylic solvents like water and methanol was essentially the same as that for the complex between triplet-excited retinal and hydroxylic solvent, it was concluded that the electron distribution about the carbonyl oxygen in the two cases was the same and the triplet must therefore have been $^3(\pi,\pi^*)$[90]. Flash photolysis of retinol, however, produced no such triplet (Dawson, 1962). Nevertheless, the production of triplet from retinal makes this a possible pathway for lessening the double bond character which is probably necessary for isomerization. Abrahamson and Ostroy (1967) note that its low quantum

---

[89] Livingston, R. and Ryan, V. A., "The phototropy of chlorophyll in fluid solutions," (1953), *J. Am. Chem. Soc.*, *75*, 2176-2181; Abrahamson, E. W. and Linschitz, H., "Reversible flash bleaching of chlorophyll," (1955), *J. Phys. Chem.*, *23*, 2198-2199; Linschitz, H. and Sarkanen, K., "The absorption spectra and decay knetics of the metastable states of chlorophyll A and B," (1958), *J. Am. Chem. Soc.*, *80*, 4826-4832.

[90] Abrahamson, E. W. and Ostroy, S. E., "The photochemical and micromolecular aspects of vision," (1967), *Progress in Biophysics and Molecular Biology*, 179-215.

efficiency (around 11% as found by Dawson and Abrahamson) in light of the high quantum yield of the photobleaching of rhodopsin (around 0.5-0.6) would appear to rule out participation of the triplet state. Nevertheless, these studies were carried out in retinal, and its intricate binding with opsin may well change some of the factors affecting quantum yield.

Hubbard (1966) studied several thermodynamic properties of the stereoisomerization reaction of 11-cis retinal, and found E_a (Arrhenius activation energy) to be between 22.4 and 26.2 kcal/mole and A (the Arrhenius frequency factor) to be between 10^8 and 10^{11} sec^{-1}. She noted that in the presence of iodine, the frequency factor increased but there was little change in the activation energy. This lack of change in the activation energy, she noted, probably implies that iodine does not, as previously thought (Hubbard and Wald, 1952), act by forming a frank addition product. Rather, it is more likely to act by forming $I^{\bullet}$ radicals, which she notes would be more likely to form in the light, thus explaining the increased effectiveness of iodine as a catalyst for this reaction in light. Comparing the isomerization of retinal with that of ethylene, Hubbard (1966) notes that Magee _et al._ (1941) have interpreted the data of Kistiakowsky and Smith (1934, 1935, 1936) on the stereoisomerization of ethylene as implying two alternative pathways of isomerization - "One with an activation energy (E_a) of about 45 kcal $mole^{-1}$ and a frequency factor (A) of about 10^{12} sec^{-1} is assumed to proceed...in a singlet electronic state, whereas the other, with values of E_a of the order of 25 kcal $mole^{-1}$, is assumed to involve an uncoupling of the spins of the π-electrons to a triplet state, which accounts for the low values of A (about 10^5 sec^{-1}) encountered in this class of stereoisomerizations.[91] Hubbard goes on to point out that her data do not yield an unequivocal assignment of reaction mechanism either, since if one assumes a triplet mechanism, the values of A are too high, and if one assumes a singlet mechanism, the values of E_a are too low. Abrahamson and Ostroy (1967), on the other hand, interpret her data as implying that the isomerization is occurring via the triplet state because of the large negative value of the entropy of activation (-21.4 to -10.0 e.u.), which they state is characteristic of triplet isomerization, citing the work of Mullikan (1932); and the cis-trans isomerization of several 2-olefins by light has been shown to proceed via a triplet state[92].

The mechanisms of isomerization of retinal by heat and light in the absence of iodine may or may not be identical as in its presence (Hubbard, 1966), and other investigators (Abrahamson and Ostroy, 1967) feel that they are indeed different. Obviously, in the rod outer segment the isomerization is not catalyzed by iodine. As noted earlier, with or without catalysis, each bond is isomerized independently of the others (Zechmeister and Pinckard, 1954) and each bond has its own excited state[93].

---

[91] Hubbard, R., "The stereoisomerization of 11-cis retinal," (1966), J. Biol. Chem., 241, 1818-1841.

[92] Golub, M.A., Stephens, C. L. and Brash, J. L., "Photo-and radiation--induced cis trans isomerization of several 2-olefins," (1966), J. Chem. Phys., 45, 1503-1505.

[93] Hubbard, R. et al. op. cit. (1965), 301-315.

Since the triplet state is known to be a precursor of free radicals in some systems[94], triplet and free radical pathways are not mutually exclusive. It is possible that an electron could be promoted to a triplet orbital, leaving a low lying positive hole, which may thus serve to trap available free electrons in the system. Chemically, this would result in formation of a free radical anion, a possibility which will be dealt with later in the discussion.

Previous Investigation

The possibility that a free radical might be produced when light excites the rhodopsin molecule is not a new thought; it had been considered by Wald *et al*. (1950) and Collins and Morton (1950). Looking for such a free radical, Polis and Wyeth (1962) were able to demonstrate faint electron paramagnetic resonance (EPR) signals from rhodopsin solutions in 2% aqueous digitonin and from rod outer segments suspended in 0.3 molar sucrose *at room temperature*, employing a computer of average transients (CAT) to summate several hundred time curves in order to bring the signal out of the background noise. Even then, however, they considered the signal too weak to scan an actual electron paramagnetic resonance spectrum[95].

---

[94] Sogo, P.B., Pon, N.G. and Calvin, M., "Photo spin resonance in chlorophyll-containing plant material," (1957), *Proc. Nat. Acad. Sci.*, *43*, 387-393; Ilten, D. F. and Calvin, M., "Correlations between photoinduced EPR and photoconductivity in TCNE-THF solution charge transfer complex," (1965), *J. Chem. Phys.*, *42*, 3760-3766.

[95] At a recent conference (10/68) Polis indicated that this finding was not reproducible, and the signal may have come from eye melanin.

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. I, 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 determinging 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 may be more easily detected.

This approach had been tried unsuccessfully (and therefore not published) by Lockhurst and Abrahamson (1965, cited in Abrahamson and Ostroy, 1967). They employed purified solutions of rhodopsin and rod segment suspensions, (but not pure carotenoids) and were unable to detect an EPR signal after illumination at liquid nitrogen and dry ice temperatures. Similar negative results were obtained by Pitt and Tinkham (1965) emplying freeze-dried preparations or rod outer segments illuminated at 90°K. Hence, the question of whether a free radical could be elicited from rhodopsin and rod outer segments was unsettled, Polis and Wyeth (1962) believing that a faint signal was obtainable, Pitt and Tinkham (1965) and Lockhurst and Abrahamson (1965) having had negative results. In any case, if a signal occurred in rod outer segments on illumination, it was faint indeed.

As pointed out above, however, this does not make a free radical unlikely, since if it were short-lived and nontrapable, the concentration might never build up to detectable levels. Also, recalling that rhodopsin is a protein, its molar concentration is not very high in digitonin extracts nor in rod outer segment suspensions. Neither of the sets of authors cited above report the concentrations which they employed.

While a free radical signal from rhodopsin and rod outer segments was difficult for these authors to detect at liquid nitrogen temperature (ca. 77°K), Smaller (1960) had no such difficulty in demonstrating the production of a free radical on illumination of beta-carotene under similar conditions with an incandescent light, as previously noted. He employed solid beta-carotene and a frozen 0.1% solution in carbon tetrachloride and noted an EPR signal which appeared in light and grew rapidly. It could only be destroyed by dissolving the crystalline beta-carotene (e.g. in methanol), and upon redrying the samples' photosensitivity was restored. The frozen solution lost its signal on annealing. Those studies are of interest since, as noted earlier, beta-carotene is a "dimer of retinal" with the aldehyde group split out.

Rosenberg (1958, 1959, 1961a, 1961b) was able to show that melted beta-carotene between two conductive glass plates increased its conductivity when irradiated through the positive electrode. This photoconductivity was found to be greater in the hindered-cis isomer of beta-carotene than in the unhindered cis isomers, and these, in turn, are more photoconductive than the all-trans isomer

(Rosenberg, 1961b), the actual photoconductivity ratios being $10^4:10^2:1$, respectively (Rosenberg, 1966). Having determined that beta-carotene is photoconductive, Rosenberg, working with Orlando and Orlando (1961), noted that dried sheep rods exhibited similar photoconductivity which diminished on continued light exposure. Photoconductivity was also noted in rhodopsin by Hara (1963).

Rosenberg believes that in his system positive holes are the dominant carriers of charge, because the photocurrent is 10^5 times as large (Rosenberg, 1966) when the positive electrode is irradiated than when the negative electrode is irradiated. He further believes that excitation of the molecule to the triplet state is "a kinetic intermediate step in the process leading to charge carrier production"[96]. When the positive electrode is irradiated, the action spectrum for photoconductivity conforms to the absorption spectrum of whatever is between the plates: beta-carotene, retinal, or rod outer segments; but this relationship to the absorption spectrum does not hold if the negative electrode is irradiated.

Interestingly, Rosenberg and Harder (1967) found that the average photoconduction activation energy for crystalline 13-cis and 11-cis retinal were essentially identical, within experimental error, being 0.25 and 0.24 electron volts respectively, and that for 13-cis retinal m-nitroaniline hydrochloride (a rhodopsin model in which the retinal is combined in a Schiff base linkage) was considerably lower, i.e. 0.15 electron volts. They found all-trans

---

[96] Rosenberg, B., "A physical approach to the visual receptor process," (1966), Adv. in Radiation Biology, 2, 193-241.

and 9-cis retinal less conductive by orders of magnitude, a fact which will be dealt with in the discussion.

Retinals possess yet another interesting property when irradiated with visible light. Jurkowitz et al. (1959) had observed that when they irradiated solutions of all-trans retinal at -185° to -192°C in ether, isopentane, ethyl alcohol (5:5:2) (EPA) or ether, isohexane, ethyl alcohol (5:5:2) (EHA) glass the peak extinction at 387 nm fell as much as 80% after one hour of irradiation, and two smaller peaks arose at 350 nm and 412 nm. 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. The whole process, however, was repeatable if the retinal was recooled and reilluminated. Jurkowitz postulated that the spectral change was due to production of a metastable state, possibly a pair of radicals or ions.

Further suggested evidence for the production of a free radical on irradiation of carotenoids with visible light was the finding of Mousseron-Canet _et_ _al_. (1966) that a 60% yield of dimer is produced upon irradiation of retinol in hexane with light at 325 nm.

The above experiments - Rosenberg's photoconductivity, Mousseron-Canet's dimer, Jurkowitz' frozen-in labile species and Smaller's finding of a free radical on illumination of beta-carotene--all made the author feel that the likelihood of finding a free radical on illumination of retinal and retinol was high. Since free radical production from these substances had not previously been reported, and the results of searches for free radicals in rhodopsin were equivocal, it was felt that if a free radical could be identified from these pure substances, one might learn which conditions were best suited for its demonstration and might apply this knowledge to the less ideal and more complex rhodopsin system. The biological implications of a free radical--especially a charged free radical--product of rhodopsin bleaching are considerable, and will be carefully considered in the discussion.

METHODS AND MATERIALS

Solutions of 1 mM (chosen on the basis of the EPR signal) all-trans retinal (purchased from Sigma, St. Louis, Mo., lot No. 116B-0440) were made up in heptane, carbon tetrachloride, acetone, acetonitrile, ethanol and diethyl either (all reagent grade) and 2% aqueous digitonin (also purchased from Sigma, lot No. 96B-1670). The retinal was stored in the dark at -40°C in sealed ampules under nitrogen, and was used shortly after opening. Since digitonin is rather insoluble in water, and the small amount which will dissolve goes into solution slowly, it is necessary to boil the water in order to get it into solution more rapidly. 11-cis retinal was not available to us, but 1mM solutions of 9-cis and 13-cis retinal (Sigma, lot No. 25B-1670 and 35B-0720 respectively) were also made up in heptane. All-trans vitamin A (retinol) (Sigma, lot No. 115B-1390) was dissolved in acetone and acetonitrile to a final concentration of 0.1M. All of the above solutions were carefully flushed with nitrogen for ten minutes before examination, because Abrahamson _et al_. (1959) had shown that oxygen quenches the triplet state of retinal.

The rhodopsin preparations were made by the method of Collins et al. (1952). Fresh cattle eyes within one half hour of slaughter were obtained from the Insel and Insel Company of Newark, New Jersey. For each preparation, retinae were used from fifty cattle eyes which had been iced and dark adapted after enucleation (since Tronche _et al_. (1965) had demonstrated that rhodopsin resynthesis in enucleated cattle eyes was maximal if the eyes were kept in the dark thirty to ninety minutes post mortem). All further processing occurred in darkness or red-light illumination. The retinae were then lightly ground in an ice-cold mortar with 40% (w/v) sucrose-0.05 M Tris-HCl buffer, pH 7.2, 0.8 ml. per retina and passed ten times through a No. 15 needle (flattened at the end) to effect separation of the outer segments from the inner segments at the anatomical constriction.

The homogenate was layered under buffer in 40 ml. plastic ultracentrifuge tubes, forming a discontinuous gradient. The tubes were then spun at 8,000 RPM in a Beckman Spinco Model L ultracentrifuge for ten minutes, and the rod outer segments, which are isopycnic with about 30% sucrose (Collins et al., 1952) were collected at the interface between the plain buffer and the 40% sucrose buffer. These were then harvested by diluting 3:1 with sucrose-free buffer and recentrifuging at 14,000 RPM for twenty minutes. After decanting the supernatant, the rod outer segment pellet was suspended in 4% alum $KAlSO_4 . 12\ H_2O$ to render proteins other than rhodopsin permanently insoluble (Radding and Wald, 1956), and left overnight at 4° C. The suspension was then recentrifuged, and washed twice with buffer to remove water-soluble substances. Lastly, it was extracted with 2% aqueous digitonin solution overnight to extract the rhodopsin, and centrifuged, the final solution (supernatant) being about 9×10^{-6} M in rhodopsin, based on $\varepsilon \simeq 40,600$ for cattle rhodopsin[97].

---

[97] Wald, G. and Brown, P.K., op. cit., (1953), 180-200.

For some experiments, the rod outer segment pellet was suspended in the sucrose buffer and used without further extraction.

Although it possesses 54 titratable groups per molecule, 34 base binding and 20 acid binding (Radding and Wald, 1956), rhodopsin is insoluble in water. Like many other proteins, it is denatured by alcohol, acetone, and chloroform (Dartnall, 1962b). Digitonin ($C_{56}H_{92}O_{29}$) is a non-ionic detergent with molecular weight 1229.3, and was first used by Tansley (1931) to stabilize rhodopsin (although other substances have been used to make rhodopsin more soluble, e.g., bile salts by Kühne (1879), amyl- and cetyl-trimethylammonium -chloride and many other cationic detergents (Bridges, 1957). Digitonin, nevertheless, remains the most frequently employed, and was utilized in this study.

The form of the digitonin extract with which I worked was shown by Hecht and Pickels (1938) and Hubbard (1954) to have a molecular wight of approximately 260,000 to 290,000 by sedimentation studies in the ultracentrifuge, and as noted earlier, these particles contain one molecule of rhodopsin with approximately 200 molecules of digitonin.

Having thus obtained preparations of rhodopsin, 0.2 ml. aliquots of these and of the previously described solutions of the geometrical isomers of retinal and of all-trans retinol and of suspensions of rod outer segments were in thin quartz sample tubes of 3 mm inner diameter and 4 mm outer diameter. Although we were unable to do spectrophotometry on the rod outer segment preparation, its color (to the eye) was approximately as dense as that of the rhodopsin solutions. These tubes and their contents, in the case of the non-aqueous solvents, were then placed in a Varian liquid-nitrogen-filled dewar with a clear quartz finger through which the sample could be irradiated. Since the tubes frequently cracked when aqueous solutions were quickly frozen to -196° C, it was necessary to precool aqueous samples rapidly in a dry ice-acetone mixture before placing them in liquid nitrogen to prevent this cracking.

Solid samples of all-trans retinal and all-trans retinol were also examined, as were solutions containing both a carotenoid and an electron scavenger. Having found that a free radical can be elicited from the carotenoids, various electron acceptors known to form radical anions were dissolved with the carotenoid before freezing to observe if the free electron could be trapped by the acceptor and what the effect of this would be on the resultant EPR spectrum: would one see the (known) spectrum of the acceptor free radical anion? Would one see any change in the observed EPR spectrum of the carotenoid free radical? To this end acetone solutions 10^{-3} M in all-trans retinal and 4×10^{-3} M in chloranil (tetrachlorobenzoquinone) (Baker, lot No. 8-579) were examined in the same manner as has been described for the plain retinal and retinol solutions. A preliminary extension of this study was carried out employing other isomers of retinal beside the all-trans isomer, e.g. 9-cis retinal plus chloranil, 13-cis retinal plus chloranil, and all-trans retinol plus chloranil. Some very preliminary observations have also been made with other acceptors, viz. tetracyanoethylene (TCNE) (obtained from K and K Laboratories, lot No. 19182) and 1,4-naphthoquinone (practical grade, Distillation Products, lot No. P1704). In all cases in which a solution containing an electron acceptor and a carotenoid was brought to liquid nitrogen temperature, the color of the mixture was carefully noted. Optical spectra were not taken because the frozen solutions were essentially opaque due largely to scatter, and facilities were not available to us for spectroscopy of "opaque" solutions at -196° C.

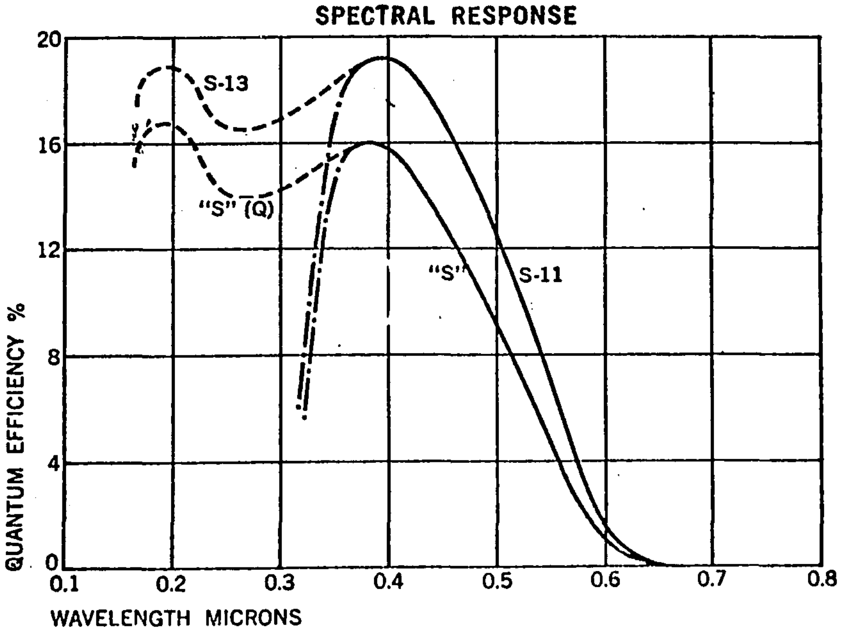
As we noted above, once the samples had been prepared and placed into the thin quartz sample tubes, these were placed in the Varian liquid-nitrogen quartz dewar. The dewar, in turn, was placed within a Varian V4531 microwave reflection cavity. The front wall of the cavity consisted of a grid capable of transmitting 50% of the incident light, thus enabling the sample to be examined as it was being irradiated.

A Varian V4502 EPR spectromenter operating with 100 kHz modulation frequency and equipped with Fieldial ® magnetic field control was employed. For this study, it was operated at X-band (9.5 GHz; 3cm. wavelength).

The light source consisted of a projector with a 500 watt incandescent bulb and a glass lensing system by which the sample could be irradiated. For some experiments, a Bausch and Lomb grating monchromator No. 33-86-25-02 was used between the light source and the lensing system in order to study the action spectrum for free radical generation, and corrections were made for both source emission and monochromator efficiency using a photocell (EMI 6256B) with an almost flat spectral sensitivity in the region 300-450 nm (See Figure 5A). With the monochromator before the projector and the beam focused on the photocell and room light excluded, the output of the photocell was measured as a function of wavelength using a cathode ray oscilloscope. Hence, the relative number of photo electrons from the source-monochromator-detector system at various wavelengths may be seen from Figure 6.

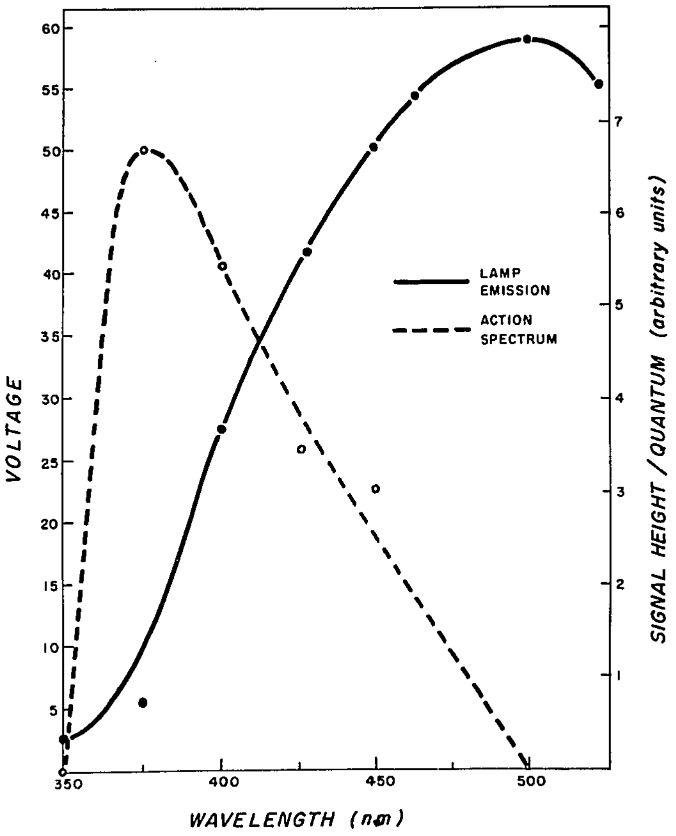
If one corrects the data in Fig. 6 for the sensitivity of the detector at various wavelengths (from Fig. 5A), one obtains a value proportional to the number of quanta issuing from the source-monochromator system at each of the studied wavelengths. A rough action spectrum for the production of free radicals from retinal was obtained by irradiating 10^{-3}M retinal in heptane glass for 20 minutes at each wavelength setting of the source-monochromator system. The peak to peak height of the EPR spectrum was measured and then divided by the number obtained as described above for a given wavelength, thereby yielding a number proportional to the efficiency of quanta at a given wavelength to produce free radicals from retinal under these conditions. The results are seen in Table I in the Result section, and in Figure 6.

For fluorescence studies, solutions of 10^{-3}M all-trans retinal and all-trans retinol in acetone were made up and examined at room temperature with an Aminco Bowman spectrofluorimenter. pH measurements were made with a Beckman Research pH meter.



Spectral Response of Phototube

Fig. 5A



Lamp Emission and Action Spectrum for Production of Free Radical from Retinal

FIGURE 6

Although the EPR spectrometer which was used for these studies was a commercially available model upon which no special modifications were made for these experiments, and although the technique of electron paramagnetic resonance spectroscopy is not a new one in biology, a short, nontechnical description of the electron spin resonance spectrometer and its function follows since most biologists are relatively less familiar with EPR spectroscopy than with the other techniques employed.

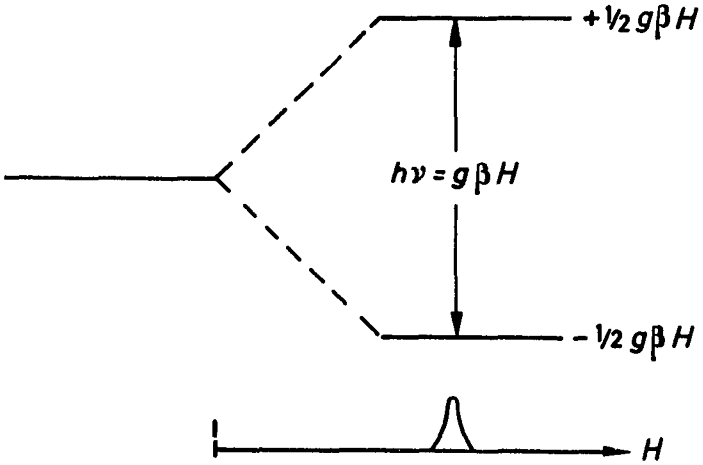
The purpose of electron paramagnetic resonance spectrometry (EPR)--also called electron spin resonance (ESR)--as a technique is basically to detect species with unpaired electrons. Substances with unpaired electrons include free radicals, triplet excited molecules, and transition metals (with unfilled shells of electrons within the valence or "optical" shell) such as iron, chromium, manganese, etc. These molecules are paramagnetic. Since each electron in a molecule can exist in one of two quantized spin states, when more electrons are in one state than the other, we call the molecule paramagnetic and refer to the excess of electrons in one state over the other as unpaired. Within a molecule, electron motion may be considered to consist of both orbital rotation about atoms and spin about its axis, much as the earth revolves in its orbit around the sun and rotates about its axis.[98] Since the electron is negatively charged, it possesses a magnetic moment to which both kinds of motion contribute.

---

[98] Although this is a useful analogy to provide a mental picture, it is not strictly true.

In the absence of a magnetic field, the magnetic moments of the unpaired electrons are randomly oriented (as a first approximation), the electrons possessing an average energy, E_o. When the electron is placed in a magnetic field, however, its magnetic moment interacts with the magnetic field such that, because of spin quantization, its magnetic moment must precess about an axis that is either parallel to or antiparallel to the direction of the superimposed magnetic field. These electrons whose magnetic moments "lie" antiparallel to the magnetic field possess greater energy, E_1. Those whose magnetic moments "lie" parallel to the field possess less energy, E_2. As may be noted from Figure 7, the difference in energy, ΔE, between these two states is proportional to the strength of the magnetic field. Specifically, it is equal to the product $g\beta H$, where g is the "spectroscopic splitting factor," β is a constant, and H is the value of the magnetic field. The "spectroscopic splitting factor" or "g-value" is a measure of the contribution of the spin and orbital motion of the electron to its angular momentum. For a free electron, the "g-value" is 2.0023. The constant β, also called the Bohr magneton, is equal to $eh/4\pi mc$, where e is the charge on the electron, h is Planck's constant ($6.6252\pm0.005 \times 10^{-27}$ erg sec), m is the mass of the electron, and c is the speed of light (2.9979 ± 10^{10} cm/sec).

The system is said to be "at resonance" when it is interacting with an energy source such that the value of an energy quantum, $h\nu$, where ν is the frequency, satisfies the following equation:



Resonance condition :- $h\nu = g\beta H$

$\therefore\ \nu = 2{\cdot}8 \times 10^{6}\,H$ for free electron

FIGURE 7

The resonance condition for EPR

$$E = h\nu = g\beta H \qquad (1)$$

For the values of magnetic field usually employed for EPR, this places the frequency in the microwave region. Phenomenologically, this equation means that there is an interaction and exchange of energy between unpaired electrons aligned "parallel" or "anti-parallel" to a magnetic field and an energy source such as to induce with equal probability both absorption of energy by the electrons and emission of energy by the electrons. The former process enables the electrons to go from the lower quantized spin state to the high, while the latter results in the opposite transition. Since the probability of absorbing or emitting energy is equal for any given electron, the only reason there is a net absorption of energy by a population of resonating centers is that there are more electrons in the lower state than in the higher.

Recalling that the difference in energy between the two electronic states, ΔE, is finite, the number of electrons in the lower state will be greater than that in the higher state, following a Boltzmann distribution,

$$\frac{n_1}{n_2} = e^{-\Delta E/kT} \qquad (2)$$

where n_1 is the number of electrons in the higher state, n_2 is the number in the lower state, e is the base of natural logarithms, k is the Boltzmann constant ($1.3804 \pm 0.0001 \times 10^{-16}$ erg deg $^{-1}$) and T is the absolute temperature in degrees K. At room temperature for a typical free radical, this ratio is about 0.998 for the usual X-band EPR[99], and hence there is a small absorption of energy.

The detection of resonance depends on this small excess of stimulated absorption over stimulated emission that results from the slightly greater number of electrons in the lower energy state. Theoretically, therefore, sensitivity of detection can be enhanced by increasing the discrepancy between n_1 and n_2, as will be described later.

Obviously, to maintain a net absorption of power, the electrons in the higher state must have some other means to lose their energy, otherwise there would soon be equal numbers in both states and no net energy absorption would occur. The processes by which this energy loss occurs are called "relaxation processes," and spin energy is shared with the rest of the molecule or "lattice" by spin-lattice interaction, i.e. there is coupling between the magnetic moments of the unpaired electrons and the moving electrical charges in their vicinity so that the energy of the excited ("antiparallel" or higher energy) electron spins can be converted into thermal energy of the molecule, resulting in repopulation of the lower state at the expense of the upper. Most spin-lattice interaction takes place via the spin-orbit coupling, since the thermal vibrations of the molecule or lattice are more closely coupled to the orbital motion of the electron than to its spin. Nevertheless, the spin-orbit coupling in free radicals is usually small, except in those cases where the free

---

[99] X-band (See later discussion) corresponds to ~9.5 GHz microwave radiation, so $\Delta E \cong 6.3 \times 10^{-17}$ Erg.

electron is strongly localized on atoms which have high atomic numbers resulting in high spin-oribt coupling, such as on halogens, sulfur, and oxygen. Because relaxation processes and spin-orbit coupling affect the width of the EPR signal as well, they will be discussed in that connection later.

The resonance equation (1) stipulates that energy may be absorbed whenever the equality is achieved, where h and β are physical constants, and the value of g is a characterisitic of the system being examined. Theoretically, then, this resonance condition can be satisfied for a family of values of ν and H. In practice, however, the frequency, ν, is set at a fixed value for instrumental reasons during a given run, and the value of the magnetic field, H, is varied until the equation is satisfied, viz., until resonance occurs.

In these experiments, X-band EPR was used, having a frequency, ν, of about 9.5 GHz (giga hertz). This corresponds to a wavelength of approximately 3 cm. and a magnetic field for electron resonance of approximately 3400 gauss. As may be noted from the equation above, other combinations of ν and H are possible. At Q-band (occasionally denoted K_a-band), characterized by a frequency of about 35GHz, the corresponding magnetic field at resonance has a strength of approximately 12,500 gauss.

To return to the question of sensitivity, it is clear from the equation (2) that the difference between n_1 and n_2 increases as ΔE increases and as T decreases. As seen from equation (1), ΔE is proportional to ν, hence there is potentially greater sensitivity at higher frequency and at lower temperature, because both increase the difference between n_1 and n_2.

This increased sensitivity at low temperature is in addition to the function of low temperature to "freeze in" or "trap" reactive free radicals by preventing them from obtaining the necessary activation energy for their decay and by partially immobilizing them to prevent their interaction with each other. Both high frequency and low temperature are, however, not unmixed blessings, because due to the smaller dimensions of detection cavities at high frequencies (hence short wavelengths), the former limits the volume of sample which can be examined, while the latter, by restricting molecular tumbling in frozen samples, results in broadening the signal and loss of hyperfine structure (hence loss of information) resulting from failure to average out anisotropic hyperfine intereactions; low temperature also decreases the spin-lattice relaxation processes described earlier, which may further decrease detection sensitivity.

In practice the sample is placed in a microwave reflection cavity to which microwave power is brought, usually via a waveguide from a klystron oscillator. The cavity which is situated between the poles of a large electromagnet (whose field is homogeneous and can be varied linearly) serves to concentrate the microwave power (up to several thousand times) on the sample placed at its center. Magnet stability may be maintained by a feedback mechanism emplying a sensor based on the Hall effect (e.g. Fieldial ®).

As the magnetic field is swept, some point will be reached where its value will be such that the resonance condition, $h\nu = g/\beta/H$, is satisfied. At this point, the energy content of the quanta of the microwave field, $h\nu$, will just correspond to the energy difference between the two allowed quantum states of the magnetically aligned electrons, and energy absorption will occur.

There are two basic ways of detecting energy absorption as exemplified by 1) transmission spectrometers and 2) reflection spectrometers (see Figure 8). The former is the simpler, since the determination of microwave energy detected as a function of magnetic field strength results in a constant level of transmitted energy, except at resonance, where the sample absorbs energy and a dip in transmitted energy is evident. In a reflection type cavity, a hybrid tee or circulator balances the microwave circuit such that the energy is divided between the sample cavity (2) (see Figure 8) and the resonating compartment (3); with a balanced microwave bridge no (little) energy reaches the crystal detector (4). If the delicate balance between compartments (2) and (3) is upset by energy absorption by the sample in (2), energy proportional to this amount is reflected to the crystal detector (4), and may be recorded as a function of the field strength at which it occurs by an X-Y recorder, or an oscilloscope. A reflection type of cavity is usually preferred to a transmission type because in the former crystal sensitivity can always be maximized regardless of the power absorbed by the sample while this is not possible in a transmission type spectrometer.

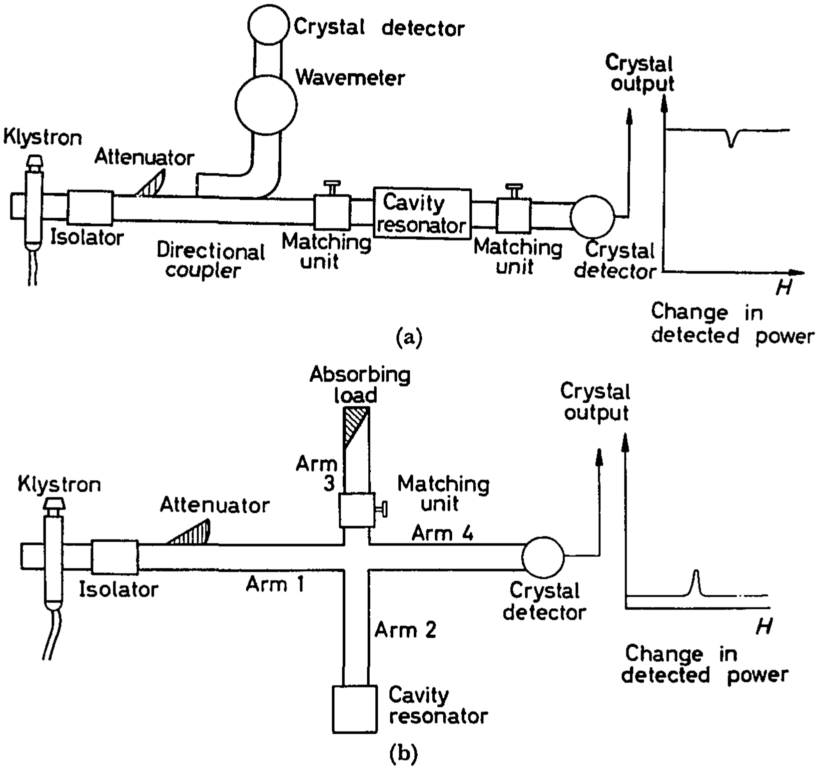


FIGURE 8

Block diagrams of

(a) transmission mode spectrometer

(b) reflection mode spectrometer

Although, as indicated above, a plot of field strength against energy absorption is easily obtained, for reasons of sensitivity this simple picture is usually complicated by employing phase-sensitive detection. This involves modulating the magnetic field by a phase-locked perturbation, and employing phase-sensitive detection which significantly improves signal-to-noise ratio and therefore resolution. The absorption of microwave energy as a function of field strength in a system employing magnetic field modulation with phase-sensitive detection is directly recorded as a first derivative presentation, dA/dH, where A is the energy and H is the field strength[100]. Therefore, the number of free spins is proportional to the signal height multiplied by the width (in gauss) squared, when the data is presented in this form. Second and higher order derivative presentation is also possible for more precise definition of line centers ("g-values"), but there is a concomitant loss of sensitivity.

Although audiofrequency field modulation may be used, the crystal diode detectors used for microwave detection in most sensitive EPR spectrometers are less noisy at higher frequencies. The frequency-dependent components of the noise spectra of modern crystal detectors are negligible above about 100 kHz.

While too low a modulation frequency results in greater crystal noise, too high modulation frequency results in no further

---

[100] In fact, the output of the phase-sensitive detector will closely approxmiate dA/dH only if the amplitude of the modulating perturbation is small relative to line-width. (See later discussion of Fig. 9).

decrease in white noise, and modulation broadening of the signal width may occur. Accordingly, 100 kc has been found a good compromise for most work (it corresponds to approximately 0.03 gauss line width)--especially in solids, where the lines are usually already rather broad for reasons which will be described later. Where linewidth is extremely narrow, even this may provide too much modulation broadening, and another type of appratus will be required--superheterodyne detection.

To provide the phase-locked perturbation of magnetic field noted before, modulation coils are placed outside the conducting walls of the microwave cavity, and variation in the current through them results in a rapid (100 kc per second), small magnetic variation superimposed on the field from the electromagnet. One can vary the magnitude (but not the frequency) of this variation in magnetic field in most spectrometers. A smaller variation or "modulation amplitude" results in a signal more accurate in shape, but smaller in size, while a larger modulation amplitude results in a larger but distorted signal (Figure 9). In this regard the effect of modulation amplitude in EPR is analogous to the effect of slit width in absorption spectroscopy, and a compromise is made between highest sensitivity and minimal distortion of true line shape.

Up to this point, the assumption has been made that the magnetic field of the electron interacts only with the applied magnetic field produced by the electromagnet and modulation coils. However, many of the atomic nuclei in the molecule with which the

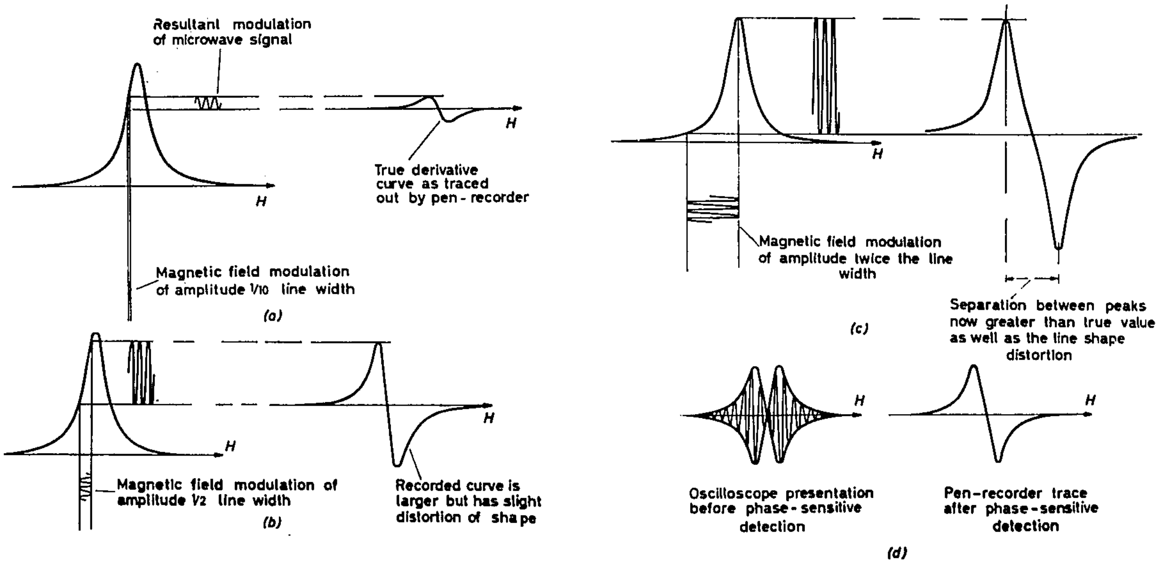


FIGURE 9

Effect of modulation
amplitude on signal site and shape

electron interacts also have spin magnetic moments, and these are also oriented in a quantized manner by the applied magnetic field. These aligned nuclear magnetic dipoles then produce slight increases or decreases in the total value of the magnetic field in the vicinity of the unpaired electron. The quantized interaction of the electronic magnetic moment with the magnetic moments of these nuclei (e.g. the dominant isotopes of hydrogen, nitrogen, and others) is called hyperfine interaction and results in changes of the spectra called hyperfine spectral detail. When present, these details can be very useful in learning something about the molecular environment of the free electron, and hence something about the nature of the free radical, as will become apparent in the following discussion.

Since hyperfine structure is so important to the interpretation of EPR spectra of free radicals, a short consideration of how it evolves will be undertaken. Examining the simple case of an unpaired electron interacting with a single proton, (with nuclear magnetic moment $M_I = \pm\frac{1}{2}$), the magnetic field produced by the quantized spin of this proton may add to or subtract from the superimposed field. Assuming approximately equal distribution between the two allowed states of proton spins, this will split each of the two energy levels of the electron into two new ones, giving a total of four electron spin energy levels. Since the proton spin does not resonate with the microwave field at the magnetic field strengths required for electron resonance, the selection rule of $\Delta M_I = 0$ holds. Hence, only two transitions between the four levels are allowed (see Figure 10) resulting in signals at two values of H or two "lines", as indicated in the figure, such that

$$H_1 = h\,\nu/g\,\beta - \Delta H \quad \text{and} \quad H_2 = h\,\nu/g\,\beta + \Delta H \qquad (3)$$

For a general case of electronic interaction with a nucleus of nuclear spin I, therefore, it can be seen that 2I + 1 lines will be produced. The separation between the lines viz. the 2 ΔH splitting, is proportional to the strength of the interaction between the free electron and the nucleus in question. This, in turn, depends on how much of the electron orbital is concentrated near the nucleus.

Since most free electrons interact with many nuclei, these spectra can grow rather complex. However, Ingram (1958) points out that most spectra can be fitted into one of two groups. First, those in which the interaction with a nucleus of spin I_1 is much stronger than that with a nucleus of spin I_2. In this case, there will be $2I_1 + 1$ lines, widely spaced in magnetic field, each of which is split into $2I_2 + 1$ sub-components which may or may not be resolvable depending upon their separation which, in turn, depends upon their strength of interaction. The second limiting case, Ingram (1958) points out, is when the unpaired electron is equally coupled to an identical nuclei of spin I. In this case, the result is 2nI + 1 lines, equally and symmetrically spaced and with a maximum intensity in the center following a binomial distribtuion. For example, in the case of two protons, the first

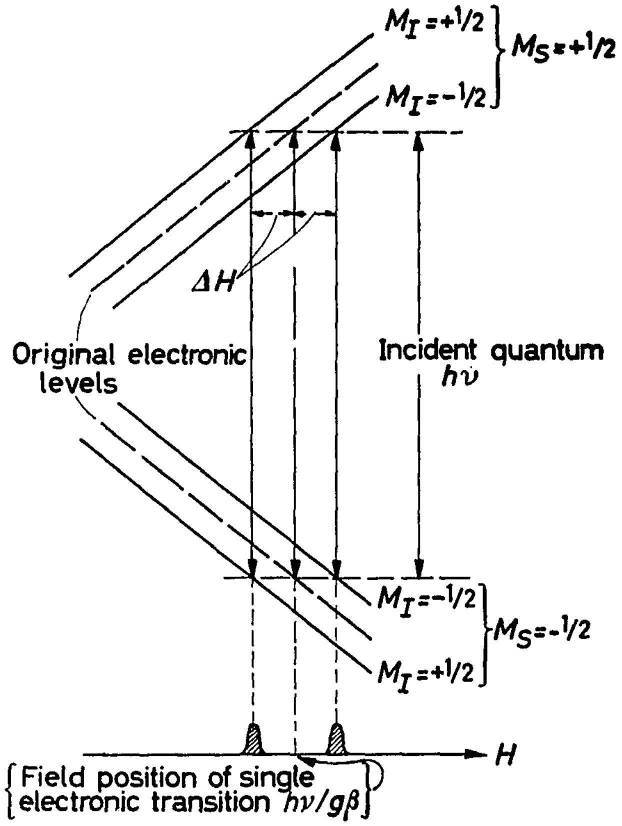


FIGURE 10

Hyperfine splitting of electronic energy levels nucleus splits the electronic absorption into two lines which in turn are split by the second proton (see Figure 11a). However, since the interactions are equal, there is overlap, and only three lines are produced instead of four, since the selection rule of $\Delta M_I = 0$ allows only the three indicated transitions. Similar treatment with larger numbers of protons yields n + 1 lines (or in the general case, 2nI + 1 lines) as is seen, for example, in the case of three protons (Figure 11b).

The hyperfine structure thus far considered is called the isotropic splitting, and the mathematical term used to describe its magnitude is called the Fermi contact term. For a free electron's interaction with a nucleus to result in isotropic hyperfine splitting, the electron's wave function must have a finite value at the nucleus. Theoretically, π-electron orbitals have two lobes of electron density (because they are modified atomic p-orbitals) above and below the molecule with a zero node at the nucleus (or at the plane of the molecule in the case of planar molecule). Therefore, as a first approximation the unpaired spin which is localized in the π-orbitals of any conjugated system should not produce any hyperfine structure, and only unpaired electrons in the molecular σ-orbitals or atomic s-orbitals would be expected to produce hyperfine spectral detail. In practice, however, there is electronic configurational interaction such that, for example, an unpaired π-electron interacts with the electrons of an adjacent σ-bond to induce polarization of that bond. The σ-bond will partially polarize to produce more spin density

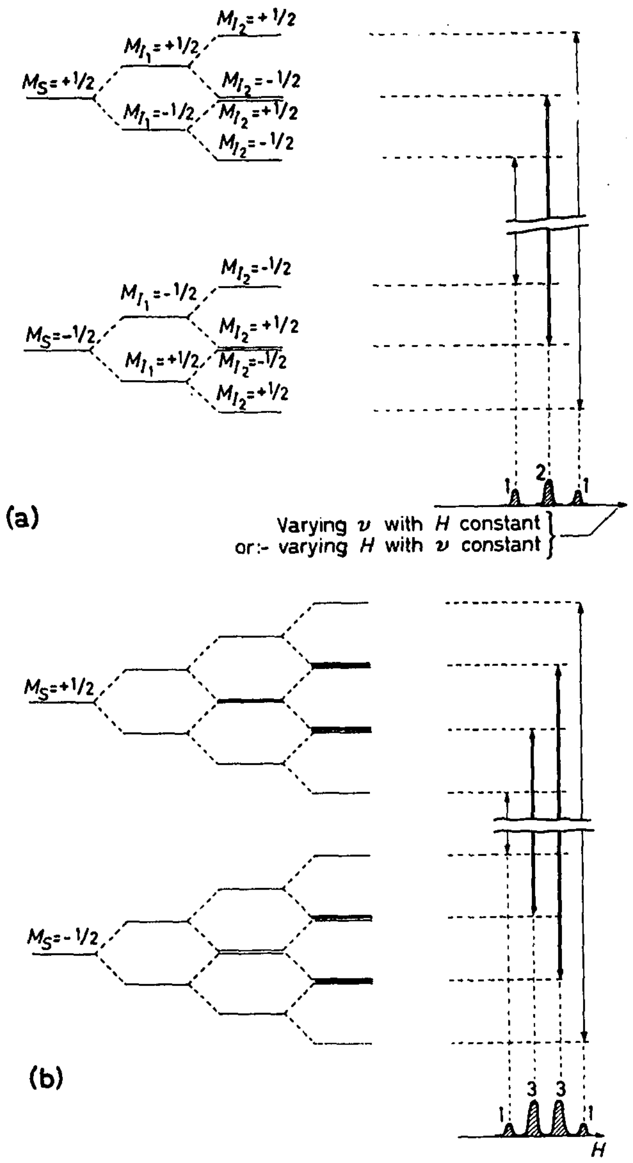


FIGURE 11

Hyperfine splitting produced by
(a) two and (b) three identical protons

parallel to an unpaired π-electron in the vicinity of that σ-electron and therefore greater spin density of opposite sign in the region of the nucleus at the other end of the sigma bond. Hence, some unpaired character is imparted to the electron of the σ-bond, and isotropic (contact) hyperfine detail results from the presence of unpaired π-electrons.

While the previous section dealt with the isotropic hyperfine interaction, there is also a second major type of hyperfine splitting: the dipole-dipole, or anisotropic interaction between the spins of free electrons and nuclei, and, in cases of high free radical concentrations, between the spins of free electrons. This effect is the "classical" influence of one magnetic dipole upon another and hence depends on their relative orientation. The magnitude of this interaction is given by the vector sum of the electron and nuclear dipoles (or two electron dipoles in the case of high free radical concentration) projected upon the magnetic field produced by the electromagnet. Thus, unlike the isotropic or "contact" interaction, the dipolar effect is directionally dependent or "anisotropic."

If the viscosity of a containing medium is low, the thermal tumbling of the free radicals will cause the net moment of coupled dipoles to reorient sufficiently rapidly to sweep out a sphere in a time short compared with the inverse of hyperfine line separation (expressed as a frequency). Since the anisotropic interaction averaged over all orientations with equal weight is zero, the effect of rapid Brownian tumbling is to average out the anisotropic hyperfine effect. In the solid state, or even in viscous liquid media where this tumbling is not rapid enough to average out the anisotropic splitting completely, this interaction is smeared out, resulting in line broadening[101]. Thus, in extremely viscous glasses near liquid nitrogen temperature, one sees apparent broadening of line width due to smeared-out anisotropic hyperfine structure.

Having previously considered the factors which determine the number and separation of lines, one can briefly examine the main factors affecting line width. As is evident from the preceding discussion, anisotropic spin-spin (dipolar) interaction can broaden a line. Spin-lattice interaction, exchange interaction, and saturation considerations also can affect line width.

Relaxation processes whereby excited spins lose energy either to other spins or to the molecule or "lattice" have already been briefly examined. These interactions can be further characterized and quantitated by the spin-spin relaxation time, T_2, and the spin-lattice relaxation time, T_1, respectively. Considering only these factors, the line width may be written as (Ingram, 1958):

$$w = 1/T_1 + 1/T_2 \qquad (4)$$

---

[101] It might be noted that in single crystals of paramagnetic materials the anisotropic interactions are directionally coherent. The result is not a smearing effect due to the random-but-fixed orientation that obtains in polycrystalline or amorphous material (such as in most biological specimens), but rather a resolved set of EPR hyperfine lines whose spectrum is a function of the crystal's orientation in the magnetic field.

In free radicals spin-orbit coupling tends to be weak, as noted before, so spin-lattice relaxation times are usually long and can be as long as several seconds (Ingram, 1958). Low temperature may further lengthen the relaxation time. Hence, free radical spectra are very seldom broadened by too strong a spin-lattice interaction. However, weak spin-lattice interaction, especially when further decreased by low temperature, may result in "saturation broadening" where the spins so not fall rapidly enough from the excited or "antiparallel" spin state to the energy-absorbing lower state to maintain a Boltzmann population difference. As might be expected, the onset of saturation depends upon the level of microwave power incident on the sample, and saturation can result not only in line broadening but in decreased signal height as well.

Unpaired electrons may exchange their spins with one another. However, this exchange is very short ranged and is usually important only when spin concentrations are high. Nevertheless, as might be expected, if such exchange occurs to any significant extent (and if it occurs between identical energy levels, as is usually the case), it results in partially averaging out of the anisotropic hyperfine interaction in solids, much as molecular tumbling does in liquids, with resultant sharpening of the spectrum. Furthermore, while it was noted that most spin-lattice coupling was via spin-orbit interaction, spin exchange also results in relaxation, with spin energy being converted to thermal energy of the lattice. Hence the approximate expression for line width given in equation (4) includes both T_1 and T_2 terms.

Unlike paramagnetic species with localized unpaired electrons (such as chromium and iron) which result in large differences in g-value, the delocalization of the electron in free radicals and their small spin-orbit interactions, as previously noted, result in g-values which are not far from that of a completely free electron 2.0023. Therefore, in comparison with hyperfine structural detail, g-values are generally of little help for identification of free radicals[102].

Having thus discussed some of the more important theoretical considerations of EPR, one can summarize by reexamination of a block diagram of the apparatus and briefly reviewing the function of each component. A block diagram of the apparatus used for these studies has already been seen in Figure 8b, which was cited previously in distinguishing between the transmission and reflection modes of detection. The remainder of its components will now be described. The klystron oscillator is the source of the microwave energy. The isolator is a microwave rectifier which serves to prevent the return of microwave energy which would damage the klystron. The attenuator serves to regulate the power incident on the sample. The sample is contained in the cavity resonator (another name for the reflection cavity), and the hybrid tee is a balanced microwave circuit (as previously described) with a crystal diode detector in one arm, the AC component of whose output is detected by the phase sensitive detector and plotted

---

[102] Precision g-value determinations are possible, but the variations to be expected in most free radicals (a few parts per 10^5) require special equipment and calibration not generally available.

as a function of field strength by an X-Y recorder.

A photograph of the apparatus is seen in Figure 12. In this photograph, the electromagnet between whose 9 inch diameter poles the sample is placed is seen at the far right. On the table above the magnet is a unit containing the klystron oscillator, the source of microwave energy, and the microwave bridge. To the left of the magnet lies the main console with its display oscilloscope for tuning the instrument. Above the console is the 100 kHz modulation control unit and the phase-sensitive detection amplifier, and to the left of the console are the output devices - an X-Y pen recorder (not seen) and a storage oscilloscope, below which the power supply can be seen.

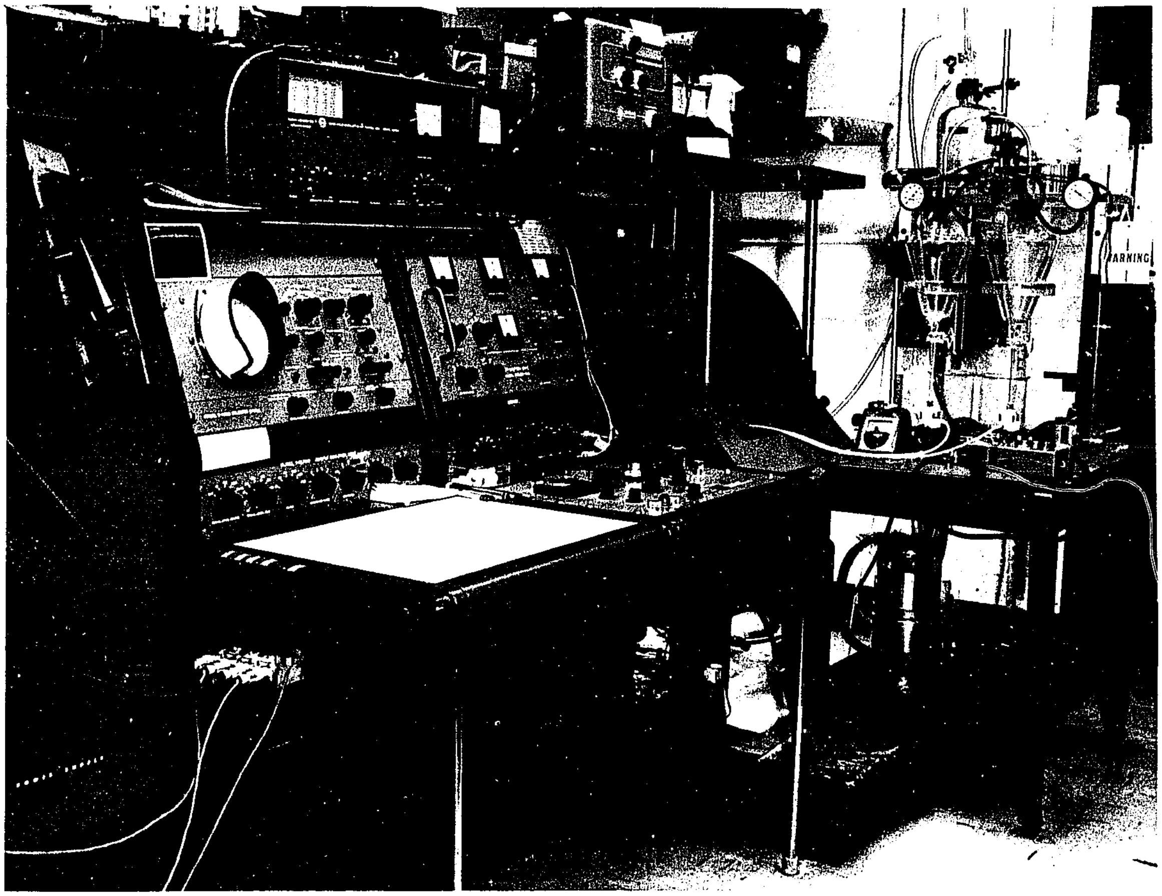


FIGURE 12

Photograph of the EPR spectrometer

RESULTS

A. Free Radicals from Retinals

On illumination with light from a 500 watt projector bulb, all of the studied isomers of retinal in rigid solution or in the crystalline state at -196° C form paramagnetic species characterized by a single broad (approximately 23 gauss) EPR signal with some suggestion of fine structure (Fig. 13) that was not further resolvable under the conditions described[103].

It was further characterized by a "g-value" or spectroscopic splitting factor, as described previously, of 2.004 ± 0.001 employing DPPH (diphenyl-picrylhydrazine) as a standard. The singlet spectrum is not accompanied by a half-field EPR signal characterisitic of the forbidden $\Delta m = 2$ transition of the triplet state (Van der Walls and de Groot, 1959; de Groot and Van der Walls, 1960; Lhoste *et al*, 1966), and it appears after less than one second of illumination, increasing in intensity with increasing time of illumination (Fig. 14). It is apparent from Fig. 13 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 (Fig. 14), with the signal strongest at -196° C 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

---

[103] Recent studies by Borg, Freed, Sack and Grady (unpublished) using liquid propane-propene as solvent at low temperature reveal that this signal can be resolved into three lines.

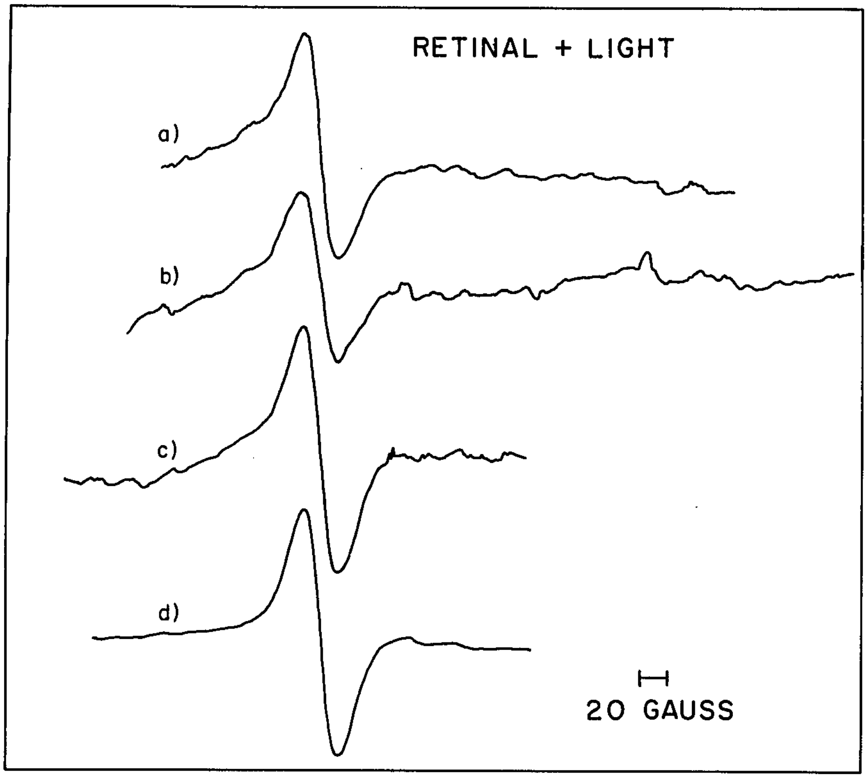


FIGURE 13

EPR spectra of $10^{-3}M$ retinal in heptane glass

(a) all-trans retinal
(b) 9-cis retinal
(c) 13-cis retinal
(d) all-trans retinal after prolonged illumination

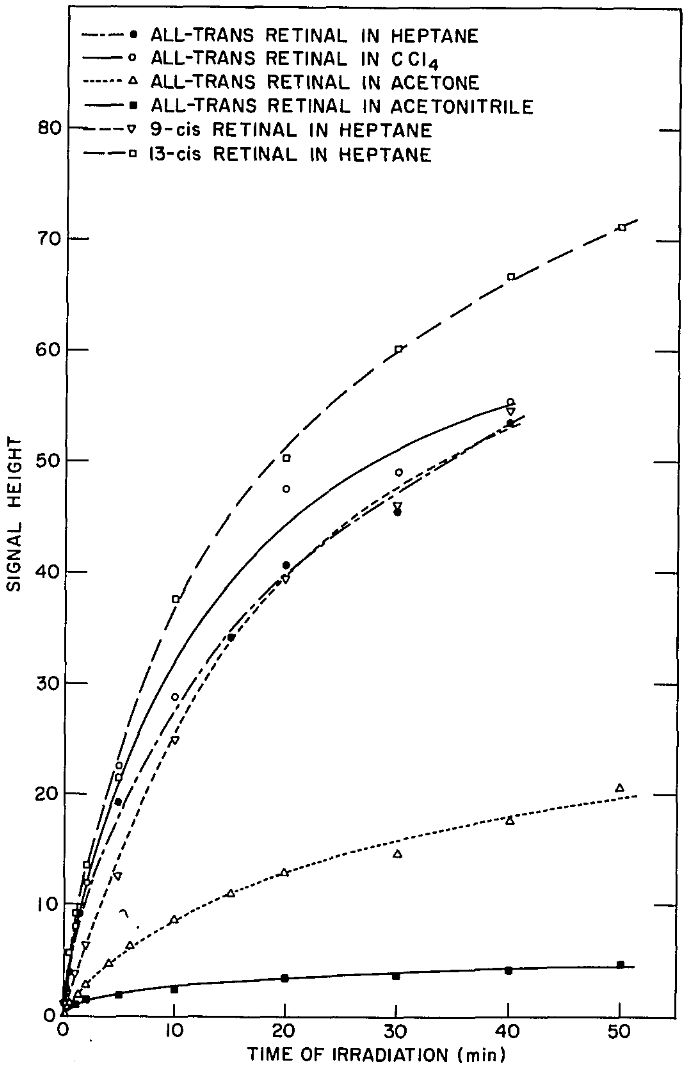


FIGURE 14

Plot of EPR signal height vs.
time of irradiation of 1mM retinal

detected from frozen ethanolic solutions.

In carbon tetrachloride the irradiated surface blackened (reversible on annealing), but this did not occur with the other solvents. In all cases, irradiation of solvent alone under identical conditons yielded no detectable signal, nor did the dissolved substances demonstrate any signal prior to irradiation.

The slow rise of the retinal EPR signal and its failure to saturate after several hours of exposure to light is seen in Fig. 14. The effect on signal formation of a 25% transmission neutral density filter was investigated and found to be exactly compensated by a four-time prolongation of light exposure.

A third point which may be seen from this figure (Fig. 14) is the relative yield of free radical from the various isomers of retinal. This is evident if one considers just the three curves representing the 9-cis, all-trans, and 13-cis isomers in heptane. Shortly after the sample is illuminated, the 9-cis isomer lags behind the others, and as time proceeds, it approaches the all-trans, while the 13-cis curve rises well above the other two.

The action spectrum for production of a free radical from retinal (Fig. 6, Table I) reveals most efficient generation at wavelengths corresponding approximately to the range of retinal's absorption spectrum, with a peak between 375 nm and 400 nm which is in the range of the absorption maximum. (Since the signal from rhodopsin was so much weaker than that from the carotenoids, I was unable to determine an action spectrum for it.) The free radical was also regenerable an indefinite number of times, if, after warming, the material was recooled and then reirradiated.

TABLE I

ACTION SPECTRUM FOR GENERATION OF RETINAL FREE RADICAL

Excitation Wavelength in nm	Photocell Output	Photocell Efficiency	Output Efficiency	EPR Relative Signal Height (Arbitrary Units)	EPR Signal Height Output/Efficiency
350	2.5	.18	13.9	0.0	0.0
375	5.5	.185	29.7	0.2	0.00674
400	27	.19	142	0.8	0.00563
425	42	.185	227	0.8	0.00352
450	49.5	.18	275	0.9	0.00328
475	54	.15	360	--	--
500	58	.125	465	0.0	0.000
525	55	.10	550	0.0	0.000
550	55	.08	687	0.0	0.000
575	53	.04	1330	0.0	0.000
600	49	.02	2450	0.0	0.000
625	42				
650	32				
675	12				
700	6				

Quanta = K voltage/efficiency

Photocell efficiency is obtained from Fig. 5A

B. Free Radicals from Retinol

Retinol (vitamin A), as noted earlier, is known to dimerize when exposed to ultraviolet light[105]. It was, therefore, not surprising that retinol was found by me to form a free radical when irradiated. What was surprising, however, was that the width of the EPR spectrum of the free radical thus produced was 210 gauss (Figure 15).

The retinol radical was less easily produced by the apparatus which was employed than was the retinal free radical, and it required over 100 times the concentration for detection (10^{-1}M versus 10^{-3}M).

As may be seen from Fig. 15, the spectrum of the free radical produced from retinol is characterized not only by its great width, but also by some line structure. When, as in the lower tracing, the spectrum is overmodulated, its symmetry and underlying three line character become clear. Overmodulation in EPR is, however, analogous to too large a slit width in light absorption spectroscopy, and results in some loss of structure with smoothing over of the general envelope and increase in signal size. In the upper tracing of Fig. 15, more fine structure is seen, although the resolution is still poor, especially on the high field side (the right).

---

[105] Mouseron-Canet, M., Mani, J. C., Favie C., and Lerner, D., "Photochemical isomerization of vitamin A," (1966), Compt. Rend. Ser. C., 262, 153-155.

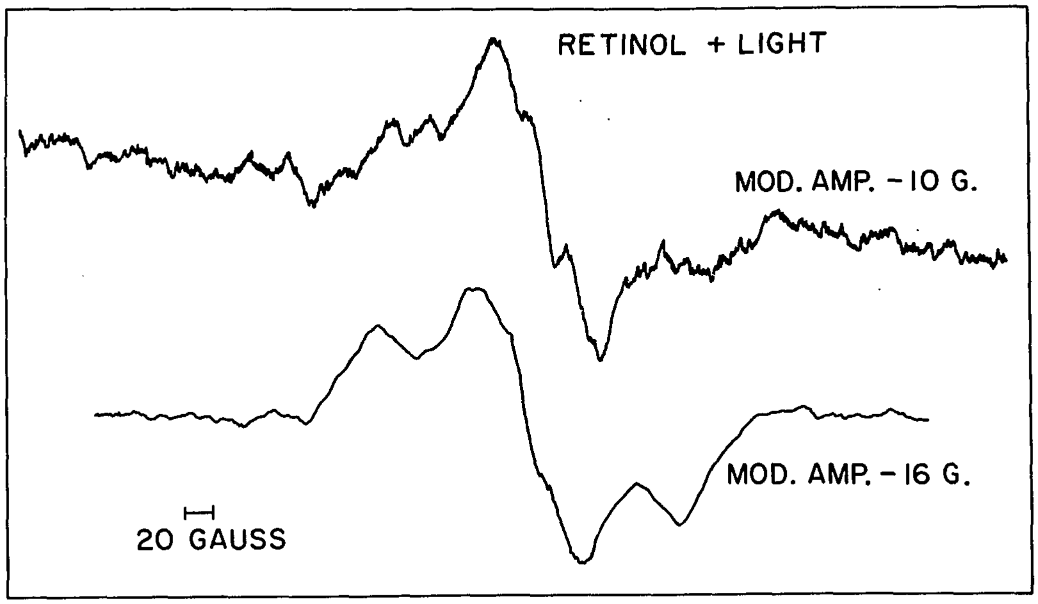


FIGURE 15

EPR spectrum of 10^{-1}M retinol in acetone glass

(a) modulation amplitude 10 gauss
(b) modulation amplitude 16 gauss

C. Free Radicals from Rhodopsin

Rhodopsin in 2% digitonin solution yields upon illumination an EPR signal qualitatively similar to that of retinal (Fig. 16), but it is considerably weaker, and it cannot be made to grow on continued irradiation. Furthermore, unlike 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° C, 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 partially regenerable upon reexposure to light at -196° C. (A digitonin blank gave no signal on similar treatment.)

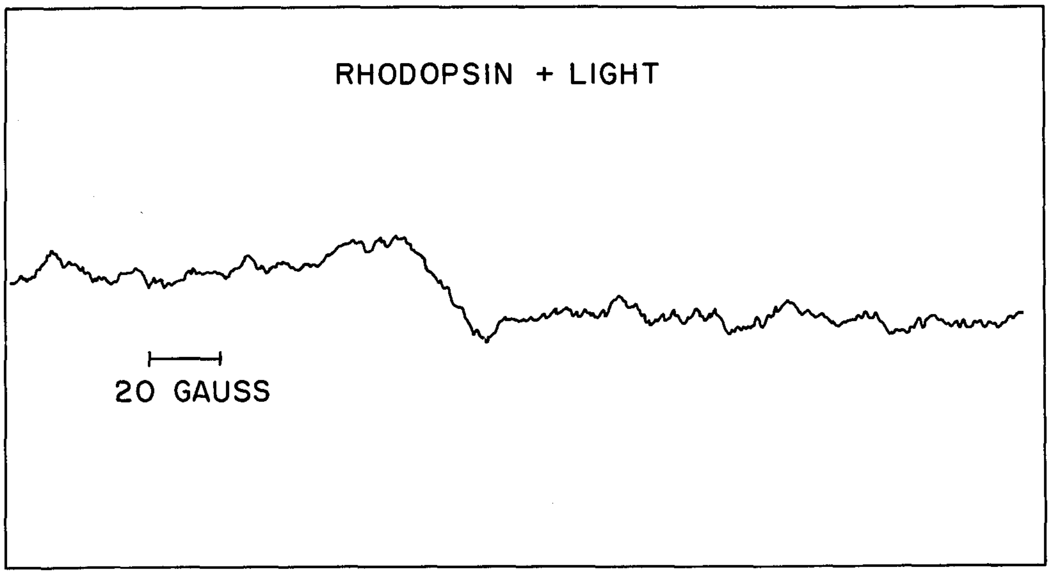


FIGURE 16

EPR spectrum of rhodopsin in 2% aqueous digitonin glass.

D. Charge Transfer Studies

It was found that solutions of retinol to which chloranil (tetrachlorobenzoquinone) was added turned green at room temperature, and became a deep blue-green when immersed in liquid nitrogen. Similar behavior characterized solutions of 13-cis retinal plus chloranil. Solutions of chloranil and the other isomers of retinal remained yellow and did not undergo any change at room temperature, but produced the same blue-green complex when cooled to liquid nitrogen temperature (-196° C), reverting to their natural yellow on rewarming.

EPR studies appear to parallel the visible changes such that an EPR signal was observable at room temperature from retinol-chloranil mixtures, and not from all-trans or 9-cis retinal-chloranil mixtures. On illumination at low temperature, however, the blue retinal-chloranil mixtures could be induced to give an EPR signal; however, this was the known characteristic signal of the chloranil free radical anion (Fig. 17), characterized by its extremely narrow width. As can be seen from the figure, this signal is not accompanied by the 23 gauss wide retinal signal. Futhermore, when (reduced) hydrochloranil was employed with retinal and irradiated at low temperature instead of chloranil, the same typical (oxidized) chloranil anion free radical spectrum was obtained, but much smaller in size.

Tetracyanoethylene (TCNE), another substance known to be a good electron acceptor (Ilten and Calvin, 1965), will also form charge-transfer complexes with retinal. These, however, are red instead of the blue-green of those with chloranil. Similarly, irradiation of these complexes yields the characteristic TCNE free radical anion spectrum rather than that of the carotenoid (Fig. 17).

Unlike the sharp, narrow singlet of the chloranil anion free radical, however, the spectrum of the TCNE anion free radical has a partially resolved 9-line hyperfine structure due to hyperfine splitting from four equivalent nitrogen nuclei, as suggested in Fig. 17.

E. Fluorescence Studies

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, while we were easily able to demonstrate (the known) fluorescence of retinol.

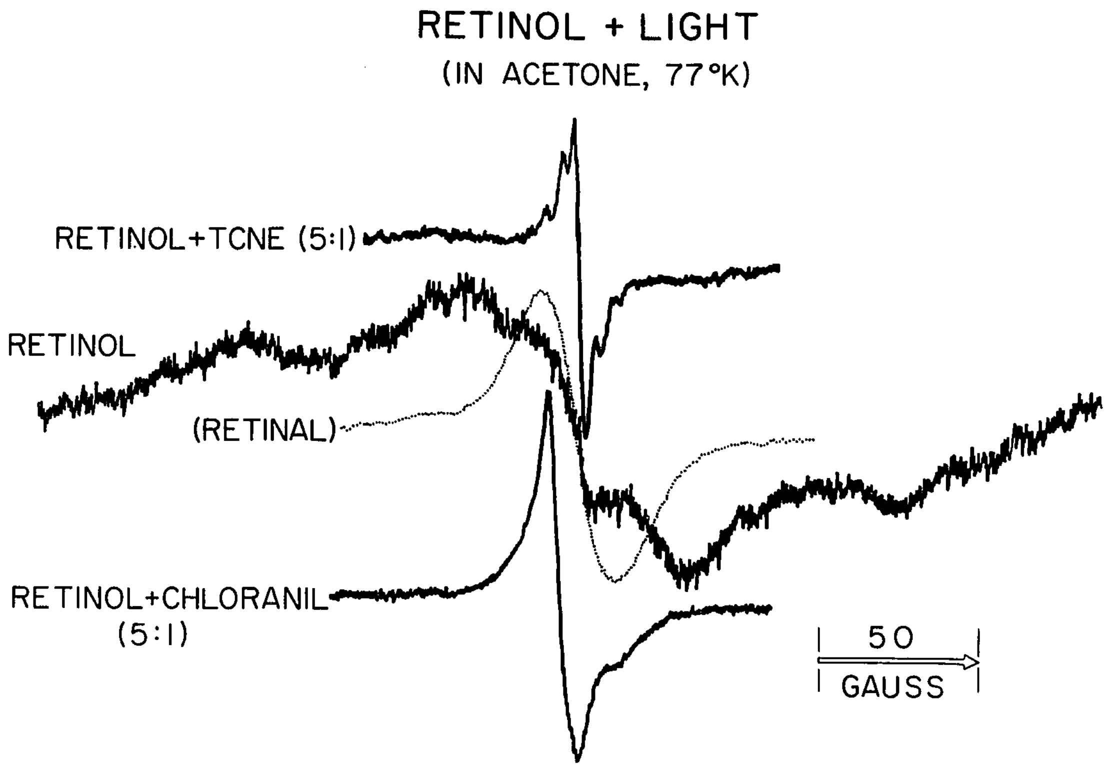


FIGURE 17

EPR spectra of irradiated charge-transfer complexes of retinol.

DISCUSSION

A. Discussion of Data

The free radical which we have demonstrated by means of EPR spectrometry in all likelihood corresponds to the labile species observed by Jurkowitz *et al.*(1959) at liquid nitrogen temperatures by means of optical spectroscopy after irradiation of retinal. The irradiation of retinal was carried out by these authors using a mercury arc lamp (GE AH5, 250 watts) at liquid nitrogen temperature and resulted in the retinal peak at 387 nm decreasing to 60% of its original height after 30 minutes of irradiation and falling to 20% of its original height after one hour of irradiation. (Fig. 18). At the same time, two new peaks were noted to arise at 350 and 412 nm. If the specimen were rewarmed, the original all-trans retinal peak returned, but was slightly less intense and shifted slightly toward longer wavelength, probably because a small amount of material had been converted to cis isomers which have smaller extinction coefficients and absorb at longer wavelengths. If the sample were recooled and reirradiated, the loss of the retinal peak could once more be made to occur - just as our signal could be regenerated after annealing, recooling, and reirradiating.

Jurkowitz *et al.* (1959) interpret their data as also showing that "neither low temperature nor rigid solvent inhibits the geometric isomerization of this molecule." Their evidence for this (Fig. 19) is that starting with (an unspecified concentration of) 11-cis retinal in EHA glass, irradiation for one minute with

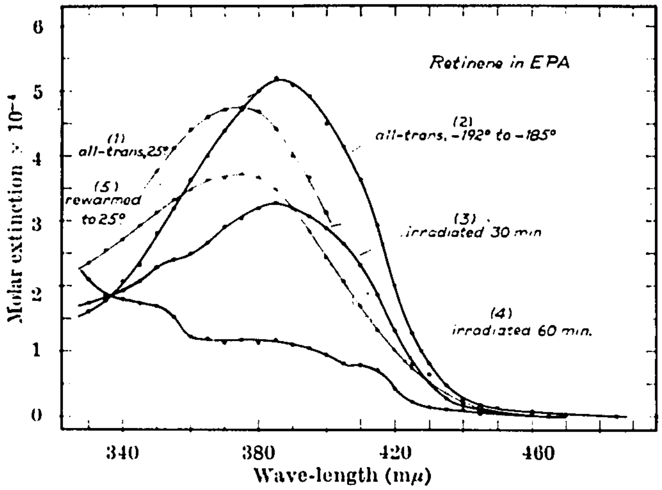


Fig. 4. Reversible photobleaching of retinene at low temperature in a rigid solvent. (1) All-*trans* retinene in *EPA* at room temperature. (2) Same brought to liquid nitrogen temperature. (3) Irradiated 30 min. in the cold; $\epsilon_{max.}$ falls 40 per cent. (4) Irradiated 30 min. longer; $\epsilon_{max.}$ has fallen 80 per cent. (5) Rewarmed to room temperature in the dark. Most of the original extinction is regained; what has been lost is due mainly to the light having isomerized the all-*trans* retinene to a steady state mixture of *cis* and *trans* forms

FIGURE 18

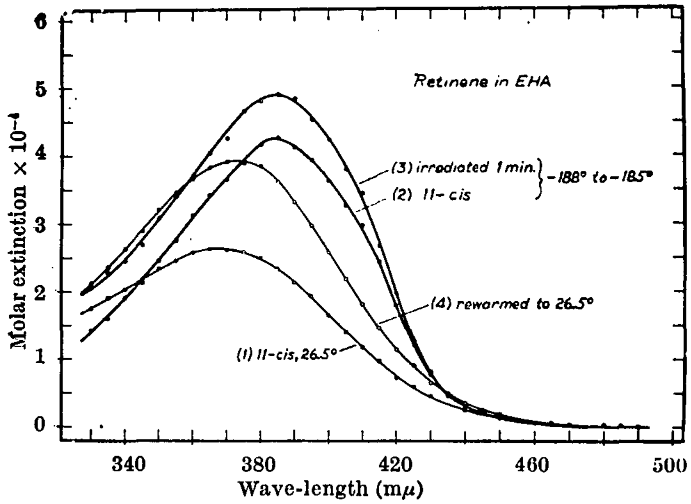


Fig. 3. Geometrical isomerization of retinene by light at a low temperature in a rigid solvent. (1) Absorption spectrum of 11-*cis* retinene in *EHA* at room temperature. (2) Same at the temperature of liquid nitrogen; the solvent is vitrified. (3) Irradiated 1 min. in the cold. (4) Returned to room temperature, in the dark. The rise of $\varepsilon_{max.}$ in the cold, accentuated in the warm, is associated with the isomerization of the hindered 11-*cis* isomer to a steady state mixture of all possible isomers, primarily all-*trans*

FIGURE 19

their mercury arc (AH 5) resulted in an increase of molar extinction of "16%, λ max remaining almost unchanged," and they go on to state that, "On warming the product to room temperature, these changes were magnified: compared with the spectrum before irradiation the extinction had risen 1.5 times and the λ max had shifted about 5 nm toward longer wavelengths, arriving close to the λ max of all-trans retinene." It is my feeling that most of the actual isomerization occurred on warming, and that an "active intermediate" (probably our free radical) might have been generated by the irradiation. Futher evidence that at low temperatures one observes a slow generation of an intermediate and not rapid intermediate formation nor actual isomerization is the already cited fact that in these authors' experiments (using a 250 watt AH 5 irradiation source whose emission in the near UV where retinal absorbs is several orders of magnitude greater than that of our incandescant source), the retinal spectrum continued to decrease over one hour of irradiation (Fig. 18), and the retinal spectrum did not return until the sample was rewarmed. As seen in Fig. 14, much of the 13-cis retinal is probably still not isomerized even after 50 minutes of irradiation; viz, equilibrium has not been attained yet. The greater ease of formation of the free radical from the residual 13-cis retinal therefore could account for the discrepancy between this curve and those from the other isomers examined in Fig. 14, even after 50 minutes of irradiation.

If, in this same figure, one examines signal height as a function of solvent, one might generalize that the lower the dielectric constant of the liquid solvent, the better the yield of free radical in the corresponding frozen samples. In the preceding statement, I mention the dielectric constant of the <u>liquid</u> solvent, because, as the temperature is dropped to 77° K, dipoles are frozen such that they cannot rotate and therefore, the dielectric constant of substances falls sharply. This is true even for substances such as water which have large dielectric constants at room temperature. I therefore make the assumption that the dielectric constants of all of the solvents are very small at these temperatures and hence not too different. Dielectric constants are also important since the dielectric constant of the solvent can affect cavity sensitivity ("cavity Q") as well.

The blackening observed in the case of carbon tetrachloride solutions may be related to the possibility that carbon tetrachloride may be reacting with the free radical (see Walling, 1957, as quoted by Samller, 1960, p. 319).

The relatively slow rate of rise of the slope in Fig. 14, showing the signal height versus time, was at first disquieting, suggesting that perhaps free radical formation was an unlikely event (e.g., one requiring simultaneous impact of two light quanta). This possibility was rendered less likely by the use of neutral density filters (a 25% transmission filter being 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. It is most likely, however, that one is dealing here with self-screening of incident radiation by the highly absorbing carotenoid solutions, since Brown and Wald (1956) had encountered similar difficulties in light penetration, while employing light to isomerize all-trans retinal to a mixture from which they could extract the neo-b (11-cis) isomer. They calculated that, "...at the absorption maximum, the most efficient wavelength for isomerization, the light falls to 10% of its incident intensity after penetrating a layer only 0.16 to 1 micron deep" (p. 868). A similar calculation for my experiments indicates that the light intensity at the absorption maximum is decreased to 10% of its incident value within 200 micra of the surface, based on $\epsilon_{-65^{o}} = 47{,}000$ for all-trans retinal (Hubbard and Kropf, 1959). Thus the slow rise in EPR signal intensity may have reflected the low statistical likelihood of reaction within the main volume of the samples due to severe attenuation of the incident light with little penetration beyond the surface. Further support for this comes from later experiments with liquid propene-propane solutions wherein the efficiency of light appears much greater, perhaps due to a greater number of carotenoid molecules reaching the irradiated surface per unit time as a result of more rapid diffusion and/or convection.

Although the rate of rise of signal height as a function of time (Fig. 14) was small for all isomers as indicated earlier, it was least in the case of 9-cis retinal and greatest in the case of 13-cis retinal. As mentioned earlier, this implies that the 13-cis isomer is most easily induced to form a free radical and the 9-cis and all-trans are less easily induced to form free radicals. This, as will be discussed later, is similar to the relative order which Rosenberg found for photoconduction of the various isomers (Rosenberg and Harder, 1967) and to that which Hubbard found for the ease of isomerization.[106] From the preliminary studies on charge transfer already discussed, it appears to correlate as well to the ease of formation of charge transfer complexes. (Although the order of bond lability and therefore ease of isomerization (11-cis $>$ 13-cis $>$ 9-cis $\cong$ all-trans) should also hold at lower temperature, the discrepancy between the 11-cis and 13-cis should be considerably reduced at lower temperatures since, as Jurkowitz _et al_ (1959) have shown, the steric hindrance of the 11-cis is markedly decreased at low temperatures, thereby allowing it to increase its stability somewhat by more closely approaching planarity.)

The charge-transfer (carotenoid-electron acceptor) experiments are taken to imply that the species whose spectra we are observing in the case of the pure carotenoids are those of the carotenoid anion free radical, since as Pullman (1960) concluded after calculating resonance energies, bond orders, and electronic charges, these substances can be both good donors and good acceptors of electrons. It is also rather ususal where a reaction produces a free radical anion and cation pair to see only one of the pair by EPR (Manzerall and Feher, 1964). Therefore, if a stronger acceptor than the carotenoid itself is made available,

---

[106] Hubbard, R. _et al_. _op cit_., (1953), 415-429; Hubbard, R., _op. cit_., (1956a), 4662-4667.

the released electron would be expected to be taken by this stronger acceptor, converting the acceptor to a free radical anion, as in the case of the chloranil and tetracyanoethylene. That the carotenoid-electron system can also act partially as a redox system is implied by the experiment with the hydrochloranil, showing that a small amount of this substance was oxidized to chloranil, which then served as an electron trap.

The "regeneration" of the rhodopsin signal deserves mention, since after the frozen solution of rhodopsin in digitonin has been irradiated and subsequently rewarmed in the dark to room temperature and frozen, there remains some rhodopsin, but the solution also contains a considerable amount of all-trans retinal. When it is recooled and reexposed to light and a very small signal is again formed, one cannot say whether this is due to the remaining rhodopsin or to the previously released retinal, but in reality, this is unimportant since both are capable of yielding paramagnetic species on illumination.

The persistance of the rhodopsin signal at room temperature for a short time remains an enigma which is still unexplained. It is possible that this or indeed the entire rhodopsin signal may be due to an impurity, but I feel, (for reasons which will be described later) that the rhodopsin signal is probably real. I cannot, however, satisfactorily explain the persistance of the signal at room temperature at this time.

Dawson (1962) and others[107] have shown that the triplet

---

[107] Abrahamson, E. W., Adams, R. G., and Wulff, V. J., "Reversible spectral changes in retinene soltuions following flash illumination," (1959), J. Phys. Chem., 63, 441-443.

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-excited molecule may lose its energy by phosphorescence (although Dawson, 1962, could detect no phosphorescence from retinal), or by non-radiative (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, as noted earlier, it is not uncommon for the triplet state to be a precursor of free radical formation.[108]

However, in our experiments retinal and retinol frozen at -196°C were induced to form their characteristic free radicals by irradiation with near u.v. and 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. As noted earlier, Pullman (1960) calculated resonance energies, bond orders and electronic charges for these compounds and concluded that they can be both good donors and acceptors of electrons. Such a mechanism of radical anion formation from the triplet state is probably the most likely explanation of the retinal and rhodopsin paramagnetic resonance, and the EPR investigation of the characteristics of charge-transfer complexes of retinal and retinol further supports

---

[108] Sogo, P. B. et al. _op_. _cit_., (1957), 387-393; Illen, D. F. and Calvin,M. _op_. _cit_., (1965), 3760-3766.

the conclusion that a free readical anion is the species whose spectrum was observed. The fact that at $10^{-3}M$ the retinal molecules, if evenly distributed, would be separated by an average of approximately 120 Å, makes one consider the possibility of electronic interaction with solvent, but the failure to demonstrate a free radical from retinal in the presence of chloranil, and the demonstration of the chloranil free radical instead argues in favor of electron transfer by some process between retinal and chloranil in this case, and between two molecules of retinal in the absence of chloranil. Since the solvent is present in large excess, if the reaction were electron acceptance from solvent, the presence of chloranil should not completely inhibit it. One may, therfore, conclude that the retinal molecules in their micro-environments probably are not uniformly distributed within the "glass", although, because of the speed at which the samples were cooled and the opacity of the "glass" no visible precipitation was observed.

Just as non-polar solvents are best for free radical production from retinal, Dawson (1962) and Grellman <u>et al</u> (1962) have pointed out that the relative percentage of triplet yield is also best in non-polar solvents and decreases as the polarity of the solvent increases. This, Dawson and Abrahamson (1962) point out, is because retinal forms hydrogen bonds with hydroxylic solvents, and the hydrogen bonded complexes are less easily promoted to the triplet state, but those in this state live longer because the hydrogen bonded triplet has a longer lifetime.

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. As noted above, 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 (and water) (Handbook, 1964). However, the relationship of liquid state polarities to the dielectric environment in the frozen solutions may be complex. The solvent influences product yield as well as yield of triplet, Brown and Wald (1956) having shown that more cis isomers are produced when retinal is irradiated in a polar solvent than is the case with a non-polar solvent. In the presumed non-polar environment of the lipid membrane of the rod cell's outer segment disc, it is perhaps not surprising that the major product of irradiation is the all-trans isomer.

That the retinal in 2% aqueous digitonin behaves as if it is in a somewhat non-polar environment was shown both by the finding that a signal was still detactable in this medium (although barely), and the study by Hubbard, Bownds and Yoshizawa 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 non-polar 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 non-polar environment. A similar relationship may well obtain between the retinal and opsin..."[109]

There is some evidence for retinal's having an even more non-polar environment in rhodopsin that in digitonin. While the retinal EPR signal is just barely detectable in 2% digitonin (and we could not be truly certain of its presence in all runs), it was behond a doubt present in 2% digitonin solutions of rhodopsin, although considerably smaller than in solutions of retinal in non-polar solvents such as heptane. In fact, using optical absorption spectroscopy, Grellmann was able to detect the retinal triplet in non-polar solvents with ease (up to 28% yield), but could not detect it in 1% aqueous digitonin solutions[110]. Another factor, however, leading to a larger signal from rhodopsin than from corresponding concentrations of all-trans retinal in digitonin is that the retinal isomer in rhodopsin is the more labile 11-cis, which presumably may be more easily induced to form a free radical.

Rosenberg (1958, 1966) and his associates (1961) have shown beta-carotene and dried rod outer segments to be photconductive, 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 beta-carotene will form free radicals on

---

[109] Hubbard, R. et al. op. cit., (1965), 301-315.

[110] Grellmann, K-H., Livingston, R. and Pratt, D., "A flash photolytic investigation of rhodopsin at low temperatures," (1962), Nature, 193, 1258-1260.

irradiation with visible light[112].

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

a) C=C-C=C-C=C (cis)
↓ hν
b) ·C-C=C-C=C-C· (one of several possible contributions
↓ Δ to the triplet state)
c) ·C-C=C-C=C-C· (90° twist)
↓
d) C=C-C=C-C=C (trans)

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 promotion of an electron to a triplet oribtal 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. However, as suggested earlier, the <u>in vivo</u> environment of the retinal molecule may be such as to alter its electronic character and thereby enhance the likelihood of triplet formation. Alternatively, the isomerizing intermediate might be the free radical itself, formed from an excited singlet precursor according

---

[112] Smaller, B., "Photoinduced free spin species in plant pigments," (1960), Free Radicals in Biological Systems, 315-323.

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 EPR 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 EPR data are not inconsistent with there being similar but not identical excited states for the different isomers, and these may give rise to either similar or different free radical products.

The retinol free radical was, as indicated earlier, less easily generated by our system than that from retinal. The reasons for this are at least partially evident. First, the absorption maximum of all-trans retinol is approximately 335 nm in a low temperature glass, while that of all-trans retinal is 385 nm, and I was irradiating through glass lenses with an incandescent source, resulting in many fewer quanta at 335 nm than at 385 nm. Thus, the most effective light presumably is that small area of overlap at the upper wave lengths of the source. Secondly, the retinol signal is about 210 gauss wide (see footnote),[106]

---

[106] The anisotropy of triplet state magnetic resonance gives rise to very broad EPR 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.

(Fig. 15) compared with 23 gauss for retinal, necessitating many more unpaired spins before the signal can be detected, since the number of spins is proportional to the product of the height times the square of the width. Since the retinol signal is about nine times as wide as the retinal signal, this alone accounts for a factor of about 81. Yet another possible contributing factor, the fluorescence of retinol, will be dealt with shortly.

Although at the present I can find no biological correlation between the formation of a free radical from retinol and its biological function (since, for example, the mechanism of its role in maintaining the integrity of epithelial structures is completely unknown), the nature of the spectrum itself is intriguing from a chemical point of view. The signal is extremely wide (ca. 210 gauss--see Fig. 15) in contrast to the "normal", ca. 23 gauss signal from the retinals and rhodopsin. This latter width corresponds exactly to the expected signal width from a hydrocarbon free radical whose unpaired electron interacts with a majority of protons (Bersohn, 1963; Ingram, 1958). 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, I am 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 retinal 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"[113].

Since excitation energy localized to a part of a molecule not strongly coupled to the matrix is less apt to be dissipated by non-radiative decay, localization and relative sequestration of excitation energy by retinol is also suggested by its fluorescence (at room temperature) following near-ultraviolet light stimulation. On the other hand, ultraviolet-irradiated retinal (at room temperature) discharges its excitation nonradiatively, via triplet states or other mechanisms, including chemical pathways. Indeed, it has already been noted that in the presence of iodine

---

[113] Dartnall, H. J. A., "Indicator yellow and retinene," (1948), Nature, 162, 222.

and light all isomers of retinal isomerize faster than the corresponding isomers of vitamin A (Hubbard, 1956a). Functionally, therefore, the delocalization indicated by the EPR and fluorescence data may suggest a 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 (aqueous) 2% digitonin instead of one of the non-polar hydrocarbons that enhanced photosignal formation (Fig. 16) (although no doubt the one hundred-fold lower concentration of carotenoid in the rhodopsin solutions also contributed to the difference.

It is extremely difficult to tell how long the signal persists at room temperature since it is at the lower limits of detection when it is maximal, and when it diminishes it is barely perceptible. The extreme weaknesses of the signal is in agreement with the findings of Pitt and Tinkham (1965), and further study employing a computer of average transients at low temperature might prove very profitable.

Since the lamellae of the rod outer segments contain a great deal of lipid (Wolken, 1961), rhodopsin *in vivo* is almost certainly in a relatively non-polar 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. Nevertheless, I was unable to demonstrate an EPR signal from suspensions of rod outer segments in sucrose, presumably because of the semi-opaque nature of the suspension in contrast to the translucent rhodopsin glass, 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 (Fig. 16) could be obtained in any case.

The inability to elicit an EPR signal from the outer segment preparations is in agreement with the finding of Pitt and Tinkham (1965), who also were unable to demonstrate a signal from freeze-dried rod outer segments irradiated at 90° K. However, as was noted earlier, time-averaging techniques enabled Polis and Wyeth (1962) to report the presence of EPR in illuminated rod outer segments and rhodopsin solutions at room temperature. It was also mentioned that these investigators were unable to demonstrate similar free radical signals from retinal and retinol, but it must be re-emphasized that they were working at room temperature, and perhaps these species decayed too rapidly at this temperature and thus escaped detection. Examining retinal, retinol, rhodopsin, and rod outer segments, we were unable to demonstrate EPR signals from any of these after irradiation at room temperature, and it is possible that the signal extracted by Polis and Wyeth using time averaging may be due to an impurity.

In the unpublished study of Lockhurst and Abrahamson (1965, cited by Abrahamson and Ostroy, 1967) which was referred to earlier, the authors, "...tried to detect ESR signals in carefully purified solutions of rhodopsin and rod segments suspensions at liquid nitrogen and dry ice temperatures. The results, however, were negative". They concluded that, "Such a result does not rule out electron transfer processes, but does render them rather unlikely candidates for the primary process"[115].

My results are in agreement with Polis regarding the generation of a free radical from rhodopsin, but as noted above, I, like the other authors, was unsuccessful in my attempts to confirm the Polis report of a signal from rod outer segments. This, more than any other factor, raises the question whether the free radical which I observed when retinal and rhodopsin molecules were examined in less physiological environments actually does play a role within the body, for within the rod outer segments the rhodopsin molecule should be in its natural (non-polar) environment. Could it be, as I have asked above, that my suspensions were too opaque (due to optical scatter) or too dilute to detect the free radical, or is the free radical from rhodopsin not generable in its physiological environment? Regarding the dilution, the optical density of the frozen rod outer segments appeared about the same (to the eye) as the frozen rhodopsin solution. However, even the signal from the frozen solution was very weak, as noted earlier. Because of the extreme weakness of the EPR signal from rhodopsin in contrast to the strong signal from the pure carotenoids, one must further consider the possiblity of its arising from an impurity. In an attempt to examine this, we have compared the relative signal height from irradiated rhodopsin

---

[115] Abrahamson, E. W. et al. op. cit., (1967), 179-215.

and retinal. This measurement was made with both samples examined under the same conditions, including the same power setting and the same modulation amplitude. The signal height was measured with a ruler and corrected for the gain setting. We have found (surprisingly) that the signal from the rhodopsin (per mole of retinal) after 40 minutes of irradiation is one to three times as large as that from the pure carotenoid. While this figure is of the correct order of magnitude, it is indeed surprising that the rhodopsin (in 2% aqueous digitonin) signal is larger (per mole of retinal) than that of the retinal (in heptane), considering the studies of signal height as a function of solvent (Fig. 14). As Hubbard et al. (1965) note, however, it is possible that the opsin provides a strongly hydrophobic environment for the retinal, similar to that in heptane. Furthermore, the more dilute nature of the retinal in the rhodopsin glass (approximately 10^{-5}M) makes a given irradiation time more efficient due to less self screening of incident radiation--a phenomenon which has been noted earlier to be significant in the 10^{-3} M retinal.

The question whether the rhodopsin free radical is truly from the rhodopsin or from an impurity is still unanswered, but the above data and the similarity of the free radical signal shape, width, and location to that of retinal lead me to believe that the signal is probably truly from rhodopsin. As to why we were unable to demonstrate a signal from rod outer segments, this question also still remains unanswered, but electrophysiological phenomena such as the early receptor potential (ERP) of the electroretinogram (ERG)[116] and the correlation (which the finding of a charged free radical species provides between the photoconductive theories of Rosenberg (1966) and photoisomerization theory of Wald (1958)) lead this author to believe that despite our inability to detect the free radical in the rod outer segments, it may still be generated there.

B. Implications for Vision

1. Signal Amplification and Nervous Stimulation

It is known that a dark adapted rod cell can be stimulated by the absorption of a single quantum of light.[117] Similarly, looking at the system electrophysiologically, "...the effects of light on a single molecule of rhodopsin...are enough to stimulate a dark adapted rod into the multielectron change recorded at the level of the a-wave of the electroretinogram and the propagated impulse".[118]

This is a small stimulus even for a single cell. Clearly the conversion of one molecule requires an amplification factor of "thousands of millions" (Wald *et al*, 1963) before it can effect even the earliest perceptible electrical response. Wald (1965) notes that a similar problem exists in photography where a whole grain becomes developable after one or a few photons is absorbed. He points out that in this case the mechanism is known,

---

[116] The ERG and the ERP are described more fully on page 150 ff.

[117] Hecht, S. *et al.*, *op. cit.*, (1941-2), 819-840.

[118] Hubbard, R., *et al. op. cit.*, (1965), 301-315.

viz. catalysis; one photon reduces a silver ion to an atom of metallic silver which serves as a catalyst for further chemical reduction and thereby results in conversion of the whole grain.

In biology, the clotting mechanism requires extremely large amplification between stimulus and response, an amplification which proceeds via a proenzyme-enzyme cascade whereby an inactive substance is converted to an enzyme with a high turnover number which catalyzes the production of another enzyme which, in turn, serves as a catalyst for the production of another enzyme, etc. This obviously results in considerable amplification. Accordingly, Wald compared visual excitation to blood clotting (Wald, 1965a), and had postulated a similar cascade for vision (Wald, 1954, 1956). He had proposed that rhodopsin might be a proenzyme or zymogen whose reactive site was covered by 11-cis retinal or by the folded protein, and on isomerization of the retinal and unfolding of the protein, the site might be exposed to catalyze the production of another enzyme.

While Wald had at first noted that, "...every instance of zymogen activation we know involves the breaking off from the inactive protein of a small fragment of its structure, one or a few amino acids in length, so exposing the active catalytic center,"[119] and had assumed that they hydrolysis of retinal from opsin might serve a similar "uncorking" function, it has been shown that this hydrolysis is both too slow (Wald and Brown, 1965)

---

[119] Wald, G., _op_. _cit_., (1958), 401.

and does not occur in some crustaceans, in which the isomerized carotenoid remains attached to the opsin in the fully bleached state.[120] Thus, if we assume a similar mechanism in man as in the crustacean, hydrolysis of the retinal from opsin cannot be significant. Indeed, vision is now generally believed to be triggered not by the bleaching of rhodopsin, but somewhere on the way to bleaching, such as its conversion to lumirhodopsin or metarhodopsin.[121]

No matter what the trigger mechanism, amplification is, as noted earlier, very necessary, and another suggestion regarding the mechanism of the amplification process is as follows: The transverse membranes in the rod outer segments are composed in large part of visual pigments, and the light conversion of a single molecule of visual pigment might result in a unimolecular hole in a membrane. If, for example, this hole allowed the flow of ions between two differently charged layers (as in nervous conduction), it would result in a measurable electrical phenomenon (Wald, 1958). Unfortunately, there is as yet no evidence for either this mechanism or for the enzyme cascade, as both opsin and retinal have been shown to be

---

[120] Wald, G. and Hubbard, R., "Visual pigment of a decapod crustacean: the lobster,: (1957), Nature, 160, 278-280; Hubbard, R. and St. George "The rhodopsin system of the squid", (1958), J. Gen. Physiol, 41, 501-528. Brown, P.K. and Brown, P. S., "Visual pigments of the octopus and cuttlefish," Nature, 182, 1288-1290.

[121] Hubbard, R. and Kropf, A., "The action of light on rhodopsin," (1958) Proc. Nat. Acad. Sci., 44, 130-130; "Molecular aspects of visual excitation," Ann. N.Y. Acad. Sci. 81, 388-398, (1959).

relatively inert.[122]

Yet another possibility is attractive, namely that retinal might be a biological trigger molecule like acetylcholine, but Wald (1959) has stated that he believes retinal to be a "bland, relatively inert substance, hardly capable of initiating a nervous excitation".[123]

The small changes which occur in the rhodopsin molecule, viz. the exposure of two to four sulfydryl groups and a proton binding group (probably the imidazole group of histidine), as noted earlier, also have not been shown to be directly connected to triggering the nervous impulse. Wald's finding that the sulfydryl groups were not yet detectable at the metarhodopsin stage, to which reference was made before, led him to conclude that the exposure of sulfydryl sites "cannot play a part in visual excitation" (Wald, 1964a, cited by Rosenberg, 1966). Similarly, the exposure of the proton binding group has tentatively been identified with the metarhodopsin I-to-metarhodopsin II

---

[122] Although McConnell and Scarpelli (1963) have suggested that rhodopsin in the dark can act as an enzyme (not a proenzyme), an ATPase, the turnover rate is not sufficiently different in light and in darkness to be significant in the visual process (Rosenberg, 1966), and the ATPase activity which McConnell and Scarpelli found has been attributed by others (Frank and Goldsmith, 1965) to impurities in their preparation, since these latter authors found the ATPase activity of pig retinas exclusive of rod outer segments to be roughly the same per unit dry weight as that of the segments alone, and they also noted that digitonin extraction effects a partial separation of rhodopsin and ATPase activity. Opsin, however, has not been shown to be an enzyme.

[123] Wald, G., "The photoreceptor process in vision," (1959), Handbook of Physiol., Section I, Vol. I, 671-692.

transformation (Matthews et al, 1963-64), and as such is probably not involved in triggering the nervous excitation either. Since it has been shown that the generation of the early receptor potential (ERP) of the electroretinogram occurs prior to metarhodopsin I (Pak and Ebrey, 1965), the exposure of the proton binding group cannot be significant for the ERP, and if the ERP is significant for later electrical events, this exposure then cannot be significant with regard to nervous stimulation, as concluded above. Hubbard, Bownds, and Yoshizawa (1965) believe that,

> "...it is more attractive to think of changes in the distribution of charges on opsin as a source of excitation, rather than of the exposure of specific reactive groups. Recent discussions of enzyme action have stressed the role of conformational changes as modulators of enzyme activity (Lumry and Eyring, 1954; Koshland, 1963; Schachman, 1963; and others). This type of effect, for example, now believed to be responsible for the Bohr effect in hemoglobin, a physiological expression of the fact that hemoglobin becomes a stronger acid upon combination with oxygen.... An allosteric transition....would not necessarily involve a conformational alteration _sensu stricto_. It might conceivably be due, for instance, to a redistribution of charge within the molecule without detectable alteration of its spatial configuration... In this terminology we might think of the retinal _chromophore_ as the allosteric effector, which upon stereoisomerization induces an allosteric transition in opsin--initially perhaps only a redistribtuion of charges.... The electrical signal might be triggered by the very first of these, and hence might even precede frank conformational alterations."[123a]

The demonstration that the effect of light on retinal is to induce formation of a charged free radical species is thus attractive in that it provides one means of creating charge redistribution on

---

[123a] Hubbard, R. _et al._, _op._ _cit._, (1965), 313.

the retinal and presumably this would occur also when the retinal was combined with rhodopsin. (Although the free radical signal appeared the same in rhodopsin and retinal I cannot be sure how similar the represented species were, since, as noted earlier the low temperature at which the system was studied makes it very difficult to observe "fingerprinting" fine structure.)

While retinal and opsin are acknowledged to be "bland and inert", free radicals, especially charged free radicals, are in general known for the opposite characteristic, and the demonstration of a free radical in the rhodopsin system is the first demonstration of a truly reactive species in this scheme. The possibilities for charged free radicals to depolarize biological membranes, or for amplification via a free radical chain reaction are therefore brought to the forefront. Furthermore, it is necessary that any triggering substance be rapidly generated and rapidly destroyed. At nervous synapses acetylcholine is postulated to be rapidly liberated from small vesicles, and it is then rapidly destroyed by acetylcholinesterase. If indeed, a free radical is the trigger mechanism, the naturally short life of the free radical (due to its high chemical reactivity) would obviate the necessity for a separate destructive mechanism.

2. Photoconduction versus Photoisomerization

As described earlier, Rosenberg has shown that a current many times the dark current will flow if a carotenoid is melted and allowed to solidify between two electrically conductive glass plates across which a potential has been placed, and this system is then irradiated with a tungsten lamp (Rosenberg, 1958, 1966). This photoconduction is felt, by Rosenberg, to be primarily due to positive holes acting as charge carriers, since irradiating the anode is 10^5 times as effective as irradiating the cathode. Rosenberg et al (1961) demonstrated this phenomenon on dried sheep rods and found that the photoconduction excitation spectrum corresponded roughly to the absorption of the alpha and beta bands of rhodopsin. Based on his findings, Rosenberg has proposed a photoconduction theory of vision which postulates that visual excitation is initiated by the creation of a mobile electronic charge carrier from the excited chromophore, resulting in a current (Rosenberg, 1966). He recently studied photoconduction in the retinals (Rosenberg and Harder, 1967) and found that 11-cis and 13-cis retinal exhibited photocurrents that were greater than the dark currents "by at least an order of magnitude", and that the 13-cis retinal m-nitroaniline hydrogen chloride, a Schiff base model of rhodopsin, was even more photoconductive. On the other hand, 9-cis retinal was only slightly photoconductive and all-trans retinal was not measurably photoconductive. (Perhaps, if they had irradiated longer and with greater light intensities, they might have been able to detect some photoconductivity from this isomer, since his work with beta-carotene (Rosenberg, 1966) showed that the hindered 11', 12', 11, 12, di-cis beta-carotene is approximately 10^4 times as photoconductive as is all-trans beta-carotene and around 10^2 times as photoconductive as the unhindered 15, 15' mono-cis beta carotene.) The increase in conductivity of the 13-cis retinal may, Rosenberg and Harder note, be due to its more efficient absorption of white light due to its absorption maximum at 555 nm instead of 380 nm.

Rosenberg states that, "the very low photocurrents of 9-cis retinal are neither understood nor expected because the other cis isomers are fairly good conductors,"[124] but as shown by Hubbard (1956a), the 9-cis isomer, like the all-trans isomer, is a relatively stable species which does not isomerize readily. Similarly, this study demonstrates that the 9-cis isomer, like the all-trans, is less readily induced to form a free radical than is the 13-cis. In agreement with this, preliminary experiments on the ease of formation of charge-transfer complexes appear to indicate that the 13-cis isomer of retinal forms charge-transfer complexes more readily than the all-trans or 9-cis isomers, but this experiment must be repeated in order to confirm the results as it has been carried out only twice. It is at once striking that the order 13-cis, 9-cis, all-trans holds for ease of isomerization, photoconductivity, photogeneration of free radicals and perhaps charge-transfer complex formation. It is thus possible that these processes might share a common mechanism. For example, if isomerization proceeded via a free radical pathway and these free radicals were charged species (as those produced from

---

[124] Rosenberg, B. and Harder, H. C., "Semiconduction and photoconduction activation energies of the retinals," (1967), Photochem. Photobiol., 6, 629-641.

carotenoids appear to be), then these free radical ions may be the charge carriers responsible for Rosenberg's photoconduction. The fact that photoconduction is 10^5 times as efficient when the anode is irradiated further supports this possibility. If, as postulated, light assists isomerization by driving off an electron as a primary process, then an anodic current might be synergistic in helping to remove the electron while a cathodic current would be expected to be antagonistic.

If the free radicals from the retinal prosthetic group are indeed the same species responsible for photoconduction, this would tend to resolve some of the conflicting points between Rosenberg's photocoductivity theory of the visual process and Wald's isomerization theory. "Photoisomerism and subsequent dark reactions leading to bleaching are postulated (by Rosenberg) to be part of an adaptive mechanism which is competitive with the (photoconductive) receptor mechanism instead of being the initiator of visual excitation itself."[125] Thus, if the photoisomerization of 11-cis retinal in rhodopsin to the all-trans isomers of the subsequent intermediates proceeded via a pathway which involved ionic free radicals similar to those produced from retinal, the two processes of photoconduction and photo-isomerization could go on <u>pari passu</u> rather than competitively. Rosenberg (1958a) has suggested a triplet state model of photoconduction for his system in which it is postulated that the triplet state serves

---

[125] Rosenberg and Harder, <u>op. cit.</u>, (1967), 629.

as a necessary kinetic intermediate in the creation of a mobile charge carrier, but this postulation is based on a comparison with other systems rather than on demonstration of a triplet in this (photoconductive) system.

3. The Spectra of Receptor Pigments for Color Vision

The spectrum of each of the visual pigments is composed of three main extinction bands--one in the visible (around 500 nm in rhodopsin), a second in the near ultraviolet (the cis peak), which has 20 to 30% of the height of the main band (and lies around 340 nm in rhodopsin), and an ultraviolet peak at approximately 280 nm in all of the pigments.

The visible peak is called the alpha peak, the cis peak is designated beta, and the ultraviolet peak is called the gamma peak. The alpha and beta peaks are separated by an almost constant distance in wave numbers (9700 ± 400 wave numbers), suggesting that they both arise from the same structural component of the molecule. Light absorbed by either the alpha or beta band is capable of bleaching the molecule, while that absorbed by the ultraviolet peak (attributed to aromatic amino acids of the protein moiety) does not result in bleaching.

One of the most puzzling facets of research into the biophysical problems of vision has been the explanation of the λmax of the visual pigments--how retinal, which has a λmax of 384 nm in aqueous digitonin (Dartnall, 1962b), results in a compound with λmax of 498 nm in cattle rhodopsin (Dartnall, 1962b), one with a λmax around 438 nm in human blue-sensitive pigment or cyanolabe (Wald, 1964), one with a λmax between 548 nm (Wald, 1964) and 535 nm (Wald and Brown, 1965) in human green-sensitive pigment (chlorolabe), and one with a λmax between 565 and 580 nm in human red-sensitive pigment or erythrolabe.[126]

The hypothesis that the increased λmax was accounted for by more than one retinal molecule per rhodopsin,[127] and hence elongation of the conjugated system, was ruled out by Hubbard's demonstration (1954) that there is only one retinal molecule per rhodopsin. Noting this problem, Dartnall (1962b) inquires, "Is the answer to be found in Hubbard's (1958) conjugate acid hypothesis or by supposing that there is an extension of conjugation from the isoprenoid chain into the polypeptide?"[128] Currently, neither of these possibilities is thought to be an adequate explanation,[129] and if all these compounds have only one chromophore of 11-cis retinal, Dartnall (1962b) concludes that, "...one must place the whole burden of this versatility on the protein moiety".[130]

---

[126] Thompson, L. C., _op_. _cit_., (1947), 368-377; (1951), 114-132; Hsia and Graham, _op_. _cit_., (1952), 80-85; Wald, G., "The receptors of human color vision," (1964), _Science_, _145_, 1007-1017; Wald and Brown, _op_. _cit_., (1965), 345-361.

[127] Wald, G., "Photochemistry of vision", (1949), _Doc. Ophalmoloica_, _3_, 94; Collins, F.D. and Morton, R.A., "Studies on rhodopsin, 3: Rhodopsin and transient orange," (1950a), _Bioch_. _J_., _47_, 18-24.

[128] Dartnall, H. H. A., _op_. _cit_., (1962b), 427-471.

[129] Wald, G., (1967), personal communication.

[130] Dartnall, H. J. A., _op_. _cit_., (1962b), 427-471.

At present, there are two main theories which try to account for the bathochromic (red) shift of the spectrum of rhodopsin from that of retinal. One, the Morton-Pitt-Kropf-Hubbard theory (MPKH, as designated by Abrahamson and Ostroy, 1967), assumes that the combination of retinal with opsin as a Schiff base linkage shifts the λmax to about 367 (Hubbard, 1958), and the protonation of this Schiff base raises the λmax to about 440 nm.[131]

The further shift of the λmax to 498 nm is felt to be associated with the lowering of the energy level of the excited state due to the interactions between the 11-cis retinal and components of opsin, such as negatively charged groups adjacent to the polyene chain (Kropf and Hubbard, 1958). This assumes that optical excitation induces a transition dipole moment along the chain in which an electron moves toward the positively charged nitrogen. In this case, the energy of the transition would be lowered by properly placed negative groups along the way, thus resulting in a red shift.

The second theory is Dartnall's lock and key theory[132] which assumes that the primary Schiff base linkage is not protonated, and that secondary binding of the polyene to the lipoprotein backbone is accomplished by a pair of charges on opsin, one at either end

---

[131] Ball, S., Collins, F. D., Dalvin, P.D. and Morton, R.A., "Studies on vitamin A, 11: Reactions of retinene, with amino compounds", (1949), Biochem. J., 45, 304-307.

[132] Dartnall, H.J.A. and Lythgoe, J.N., "The spectral clustering of visual pigments," (1964), Visual Res., 5, 81.

of the polyene chain such that a permanent dipole is induced by polarization of the chain: this would result in dipole-dipole binding of the carotenoid to the lipoprotein such as the interaction of sulfhydryl groups with the pi electron cloud of retinal to form new orbitals, thus changing both the ground and excited states, and therefore producing shifts of λmax (and, perhaps, altering the ease of triplet generation, as suggested earlier).

Rosenberg's recent work with models employing substituted anilines (Rosenberg and Krigas, 1967) point up the fact that the λmax values in the protonated Schiff bases can be red shifted by increasing the positive charge on the nitrogen of the $C=\overset{+}{N}$ bond due to the inductive effect of substituents on the ring. This would tend to support the MPKH theory that the Schiff base linkage is protonated, as does the work of Blatz (1965) on the red shifts produced by protonation of polyenes. One should note, however, that the range over which Rosenberg could vary the spectra by changing the ring substituents was small, the extremes of λmax being 490 and 530 nm, as compared with 430 to 580 nm which are the limits of the range of observed (human) visual pigments. This latter range is presumably due to variation of the interactions between retinal and the various photopsins--between the carotenoids and various electron acceptors.

The variety of colors which characterized the charge transfer complexes presents another interesting model for explaining how a chromophore with λmax of approximately 380 nm (depending on the solvent and the temperature) results in substances whose peak abosrption lies anywhere in the range of 430 to 580 nm. Unfortunately, the charge-transfer complexes were observed mainly at low temperature in the solid phase, so absorption spectroscopy was not done, but the complex of retinals with chloranil was a blue-green, and that with TCNE was red. While electron donor and acceptor properties are certainly important, as was shown by Rosenberg with the substituted anilines, it is also well-known that fit and proximity are also important in determining absorption maxima (due to the effect on electronic energy levels). It is known that fit and proximity are very important in determining the characteristics of charge-transfer complexes, as are electron-donor and acceptor properties. Similarly, it is known that configuration is very important for determination of the spectral properties of retinal-protein complexes: both indicator yellow and rhodopsin seem to possess retinal attached to opsin via a Schiff base linkage but their λmax values differ considerably (acid indicator yellow being about 440 nm, alkaline indicator yellow about 365 nm, and rhodopsin about 498 nm). Perhaps, then, as Rosenberg and Kriga's study and my own observations suggest, charge transfer may explain the puzzling λmax shifts of the visual pigments, different proteins forming different charge-transfer complexes with retinal and thus having different spectra, and the same protein forming a different charge transfer complex with retinal at different times, depending on the configuration of the protein (i.e. the state of unwinding) and the geometrical configuration of the retinal, both of which affect the closeness of the fit. As the bleaching process proceeds, the general trend is toward blue, (except for pre-lumirhodopsin), due to changes which progressively worsen the fit, as Hubbard proposes. The Harvard group's explanation for the red-shifted spectrum of pre-lumirhodopsin (λmax 517 nm) is that it is unstable, the strained condition of the molecule due to the poor fit between the all-trans retinal and opsin resulting in a large rise in the energy of the ground state, shortening the transition to the first excited state, and thus resulting in a shift in its spectrum toward the red.[133]

4. Early Receptor Potential of the Electroretinogram

The electroretinogram (ERG) is a recording of the mass potential produced when light strikes the retina and the nervous elements within it. It is recorded with the sensing electrode on the cornea or in the vitreous and the reference electrode on the skin or, in the isolated eye, on the back of the sclera. With electrodes placed in this manner, the recording convention is that positive potential deflections are recorded as up, negative deflections downward. Since the study of ERG has been quite detailed, I shall not attempt to review it here, but rather will present a brief outline to serve as background for subsequent discussion of the early receptor potential. Good recent reviews have been published by Granit (1962) and by Brindley (1960).

The various components of the vertebrate ERG have been

---

[133] Yoshizawa, T. and Wald, G., *op. cit.*, (1963), 1279-1286.

labelled the a-, b-, c- and d- waves. As Brindley (1960) points out, ERG's from all vertebrates are quite similar, except that the d-wave or "off-effect" is more prominent in non-mammalian species. With regard to the origin of the components, he goes on to point out that since a micro-electrode penetrating the retina from the inside out records no changes in the amplitude of the ERG as the electrode passes through the ganglion cell layer, these cells contribute nothing to the ERG. This point of view is held as well by Granit (1959).

Although Brindley believed that both the a- and b-waves originate in the same retinal layer, the bipolar cells, Brown and Wiesel (1961), using micro-electrodes, demonstrated that the a-wave originates in the rods and cones while the b-wave originates slightly distal to the inner nuclear layer (in the bipolar cells--see review of retinal anatomy in the introduction). The present feeling is that the c-wave, which is a slow positive potential outlasting the light stimulus, originates in the pigment epithelium; although it requires the receptors of the retina as well, Noell (1953) having found the c-wave to be abolished by intravenous sodium iodate which destroys the pigment epithelium preferentially, and Tomita (1959) having noted the absence of the c-wave in the isolated amphibian retina (removed from the pigment epithelium).

Recently, Brown and Murakami (1964a, 1964b) have described a very different type of potential, recorded from monkey retinas, called an "early receptor potential" or ERP. It preceeds the conventional ERG in its entirety, having a latency too small to be measured accurately, but reported to be less than one-half of one micro-second (Cone, 1967). Since the voltage of both the ERG and the ERP depend in a complex way on flash intensity, the only definite statement that can be made regarding their relationship is that the ERP is considerably smaller (orders of magnitude under comparable conditions), and, unlike most neurosensory responses which, in order to have a large range, increase with the $\log_{10}$ of stimulus intensity (approximately), the ERP is linear with stimulus intensity (Cone, 1964).

This potential is biphasic, having its large component of the same polarity as the a-wave. As noted earlier, it precedes the a-wave in its entirety and is obtained only with very high intensity flashes. Brown and Murakami (1964a) feel that this potential is the direct result of light quanta striking rhodopsin, and if so, it must be the earliest consequence, in electrical terms, of the light stimulus on the retina. Their work has been extended by Pak and Cone (1964) and by Cone (1964, 1967) to several other species, and the shape and latency were more clearly determined. Cone (1964) plotted a spectral sensitivity curve for the early receptor potential and found it to correspond to the rhodopsin absorption spectrum. This provided for the evidence that the early receptor potential is indeed closely related to the effects of light on rhodopsin. He has also written a recent review on the ERP (Cone, 1965), which is nicely supplemented by that of Brown _et al_. (1965).

In addition to its action spectrum, another reason for linking this response closely to the initial action of light on the rhodopsin molecule and not to the nervous phenomena which occur when the retina responds to light is its extremely short latency. As noted earlier, Cone reported that the latency of the ERP "...is definitely shorter than 0.5 microseconds at 25° C"[134], as opposed to the latency of the a-wave, for which Brown, Watanabe and Murakami (1965) estimate that 1.7 milliseconds is close to the minimum possible latency. These authors go on to state that,

> "The extremely short latency of the early RP indicates that it is triggered by a rapid change of molecular form, rather than by the much slower splitting of the photopigment molecule (the true bleaching reaction). This is also indicated now by study of the early RP at low temperatures (Pak and Ebrey, 1965)."[135]

Other evidence suggesting the direct connection between the ERP and rhodopsin are the previously mentioned linearity of the ERP with light intensity (Cone, 1964), its lack of sensitivity to anoxia (Brown and Murakami, 1964a), and its persistance when the retina is immersed in high concentrations of sodium and potassium salts (Brindley and Gardner-Medwin, 1965, 1966) which destroy membrane conductivity. These latter authors also noted that even soaking the retina in formaldehyde was insufficient to abolish the ERP and, similarly to other workers in this field,

---

[134] Cone, R. A., "Early receptor potential: Photoreversible charge displacement in rhodopsin", (1967), Science, 155, 1128-1130.

[135] Brown, K. T., Watanabe, K., and Murakami, M., "The Early and late receptor potentials of monkey cones and rods," (1965), Cold Spring Harbor Symposia in Quant. Biol., 30, 483-504.

concluded that, "...the early receptor potential depends on displacements of charge within the monolayers of visual pigment as they undergo a sequence of chemical changes after absorbing light."[136]

The lowest temperature at which Pak and Ebrey (1965) demonstrated the ERP was -35° C, but this was the lowest temperature they examined. Although decreasing in amplitude, perhaps it exists at even lower temperatures, and if the assumption is made that it is a reflection of the separation of charge on rhodopsin, it should indeed exist at lower temperatures. As Pak and Ebrey point out, the mere fact that it is still demonstrable at -35° C rules out the steps below metarhodopsin I for its production, since these steps require higher temperatures. These authors also rule out the first step, however, that of rhodopsin being converted to pre-lumirhodopsin, because it requires photons of "several ev". The reasoning here is that the R_1 potential (the first part of the bi-phasic ERP) has a Q_{10} of about 3, and since 10° C corresponds to only about 10^{-3} ev, they feel this is too insignificant a change to affect this reaction, and thus rule out the reaction as the cause of the ERP. However, the approximately 2.5 ev/photon, corresponding to 498 nm light absorption, is equivalent to about 57 kcal/mole for this first reaction. Hubbard et al. (1965) state that the activation

---

[136] Brindley, et al, op. cit., (1965), 1P; Brindley, G.S. and Garden-Medwin, A.M., "The origin of the early receptor potential of retina", (1966), J. Physiol., 182, 185-194.

energies for the next two steps in the bleaching of rhodopsin are: Pre-lumirhodopsin to lumirhodopsin, 10 kcal/mole; and lumirhodopsin to metarhodopsin I, 60 kcal/mole. Although 10^{-3} ev/photon (0.029 kcal/mole) is an insignificant fraction of 57 kcal/mole; it is also a small fraction of 10 or 60 kcal/mole. I therefore feel that on the basis of the Q_{10} alone, one cannot rule out the first step in the photochemical conversion of rhodopsin as opposed to the next two steps, but I must agree with the postulation of Brindley and Gardner-Medwin (1966, p. 185) that, "...at least two successive movements of charge must occur, *the second of them strongly influenced by temperature.*"

Since the ERP was not yet discovered in 1962, Rosenberg (1962) had considered that perhaps the ERG was due to charge separation on rhodopsin (a supposition which we know not to be true). Currently, Cone (1967) and Hagins and McGaughy (1967), in addition to Brindley and Gardner-Medwin (1966, cited above), believe as does this author, that the ERP is probably due to charge separation on the rhodopsin molecule. Since I propose that incident light generates charge separation on rhodopsin by means of the creation of free radical ions, these may be the charged species responsible for the ERP.

CONCLUSIONS

While I have succeeded in demonstrating that free radical species can be elicited from retinal, retinol and perhaps rhodopsin under suitable conditions, and that it is very plausible that similar species are generated during the visual process, a great many questions still remain to be answered. First and most important, this species has not been demonstrated *in vivo*--nor even in rod outer segments. This constitutes the single weakest link in the chain, but an *in vivo* test is currently well beyond the physical means at our disposal, and there are many possible reasons (which have already been discussed) for not having seen the response in the rod outer segments.

If such a free readical is indeed a part of the physiological visual process, it would help to partially explain many current puzzles; for example, it would be the first active chemical species from the visual cycle and thereby might provide a mechanism to explain both the necessary amplification and the trigger mechanism, and it should also provide a reconciliation between the theories of Rosenberg and Wald, in addition to leading to some insight as to the chemical nature of the species responsible for the early receptor potential of the electroretinogram.

Nevertheless, just as when one finds another word in a crossword puzzle, he is made increasingly aware of the empty spaces and his curiosity is heightened, so here, with one more block of data, some of the voids in man's knowledge of the visual process are more sharply outlined, and his natural eagerness to fill them (hopefully) enhanced.

BIBLIOGRAPHY

Abrahamson, E. W. and Linschitz, H. (1955), "Reversible flash bleaching of chlorophyll, "J. Phys. Chem., 23, 2198-2199.

Abrahamson, E. W. and Ostroy, S. E. (1967), "The photochemical and micromolecular aspects of vision," Progress in Biophysics and Molecular Biology, 179-215.

Abrahamson, E. W., Adams, R. G., and Wulff, V. J. (1959), "Reversible spectral changes in retinene solutions following flash illumination, J. Phys, Chem., 63, 441-443.

Abrahmson, E. W., Marquisee, P., Gavuzzi, P., and Roubie, J. (1960), "Flash photolysis of visual pigments," Z. Electrochem., 64, 177-180 (in English).

Albrecht, G. (1957), "Acetylrhodopsin," Science, 125, 70-72.

Ball, S., Goodwin, T. W. and Morton, R. A. (1946), "Retinene$_1$-vitamin A aldehyde," Bioch. J., 40, Proc. 59.

Ball, S., Collins, F. D., Morton, R. A. and Stubbs, A. L. (1948), "Chemistry of visual processes," Nature, 161, 424-426.

Ball, S., Goodwin, T. W. and Morton, R. A. (1948), "Studies on Vitamin A, 5: Preparation of retinene$_1$-Vitamin A aldehyde,: Bioch. J., 42, 516-523.

Ball, S., Collins, F. D., Dalvi, P. D. and Morton, R. A. (1949), "Studies on vitamin A, 11: "Reactions of retinene$_1$ with amino compounds," Bioch. J., 45, 304-307.

Bersohn, R. (1963), "Electron Paramagentic Resonance of Organic Molecules,: Chapter 3 in Determination of Organic Structures by Physical Methods, supplementary volume, (Nachod and Phillips, eds), Academic Press, N.Y.

Berthod, H. and Pullman, A. (1960), "Aspects of the electronic structure of retinal and its isomers of bilogical interest," Compt. Rend., 251, 808-810.

Blatz, P. (1965), "The Role of carbonium ions in color reception," J. Gen. Physiol., 48, 753-760.

Bliss, A. F. (1948), "Retinene$_1$ and indicator yellow," Nature, 162, 661-662.

Boll, F. (1877), "Zur Anatomie and Physiology der Retina," Arch. Anat. Physiol, Abt. 3 36.

Bownds, D. (1967) "Amino acids near the binding site of retinal in cattle rhodopsin," *Fed. Proc.*, *26*, 601.

Bridges, C. D. B. (1957), "Cationic extracting agents for rhodopsin and their mode of action," *Bioch. J.*, *66*, 375-383.

Bridges, C. D. B. (1962), "Studies on the flash photolysis of visual pigments, 3: Interpretation of the slow thermal reactions following flash irradiation of frog rhodopsin solutions," *Vision Res.*, *2*, 201-214.

Brindley, G. S. (1960), "The electrical activity of the retina", Chapter II in *Physiology of the Retina and Visual Pathway*, Edward Arnold Ltd., London.

Brindley G. S. and Gardner-Medwin, A. B. (1965), "Evidence that the early receptor potential of the retina does not depend on changes in the permeability of membranes to specific ions," *J. Physiol.* *180*, 1P.

Brindley, G. S. and Gardner-Medwin, A. M. (1960) "The origin of the early receptor potential of the retina," *J. Physiol.*, *182*, 185-194.

Broca, E. E. and Cocceve, C. F. (1941), "The Behavior of visual purple at low temperature," *Proc. Roy. Sec. A.*, *179*, 151-159.

Brown, K. T. and Murakami, M. (1964a),"A new receptor potential of the monkey retina with no detectable latency," *Nature*, *201*, 626-628.

Brown, K. T. and Murakami, M. (1964b), "Biphasic form of the early receptor potential of the monkey retina," *Nature*, *201*, 739-740.

Brown, K. T. and Wiesel, F. N. (1961), 'Analysis of the intraretinal electroretinogram in the intact cat eye" *J. Physiol.*, 158, 229-256.

Brown, K. T., Watanabe, K., and Murakami, M. (1965), "The early and late receptor potentials of monkey cones and rods," *Col Spring Harbor Symposia in Quant. Biol.*, *30*, 483-504.

Brown, P.K. and Brown, P.S. (1958), "Visual pigments of the octopus and cuttlefish," *Nature*, *182*, 1288-1290.

Brown, P.K. and Wald, G., (1956), "The neo-bisomer of vitamin A and retinene," *J. Biol. Chem.*, *222*, 865-877.

Brown, P. K. and Wald, G. (1963), "Visual pigments in human and monkey retina," *Nature*, *200*, 37-43.

Chase, A. M. (1937), "An accessory photosensitive substance in visual purple regeneration," Science, 85, 484.

Chase, A. M. and Smith, E. L. (1939) "Regeneration of visual purple in solution," J. Gen. Physiol., 23, 21-39.

Collins, F. D. (1953), "Rhodopsin and indicator yellow," Nature, 171, 469, 471.

Collins, F. D. and Morton, R. A. (1950), "Studies on rhodopsin, 2: Indicator yellow," Bioch. J., 47, 10-17.

Collins, F. S. and Morton, R. A. (1950a), "Studies in rhodopsin, 3: Rhodopsin and transcient orange," Bioch. J., 47, 18-24.

Collins, F. S. and Morton, R. A. (1950b), "Studies on rhodopsin, 1: Methods of extraction and the absorption spectrum," Bioch. J., 47, 3-10.

Collins, F. D., Love, R. M. and Morton, R. A. (1952), "Studies in rhodopsin, 4: Preparation of rhodopsin," Bioch. J., 51, 292-298.

Collins, F. D., Green, J. H., and Morton, R. A. (1954), "Studies in rhodopsin, 7: Regeneration of rhodopsin by comminuted ox retina," Bioch. J., 56, 493-498.

Commission on Nomenclature of Bilogical Chemistry, (1960) "Definitive rules for the nomenclature of amino acids steriods, vitamins and carotenoids," J. Am. Chem. Soc., 82, 5575-5584.

Cone, R. A. (1964), "Early receptor potential of the vertebrate retina, "Nature, 204, 736-739.

Cone, R. A. (1965), "The early receptor potential on the vertebrate eye," Cold Spring Harbor Symposia in Quant. Biol., 30, 457-482.

Cone, R. A. (1967), "Early receptor potential: Photoreversible charge displacement in rhodopsin," Science, 155, 1128-1130.

Dartnall, H. J. A. (1948), "Indicator yellow and retinene," Nature, 162, 222.

Dartnall, H. J. A. (1957), The Visual Pigments, Methuen and Co., Ltd., London.

Dartnall, H. J. A. (1962a), "Extraction, measurement and analysis of visual photopigment," Chapter 17 in The Eye, (H. Davson, ed.), 323-365.

Dartnall, H. J. A. (1962b), "The chemical structure and photochemistry of the visual pigment," Chapter 19 in *The Eye*, (H. Davson, ed.) 427-471.

Dawson, W. (1962), "The population and decay of the lowest triplet state in retinene," Ph. D. Thesis, Case Institute of Technology.

Dawson, W. and Abrahamson, E. W. (1962), "Population and decay of the lowest triplet state in polyenes with conjugated heteroatoms: retinene," *J. Phys. Chem.*, *66*, 2542-2547.

deGroot, M. S. and Van der Walls, J. H. (1960), "Paramagnetic resonance in phosphorescent aromatic hydrocarbons II: Determination of zero field splitting from solution spectra," *Mol. Phys.*, *3*, 190-200.

Denton, E. J. (1954), "A method of easily observing the dichroism of the visual rods," *J. Physiol.*, *124*, 16P-17P.

De Robertis, E. (1956), "Electron microscope observations on the submicroscopic organization of the retinal rods," *J. Biophys. Bioch. Cytol.*, *2*, 319-331.

Dowling, J. E. (1960), "Chemistry of visual adaptation in the rat .," *Nature*, *188*, 114-118.

Dowling, J. E. and Gibbons, I. R. (1961), "The effects of vitamin A deficiency on the fine structure of the retina," in *The Structure of the Eye*, (G. K. Smelser, ed. Academic Press, New York, 85-89.

Dowling, J. E. and Hubbard, R. (1963), "Effects of brilliant flashes on light and dark adaptation," *Nature*, *199*, 972-975.

Erhardt, F., Ostroy. S. E. and Abrahamson, E. W. (1966), "Protein configuration changes in the photolysis of rhodopsin, I: The thermal decay of cattle lumirhodopsin in vitro," *Bioch. Biophys*, *Acta*, *112*, 256, 264.

Ewald, A. Kuhne, W. (1878-9), "Untersuchungen uber den Sehrpurpur," in *Untersuchungen aus dem Physiologischen Institute der Universitat Heidelberg*, (W. Kuhne, ed.) Erster Band, Heidelberg, Carl Winer's universitats buchhandlung.

Falk, G. and Fatt, P. (1966), "Rapid Hydrogen ion uptake of rod outer segments and rhodopsin solutions on illumination," *J. Physiol.*, *183*, 211-224.

Frank, R. N. and Goldsmith, T. H. (1965), "Adenosine triphosphatase activity in the rod outer segments of the pig's retina," *Arch. Bioch. and Biophys.*, *110*, 517-525.

Fredericia, L. S. and Holm, E. (1925), "Experimental contribution to the study of the relation between night blindness and malnutrition. Influence of deficiency of fat-soluble A-vitamin in the diet of the visual purple in the eyes of rats," Am. J. Physiol., 73, 63-78.

Futterman, S. (1965), "Stoichiometry of retinal vitamin A metabolism during light adaptation," in Biochem. of the Retina, (C. N. G. Raymore, Ed.), Academic Press, New York, 16-21.

Golub, M. A., Stephens, C. L. and Brash, J. L. (1966), "Photo- and radiation-induced cis-trans isomerization of several 2-olefins," J. Chem. Phys., 45, 1503-1505.

Granit, R. (1959), "Neural Activity of the Retina, " Chapter XXIX in Handbook of Physiology, section 1: Neurophysiology, vol. I, (H. W. Magoun, ed.,) Washington, D. C., the American Physiological Society, 693-712.

Grellmann, K. H., Livingston, R. and Pratt, D. (1962), "A flash photolytic investigation of rhodopsin at low temperatures," Nature, 193, 1258-1260.

Grellman, K. H., Manning, R. and Livingston, R. (1962a), "Some flash photolytic and photochemical studies of retinene and related compounds, J. Am. Chem. Soc., 84 546-548.

Hagins, W. A. (1957), PH.D. Thesis, University of Cambridge, as cited by Pitt and Morton (1960).

Hagins, W. A. and McGaughy, R. E. (1967), "Molecular and thermal origins of fast photelectric effects in the squid retina," Science, 157, 813-816.

Handbook of Chemistry and Physics (1964), 4th ed., Chemical Rubber Co.

Hara, R. (1963), "Changes in the electrical conductance of rhodopsin on photolysis," J. Gen. Physiol., 47, 241-264.

Haurwitz, F. (1933), "Vitamin A and Sehpurpur. Ein Beitrag zur Kenntis der Hemeralopie," Med. Klin, 29, 1148-1149.

Hausser, K. W., Kuhn, R. and Seitz, G. (1935), "Lichtababsorption und Doppelbindung, V: Uber die Absorption von Verbindungen mit Konjugierten Kohlenstoffdoppelbindungen bei tiefer temperatur," Z. Physikal. Chemie, Abt. B, 29, 391-416.

Hecht, S. and Pickels, E. G. (1938), "The sedimentation constant of visual purple," Proc. Nat. Acad. Sci., 24, 172-176.

Hecht, S., Chase, A. M. and Schlaer, S. (1937), "The diffusion coefficient and molecular size of visual purple," *Science* *85*, 567-568.

Hecht, S., Shlaer, S. and Pirenne, M. H. (1941-2) "Energy, Quanta, and vision," *J. Gen. Physiol.*, *25*, 819-840.

Hsia, Y. and Graham, Ch. H. (1952), "Spectral sensitivity of the cones in the dark adapted human eye," *Proc. Nat. Acad. Sci.* *38*, 80-85.

Hubbard, R. (1954), "The molecular weight of rhodopsin and the nature of the rhodopsin-digitonin complex," *J. Gen. Physiol.*, *37*, 381-399.

Hubbard, R. (1955), "Retinene isomerase," *Fed. Proc.*, *14*. 229.

Hubbard, R. (1956), "Retinene Isomerase," *J. Gen. Physiol.*, *39*, 935-962.

Hubbard, R. (1956a), "Geometrical isomerization of vitamin A, retinene, and retinene oxime," *J. Am. Chem. Soc.*, *78*, 4662-4667.

Hubbard, R. (1958), "On the Chromophores of the visual pigments," in Visual Problems of Colour, *National Physical Lab. Symposium No.* *8*, H. M. Stationery office, London, 153-169.

Hubbard, R. (1958a), "Thermal stability of rhodopsin and opsin," *J. Gen. Physiol.*, *42*, 259-280.

Hubbard, R. (1958b), "Bleaching of rhodopsin by light and by heat," *Nature*, *181*, 1126.

Hubbard, R. (1966), "The stereoisomerization of 11-cis retinal" *J. Biol. Chem.*, *241*, 1814-1818.

Hubbard R. and Colman, A. D. (1959), "Vitamin A content of frog eye during light and dark adaptation," *Science*, *130*, 977-978.

Hubbard, R. and Kropf, A. (1958), "The action of light on rhodopsin," *Proc. Nat. Acad, Sci.*, *44*, 130-139.

Hubbard, R., and Kropf, A. (1959), "Molecular aspects of visual excitation," *Ann. N.Y. Acad. Sci.*, *81*, 388-398.

Hubbard, R. and Kropf, A. (1967), "Molecular isomers of vision," *Scient. Amer.*, *216 (6)*, 64-76.

Hubbard, R., and St. George, R. C. C. (1958), "The rhodopsin system of the squid," *J. Gen. Physiol.*, *41*, 501-528.

Hubbard, R. and Wald, G. (1951), "The mechanism of rhodopsin synthesis," *Proc. Nat. Acad. Sci.*, *37*, 69-79.

Hubbard, R. and Wald, G. (1952), "Cis-trans isomers of vitamin A and retinene in the rhodopsin system," *J. Gen. Physiol.*, *36*, 269-315.

Hubbard, R. and Wald, G. (1952a), "Cis-trans isomers of vitamin A and retinene in vision," *Science*, *115*, 60-63.

Hubbard, R., Gregerman, R., Wald, G. (1953), Geometrical isomers of retinene," *J. Gen. Physiol*, *36*, 415-429.

Hubbard, R., Brown, P.K. and Kropf, A. (1959), "Action of light on visual pigments, vertebrate lumi-and metarhodopsins" *Nature*, *183*, 442-446.

Hubbard, R., Bownds, D. and Yoshizawa, T. (1965), "The chemistry of visual photoreception," *Cold Spring Harbor Symposia in Quant. Biol.*, *30*, 301-315.

Ilten, D. F. and Calvin, M. (1965), "Correlations between photo-induced EPR and photocoductivity in the TCNE-THF solution charge transfer complex," *J. Chem. Phys.*, *42*, 3760-3766.

Ingram, D. J. E. (1958), *Free Radicals as studied by Electron Spin Resonance*, Butterworth's Scientific Publications.

Jurkowitz, L., Loeb, J. N., Brown, P.K. and Wald, G. (1959), Photochemical and stereochemical properties of carotenoids at low temperatures-photochemical behavior of retinene," *Nature*, *184*, 614-624.

Kistiakowsky, G. B. and Smith, W. R. (1934), "Kinetics of thermal cis-trans isomerization III," *J. Am. Chem. Soc.*, *56*, 638-642.

Kistiakowsky, G. B. and Smith, W. R. (1935), "The kinetics of thermal cis-trans isomerization, IV," *J. Am. Chem. Soc.*, *57*, 269-271.

Kistiakowsky, G. B. and Smith, W. R. (1936), "The kinetics of thermal cis-trans isomerization, VI," *J. Am. Chem. Soc.*, *58*, 2428-2430.

Kito, Y., Ishigami, M. and Yoshizawa, T. (1961), "On the labile intermediate of rhodopsin as demonstrated by low temperature illumination," *Biochem. Biophys. Acta*, *48*, 287-298.

Koshland, D. E., Jr. (1963), "The role of flexibility in enzyme action," *Cold Spring Harbor Symp. Quant. Biol.*, *28*, 473-482.

Krinsky, N. I. (1955), "Esterification of vitamin A in vitro," *Fed. Proc.*, *14*, 88.

Krinsky, N. I. (1958a), "The enzymatic esterification of vitamin A," *J. Biol. Chem.*, *232*, 881-894.

Krinsky, N. I. (1958b), "The lipoprotein nature of rhodopsin" Arch. Opth., 60, 688-694.

Kropf, A. and Hubbard, R. (1958), "The mechansim of bleaching rhodopsin," Ann. N.Y. Acad, Science., 74, 266-280.

Kuhne, W. (1879), "Chemische vorgange in der netzhaut," in Handbuch der Physiologie, pt. 1, (L. Hermann, ed.), F. C. W. Vogel, Liepzig, 3, 235-297.

Kuhne, W. (1895), "Zur darstellung des Sehpurpurs," Z. Biol., 32, 21-28.

Lewis, D. M. (1957), "Regeneration of rhodopsin in the albino rat," J. Physiol., 136, 624-631.

Lhoste, J. M., Haug, A. and Hemmerich, P. (1966), "Electron Paramagnetic Resonance studies of the triplet state of flavin and pteridine derivatives," Biochemistry, 5, 3290-3300.

Livingston, R. and Pugh, A. C. (1959), "Role of the triplet state in reactions sensitized by chlorophyll," Disc. Faraday Soc., No. 27, 144-148.

Livingston, R. and Ryan, V. A. (1953), "The phototropy of chlorophyll in fluid solutions," J. Am. Chem. Soc., 75 2176-2181.

Linschitz, H. and Sarkanen, K. (1958), "The absorption spectra and decay kinetics of the metastable states of chlorophyll A and B," J. Am. Chem. Soc., 80, 4826-4832.

Lockhurst, G. and Abrahamson, E. W. (1965), unpublished, cited by Abrahamson, and Ostroy (1967).

Lumry, R. and Eyring, H. (1954), "conformation changes of proteins:, J. Phys. Chem., 58, 110-120.

Lythgoe, R. J. (1937), "The absorption spectra of visual purple and indicator yellow," J. Physiol., 89, 331-358.

Lythgoe, R. J. and Quilliam, J. O. (1938a), "The relation of transcient orange to visual purple and indicator yellow," J. Physiol., 94, 399-410.

Magee, J., Shad, W., Jr. and Eyring, H. (1941), "Non adiabatic reactions. Rotation about the double bond," J. Am. Chem. Soc., 63, 677-688.

Magoon, E. F. and Zechmeister, L. (1955), "On the cis forms of some biphenylene derivatives of butadiene and hexatriene," J. Am. Chem. Soc., 77, 5642-5646.

Matthews, R., Hubbard, R., Brown, P.K. and Wald, G. (1963-4), Tautomeric forms of metarhodopsin," *J. Gen. Physiol.*, *47*, 215-240.

McConnell, D. G. and Scarpelli, D. G. (1963), "Rhodopsin: an enzyme?" *Science*, *139*, 848.

Michael, C. R. (1969) "Retinal processing of visual images," *Sci. Amer* . *220* (5), 104-114.

Mirsky, A. E. (1936), "The visual cycle and protein denaturation," *Proc. Nat. Acad. Sci. 22*, 147-149.

Monod, J., Changeux, J.P. and Jacob, F. (1963), "Allosteric proteins and cellular control systems," *J. Mol. Biol. 6*, 306-329.

Morton, R. A. (1944), "Chemical aspects of the visual process," *Nature 153*, 69-71.

Morton, R. A. and Goodwin, T. W. (1944), "Preparation of retinene in vitro," *Nature 153*, 405-406.

Morton, R. A. and Pitt, G.A.J. (1955), "Studies on rhodopsin, 9; pH and the hydrolysis of indicator yellow," *Bioch. J. 59*, 128-134.

Morton, R. A. and Pitt, G. A. J. (1957), "Visual pigments," *Fortschr. Chem. Org. Naturst. 14*, 244-316.

Mouseron-Canet, M., Mani, J.C., Favie, C. and Lerner, D. (1966), "Photochemical isomerization of vitamin A," *Compt. Rend. Ser. C. 262*, 153-155.

Mulliken, R. S. (1932), "Excited states of ethylene," *Phys. Rev. 41*. 751-758.

Noell, W. K. (1953), "Studies on the electrophysiology and metabolism of the retina," Publication of the U.S.A.F. School of Aviation Medicine, Randolph Field, Texas.

Oroshnik, W. (1956), "The synthesis and configuration of neo-b vitamin A and neo-b retinene," *J. Am. Chem. Soc. 78*, 2651.

Oroshnik, W. Brown, P.K. Hubbard, R. and Wald, G. (1956), "Hindered Cis isomers of vitamin A and retinene; the structure of the neo-b isomer," *Proc. Nat. Acad. Sci. 42*, 578-580.

Ostroy, S. E., Erhardt, F. and Abrahamson, E. W. (1966), "Protein configuration changes in the photolysis of rhodopsin, II: The sequence of intermediates in the thermal decay of cattle metarhodopsin in vitro," *Biochem. Biophys. Acta 112*, 265-277.

Ostroy, S., Rudney, H. and Abrahamson, E. W. (1966a), "Sulfhydryl groups of rhodopsin," Biochem. Biophys. Acta 126, 409-412.

Pak, W.L. and Boes, R.J. (1967), "Rhodopsin: Responses from transient intermediates formed during its bleaching," Science 155, 1131-1133.

Pak, W.L. and Cone, R.A. (1964), "Isolation and identification of the initial peak of the early receptor potential," Nature 204, 836-838.

Pak, W.L. and Ebrey, T.G. (1965), "Visual receptor potential observed at sub-zero temperatures," Nature 205, 484-486.

Palmer, L.S. and Eccles, C.H. (1914), "Carotin - the principal natural yellow pigment of milk fat: Its relation to plant carotin and the carotin of body fat, corpus luteum and blood serum. III, the yellow lipochrome of blood serum," J. Biol. Chem. 17, 223-237.

Pauling, L. (1939), "Recent work on the configuration and electronic structure of molecules with some applications to natural products," Fortschr. Chem. Org. Naturst. 3, 203-235.

Pauling, L. (1949), "Zur cis-trans isomeriisierüng von Carotinoiden," Helv. Chem. Acta 32, 2241-2246.

Piette, L.H.(1963), "An introduction to EPR," from the 7th annual NMR-EPR workshop, Varian Associates Analytical Division, Palo Alto, California.

Pinckard, J.H., Wille, B. and Zechmeister, L. (1948), "A comparative study of the three stereoisomeric 1,4, diphenylbutadienes," J. Am. Chem. Soc. 70, 1938-1944.

Pirenne, M. H. (1951), "Limits of the visible spectrum," Research 4, 508-515.

Pitt, G.A.J. and Morton, R.A. (1960), "Cis-trans isomers of retinene in visual processes," Biochem. Soc. Symposia (Cambridge) No. 19, 67-89.

Pitt, G.A.J. and Tinkham, M (1965), "Lumi- and metarhodopsin," Nature 176, 220-221.

Pitt, G.A.J., Collins, F.D., Morton, R.A. and Stok, P. (1955), "Studies on rhodopsin, 8: Retinylidene methylamine and indicator yellow analog," Bioch. J. 59, 122-128.

Poincelot, R.P., Millar, P.G., Kimbel, R.L. and Abrahamson, E.W. (1969), "Lipid to protein chromophore transfer in the photolysis of rhodopsin," Nature 221, 256-257.

Polis, B.D. and Wyeth, J. (1962), "Free radicals generated during the interaction of rhodopsin and visible light," Fed. Proc. 23, 384.

Pratt, D.C., Livingston, R. and Grellmann, K.H. (1964), "Flash photolysis of rod particle suspensions," Photochem. and Photobiol. 3, 121-127.

Pullman, A. (1960), "Electronic character of conjugated polyenes of biological interest," Compt. Rend., 251, 1430-1432.

Pullman, A. and Pullman, B. (1961), "The cis-trans isomerization of conjugated polyenes and the occurrence of a hindered cis isomer of retinene in the rhodopsin system," Proc. Nat. Acad. Sci. 47, 7-14.

Radding, C.M. and Wald, G. (1956), "Acid-base properties of rhodopsin and opsin," J. Gen. Physiol. 39, 909-922.

Rexroad, H.N. and Gordy, W. (1956), "Electron spin resonance in X-irradiated hormones and vitamins," Bull. Amer. Phys. Soc. 1, 200.

Robeson, C.D., Blum, W.P., Dieterle, J.M., Cawley, J.D. and Baxter, J.G. (1955a), "Chemistry of vitamin A, XXV: Geometrical isomers of vitamin A aldehyde and an isomer of its alpha ionone analog," J. Am. Chem. Soc. 77, 4120.

Robeson, C.D., Cawley, J.D., Weisler, L., Stern, M.H., Eddinger, C.C. and Chechak, A.J. (1955b), "Chemistry of vitamin A, XXIV: The synthsis of geometrical isomers of vitamin A via methyl-beta-methyl glutaconate," J. Am. Chem. Soc. 77, 4111-4119.

Rosenberg, B. (1958), "Photoconductivity and the visual receptor process," J. Opt. Soc. Amer. 48, 581-582.

Rosenberg, B. (1958a), "Evidence for the triplet state in photoconductivity in anthracene," J. Chem. Phys. 29, 1108-1118.

Rosenberg, B. (1959), "Photoconduction and cis-trans isomerism in beta-carotene," J. Chem. Phys. 31, 238-246.

Rosenberg, B. (1961a), "Photoconduction activation energies in cis-trans isomers of beta-carotene," J. Chem. Phys. 34, 63-66.

Rosenberg, B. (1961b), "Photoconductivity in a hindered cis isomer of beta-carotene and its relation to a theory of the visual receptor process," J. Opt. Soc. Amer. 51, 238-240.

Rosenberg, B. (1962), "Electron charge transport in carotenoid pigments and a primitive theory of the electroretinogram," Photochem. Photobiol. 1, 117-129.

Rosenberg, B. (1966), "A physical approach to the visual receptor process," Adv. in Radiation Biology 2, 193-241.

Rosenberg, B. and Harder, H.C. (1967), "Semiconduction and photoconduction activation energies of the retinals," Photochem. Photobiol. 6, 629-641.

Rosenberg, B. and Krigas, T. (1967), "Spectral shifts in retinal Schiff base complexes," Photochem. Photobiol. 6, 769-773.

Rosenberg, B., Orlando, R.A. and Orlando, J.M. (1961), "Photoconduction and semiconduction in dried receptors of sheep eyes," Arch. Bioch. Biophys. 93, 395-398.

Rushton, W.A.H. (1958), "Blue light and the regeneration of human rhodopsin in situ," J. Gen. Physiol. 41, 419-428.

Schachman, H.K. (1963), "Considerations on the tertiary structure of proteins," Cold Spring Harbor Symp. Quant. Biol. 28, 409-430.

Schmidt, W.J. (1938), "Polarizationoptische analyse eines eiweiss-lipoid-systems erlautert am Aussengleid der Sehzellen," Kolloid-Z, 85, 137-148.

Schichi, H., Lewis, M.S., Irrevere, F. and Stone, A.L. (1969), "Biochemistry of visual pigments. I. Purification and properties of bovine rhodopsin," J. Biol. Chem. 244, 529-536.

Shields, J. E., Dinovo, E.C., Henriksen, R.A., Kimbel, R.L. Jr. and Millar, P.G. (1967), "The purification and amino acid composition of bovine rhodopsin," Biochem. Biophys. Acta 147, 238-251.

Sjöstrand, F.S. (1949), "An electron microscope study of the retinal rods of the guinea pig's eye," J. Cell. Comp. Physiol. 33, 383-404.

Sjöstrand, F.S. (1953), "The ultrastructure of the outer segments of rods and cones of the eye as revealed by the electron microscope," J. Cell, Comp. Physiol. 42, 25-44.

Smaller, B. (1960), "Photoinduced free spin species in plant pigments," in Free Radicals in Biological Systems, (M. Blois et al. eds.) New York, Academic Press, 315-323.

Snydacker, D. and Newell, F. (1952), Refraction, Am. Acad. Ophth. and Oto., 71 p.

Sogo, P. B., Pon, N.G. and Calvin, M. (1957), "Photo spin resonance in chlorophyll-containing plant material," Proc. Nat. Acad. Sci. 43, 387-393.

Squires, T. L. (1964), "An introduction to electron spin resonance," Academic Press, New York.

Stearn, A.E. (1949), "Kinetics of biological reactions with special reference to enzymic processes," *Adv. Enzymol. 9*, 25-74.

Tansley, K. (1931), "The regeneration of visual purple; its relation to dark adaptation and night blindness," *J. Physiol. 71*, 442-458.

Thompson, L.C. (1947), "The effect of change of brightness level upon the foveal luminosity curve measured with small fields," *J. Physiol. 106*, 368-377.

Thompson, L.C. (1951), "The spectral sensitivity of the central fovea," *J. Physiol. 112*, 114-132.

Tomita, T. (1959), "Study on electrical activities in the retina with penetrating microelectrodes," *Proc. XXI Int. Cong. Physiol*., 245-248.

Tronche, P., Komor, J. and Couquelet, J. (1965), "Regeneration of the visual purple in enucleated cattle eyes," *Compt. Rend*. *Soc. Biol. 159, 1564-1566*.

Van der Waals, J.H. and de Groot, M.S. (1959), "Paramagnetic resonance in phosphorescent aromatic hydrocarbons, I: Naphthalene," *Mol. Phys. 2*, 333-340.

Von Euler, H. and Adler, E. (1934), "Beobachtungen uber den Sehpurpur," *Ark. Kemi., Mineral., Geol., 11B*, No. 20.

Wald, G. (1933), "Vitamin A in the retina," Nature 132, 316-317.

Wald, G. (1934-1935), "Vitamin A in eye tissues," *J. Gen. Physiol. 18*, 905-915.

Wald, G. (1935-1936), "Carotenoids and the Visual Cycle," *J. Gen*. *Physiol. 19*, 351-369.

Wald, G. (1937), "Bleaching of visual purple in solution," *Nature 139*, 587-588.

Wald, G. (1938), "On rhodopsin in solution," *J. Gen. Physiol. 21*, 795-832.

Wald, G. (1954), "Mechanisms of vison," in *Conference on Nerve Impulse*, 4th, (D. Nachmansohn, ed.) Josiah Macy Foundation, New York, 11-57.

Wald, G. (1955), "The photoreceptor process in vision," *Am. J. Ophth. 40*, 18-41.

Wald, G. (1956), "The biochemistry of visual excitation," in *Enzymes: Units of Biological Structure and Function*, (O. Gaebler, ed.), Academic Press, New York, 355-367.

Wald, G. (1958), "Photochemical aspects of visual excitation," Exptl. Cell, Res. Suppl. 5, 389-410.

Wald, G. (1958a), "Retinal chemistry and the physiology of vision," in Visual Problems of Colour, National Physical Lab, Sym. No. 8, H.M. Stationery Office, London, 7-61.

Wald, G. (1959), "The photoreceptor process in vision," in Handbook of Physiol. (J. Field et al, eds.), Section I, Vol. I, 671-692.

Wald, G. (1964), "The receptors of human color vision," Science 145, 1007-1017.

Wald, G. (1964a), unpublished, cited by Rosenberg (1966).

Wald, G. (1965), "Introductory lecture," in Recent Progress in Photobiology, (C.J. Bowen, ed.) Academic Press, New York, 133-144.

Wald, G. (1965a),"Visual excitation and blood clotting," Science 150, 1028-1030.

Wald, G. (1967), personal communication.

Wald, G. and Brown, P.K. (1950), "The synthesis of rhodopsin from retinene$_1$," Proc. Nat. Acad. Sci. 36, 84-92.

Wald, G. and Brown, P.K. (1952), "The role of sulfhydryl groups in the bleaching and synthesis of rhodopsin," J. Gen. Physiol. 35, 797-821.

Wald, G. and Brown, P.,. (1953), "The molar extinction of rhodopsin," J. Gen. Physiol. 37, 189-200.

Wald, G. and Brown, P.K. (1956), "Synthesis and bleaching of rhodopsin," Nature 177, 174-176.

Wald, G. and Brown, P.K. (1958), "Human rhodopsin," Science 127, 222-226.

Wald, G. and Brown, P.K. (1965), "Human color vision and color blindness," in Cold Spring Harbor Symp. Quant. Biol. 30, 345-361.

Wald, G. and Hubbard, R. (1957), "Visual pigment of a decapod crustacean: the lobster," Nature 160, 278-280.

Wald, G., Durell, J. and St. George, R.C.C. (1950), "The light reaction in the bleaching of rhodopsin," Science 111, 179-181.

Wald, G., Brown, P.K. and Smith, P.H. (1952), "Iodopsin," Fed. Proc. 11, 34.

Wald, G., Brown, P.K. and Smith, P.H. (1953), "Cyanopsin, a new cone pigment of vision," Science 118, 505-508.

Wald, G., Brown, P.K. and Smith, P.H. (1954-1955), "Iodopsin," J. Gen. Physiol. 38, 623-681.

Wald, G., Brown, P.K., Hubbard, R. and Oroshnik, W. (1955), "Hindered cis isomers of vitamin A and retinene - structure of the neo-b isomer," Proc. Nat. Acad. Sci. 41, 438-451.

Wald, G., Brown, P.K. and Kennedy, D. (1957), "The visual system of the alligator," J. Gen. Physiol. 40, 703-713.

Wald, G., Brown, P.K. and Gibbons, I.R. (1963), "The problem of visual excitation," J. Opt. Soc. Amer. 53, 20-35.

Walling, C.T. (1957), Free Radicals in Solution, Wiley, New York, as cited by Smaller (1960), p. 319.

Williams, T.P. (1964), "Photoreversal of rhodopsin bleaching," J. Gen. Physiol. 47, 679-689.

Williams, T.P. (1966), "Limitations on the use of the concept of quantum efficiency in rhodopsin bleaching," Nature 209, 1350-1351.

Wolff, E. (1961), Anatomy of the Eye and Orbit, 5th ed. (revised by R.J. Last), H.K. Lewis and Co., Ltd., London.

Wolken, J. J. (1956), "Photoreceptor structures, I. Pigment monolayers and molecular weight," J. Cell. Comp. Physiol. 48, 349-369.

Wolken, J.J. (1958), "Studies of photorecptor structures," Ann. N.Y. Acad. Sci.. 74, 164-181.

Wolken, J. J. (1961), "A structural model for a retinal rod," in The Structure of the Eye, (G.K. Smelser, ed.) New York, Academic Press, 173-191.

Wulff, V.J., Adams, R.G., Linschitz, H. and Abrahamson, E.W. (1958), "Effect of flash illumination on rhodopsin in solution," Ann. N.Y. Acad. Sci. 74, 281-290.

Wulff, V.J., Adams, R.G., Linschitz, H. and Kennedy, D. (1958a), "The behavior of flash illuminated rhodopsin in solution," Arch. Ophth. 60, 695-701.

Yoshizawa, T. and Kito, Y. (1958), "Chemistry of the rhodopsin cycle," Nature 182, 1604.

Yoshizawa, T. and Wald, G. (1963), "Pre-lumirhodopsin and the bleaching of visual pigments," Nature 197, 1279-1286.

Yoshizawa, T. and Wald, G. (1964), "Transformation of squid rhodopsin at low temperatures," Nature 201, 340-345.

Yoshizawa, T., Kito, Y. and Ishigami, M. (1960), "On the labile intermediates of rhodopsin as demonstrated by low temperature illumination," Biochem. Biophys. Acta 48, 287-298.

Yoshizawa, T., Kito, Y. and Ishigami, M. (1960), "Studies on the metastable states in the rhosopsin cycle," Biochem. Biophys. Acta 43, 329-334.

Zechmeister, L. (1944), "Cis-trans isomerization and stereochemistry of carotenoids and diphenylpolyenes," Chem. Revs. 34, 267.